University of Liège

Faculty of Medicine

Luxembourg Institute of Health

Department of Infection and Immunity

New insights into the atypical chemokine receptor network and functions

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Thesis submitted in partial fulfillment of the requirements for the degree of

Doctor in Biomedical and Pharmaceutical Sciences

2021

Luxembourg National Research Fund

FNR AFR INDIVIDUAL PHD GRANTS

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Oh kleiner Mensch stell Dich den Dingen
Die Künste magst Du zu bezwingen
Doch solcher Zauber wie geschehen
Die Welt mit Farbe neu entfacht
Erahnst Du vielleicht zu verstehen
Doch hast Dir's niemals ausgedacht...

Fjoergyn – Jera

Acknowledgments

First, I would like to thank my host Institute, the Luxembourg Institute of Health (LIH), especially my co-promoter and director of the Department of Infection and Immunity, Prof. Markus Ollert, for allowing me to carry out this project and for constantly supporting me and the entire team during my thesis. I also want to thank the Luxembourgish National Research Fond (Fonds National de la Recherche, FNR) for funding this project with an AFR grant, allowing me to pursue my career goals with this doctoral thesis.

I would like to particularly thank my supervisor Dr. Andy Chevigné for his continuous support, the constructive scientific discussions and for finding the right balance between doing good and serious science and taking more stress-free little bets on which molecule might be another ligand of ACKR3. Thank you for guiding me through this project, while still giving me a lot of freedom to test my own (non-project related) hypotheses that came up at discussions during afterwork drinks.

Next, I want to acknowledge my co-supervisor in Liège, Prof. Alain Vanderplasschen, as well as the members of my thesis committee Prof. Vincent Seutin, Dr. Julien Hanson and Prof. Bernard Rogister, who in the meantime became valued collaborators in different projects during the thesis. Thank you very much for the fruitful discussions and advice during the different thesis committee meetings. I would also like to thank Prof. Martine Smit and Prof. Marc Parmentier for making time to assess my work and for accepting to be a member of my thesis jury. I furthermore thank our valued partners Prof. Bernhard Moser, Prof. Christophe Stove, Prof. Arne Östman and especially Prof. Evi Kostenis for excellent and professional collaborations.

I also wish to thank my colleagues Dr. Martyna Szpakowska and Nathan Reynders for the science and publications we did together, for creating a stimulating atmosphere in our department during and after work and for the good times we spent together at various conferences. Thank you Martyna as well for all the high-quality figure arrangements you did for the different publications, for which I neither have the eye, nor the patience to do by myself. Also, my thanks to Christie Palmer for taking over the ACKR3 project and giving me hope that high-level research on this enigmatic receptor is sustained in this group.

My sincere gratitude also goes to Manuel Counson, Nadia Beaupain and Jean-Marc Plesseria, who helped me throughout the entire PhD project in numerous ways. Not a single project could have been completed without your invaluable assistance in molecular cloning and cell culture experiments.

Last, my absolute thanks go to my friends and family, especially my parents and my wife Noemi, who backed me throughout this exciting, but not always easy period of my life. Thank you for your patience and your support, and for understanding that sometimes, lab work was more important than other personal activities.

Summary

Chemokines, or chemotactic cytokines, regulate directed cell migration, proliferation and survival by activating seven-transmembrane domain G protein-coupled receptors. A variety of new chemokines were discovered in the 1990s, and it rapidly became clear that the chemokine network is fairly intricate, with most receptors binding multiple ligands and several chemokines binding more than one receptor. Today, there are about 50 chemokines described binding to over 20 different receptors allowing to spatiotemporally fine-tune a multitude of complex biological mechanisms. Besides classical chemokine receptors, a new family of four atypical chemokine receptors (ACKRs) has emerged as important regulators of chemokine functions. ACKRs bind chemokines without triggering G protein signaling but participate in chemotactic events by internalizing chemokines into degradative compartments or by transporting or presenting them on cell surfaces, hence contributing to chemokine gradient formation and clearance of abundant inflammatory chemokines.

Of particular interest for this thesis was ACKR3, which has crucial implications in cardiac and neuronal development, in the migration and homing of hematopoietic stem and progenitor cells, but also in cancer development. ACKR3 binds two chemokines, CXCL12 and CXCL11, which are shared with classical chemokine receptors CXCR4 and CXCR3, respectively. In a first step, we sought to get new insights into the molecular and structural determinants for ACKR3 activation and ligand binding in order to better understand the biology of the receptor. We could show that ACKR3 is a highly activation-prone receptor and that its binding and activation mode differs from its classical chemokine receptor counterparts with which it shares chemokines.

Next, the second and biggest part of this PhD project dealt with uncovering and characterizing new, non-classical ligands of ACKR3, since it became increasingly clear in the last years that ACKR3 activity is not limited to chemokine ligands CXCL12 and CXCL11. We revealed that ACKR3 is a broad-spectrum scavenger for endogenous opioid peptides, regulating their abundance in the central nervous system. Furthermore, we describe proadrenomedullin-derived peptides as additional family of endogenous non-chemokine ligands of ACKR3.

Last, we aimed to identify additional chemokine ligands of the atypical chemokine receptors 2 and 4 in order to further extend the already intertwined chemokine-receptor network. By exposing CCL20 and CCL22 as additional ACKR4 ligands, as well as CXCL10 as the first CXC chemokine ligand of ACKR2, our study demonstrated that chemokine-receptor interactions are yet far from being fully characterized and encourages to undertake additional systemic reassessments of the chemokine (and non-chemokine) ligand-receptor network.

The following thesis is divided into eight parts: a general introduction followed by six chapters, each dealing with a distinct study to which I contributed to different extents, and each having led to an accepted manuscript in a peer-reviewed journal. Each of the different chapters is preceded by a short introduction, explaining the motivation to undergo the corresponding study, followed by the manuscript itself. The highlights of the study are listed at the end of the chapter as short bullet points, and are followed by a brief concluding section and an outlook. The thesis ends with an outlook on ACKR3 research and a discussion on the hurdles to overcome to further uncover the chemokine interactome. All the manuscripts that have been accepted in the period of this PhD thesis are attached in their formatted version in the appendices (some of the attached papers are only related to a limited extent to this thesis and are therefore not discussed in detail here).

Résumé

Les chimiokines, ou cytokines chimiotactiques, régulent la migration, la prolifération et la survie cellulaire par l'activation de récepteurs couplés aux protéines G. Une grande variété de chimiokines a été découverte dans les années 1990 et il est alors devenu évident que le réseau de chimiokines est ambigu, où une majorité des récepteurs sont capables de fixer plusieurs ligands, et à l'inverse où plusieurs chimiokines fixent plus d'un récepteur. À ce jour, une cinquantaine de chimiokines sont recensées fixant plus de vingt récepteurs différents, ce qui permet une régulation spatiotemporelle précise d'une multitude de mécanismes biologiques. Outre les récepteurs classiques de chimiokines, une nouvelle famille composée de quatre récepteurs chimiokines atypiques s'est révélée critique dans la régulation des fonctions chimiokines. Ceux-ci fixent les chimiokines sans pour autant induire une réponse à travers les protéines G, mais participent aux événements chimiotactiques en internalisant les chimiokines dans des compartiments de dégradation, ou en les transportant ou les présentant à la surface des cellules. Ils contribuent ainsi à la formation d'un gradient de chimiokine ainsi qu'à l'évacuation des chimiokines inflammatoires.

Cette thèse s'est tout particulièrement intéressée à ACKR3, un récepteur qui joue un rôle essentiel dans le développement cardiaque et neuronal, dans la migration et le recrutement des cellules souches et progénitrices hématopoïétiques, mais aussi dans le développement du cancer. ACKR3 fixe deux chimiokines, CXCL12 et CXCL11, qu'il partage avec les récepteurs classiques CXCR4 et CXCR3 respectivement. Dans une première étape, nous nous sommes intéressés aux déterminants moléculaires et structurels de l'activation d'ACKR3 ainsi que sa fixation avec les ligands, dans l'espoir d'étayer ce qui est connu sur la biologie de ce récepteur. Nous pûmes montrer qu'ACKR3 est un récepteur très enclin à être activé et que son mode de fixation et d'activation diffère des récepteurs de chimiokines classiques avec qui il partage ses ligands.

Dans un second temps, la majeure partie de cette thèse s'est attelée à la découverte et la caractérisation de nouveaux ligands dits non-classiques d'ACKR3 puisqu'il est devenu évident dans les dernières années que l'activité du récepteur ne se limite pas aux chimiokines CXCL12 et CXCL11. Nous avons ainsi pu révéler qu'ACKR3 est un récepteur-leurre pour une grande variété de peptides opioïdes endogènes, capable de réguler leur abondance dans le système nerveux central. De plus, nous décrivons les peptides dérivés de la proadrenomedullin comme une nouvelle famille qui s'ajoute aux ligands endogènes non-chimiokines d'ACKR3.

Enfin, nous avons aussi identifié d'autres chimiokines pour les récepteurs atypiques 2 et 4 dans le but d'étayer et compléter le réseau complexe des récepteurs de chimiokines. En présentant CCL20 et CCL22 comme deux nouveaux ligands d'ACKR4, et CXCL10 comme la première chimiokine CXC

d'ACKR2, notre étude réaffirme que l'entièreté des interactions entre chimiokines et récepteurs est encore loin d'être caractérisée exhaustivement et encourage la réévaluation systématique de ce réseau.

Cette thèse est divisée en huit parties : une introduction générale suivie de six chapitres, relatant chacun une étude différente à laquelle j'ai participée, et où chacune a mené à un manuscrit accepté dans un journal évalué par les pairs. Chaque chapitre est précédé d'une introduction courte qui explique la motivation derrière dite étude, suivi du manuscrit comme il a été accepté. Quelques éléments clés de chaque étude sont repris à la fin de chaque chapitre sous forme de liste à puce, suivie d'une brève section de conclusion et d'une perspective. La thèse se termine avec une perspective sur la recherche autour d'ACKR3 et une discussion sur les obstacles à surmonter pour définir l'interactome de chimiokines. Tous les manuscrits acceptés pendant cette période de thèse sont attachés dans leur version formatée dans les appendices (certains des articles attachés ne se rapportent pas directement à la portée de cette thèse et ne sont donc pas discutés en détail ici).

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Abbreviations

ACTH: Adrenocorticotropic hormone

ADM: Adrenomedullin

ACKR: Atypical chemokine receptor

BRET: Bioluminescence resonance energy transfer

cAMP: cyclic adenosine monophosphate

CCL: CC motif chemokine ligand

CCR: CC motif chemokine receptor

CGRP: Calcitonin gene-related peptide

CLR: Calcitonin receptor-like receptor

CNS: Central nervous system

CRS: Chemokine-recognition site

CXCL: CXC motif chemokine ligand

CXCR: CXC motif chemokine receptor

CX3CL: CX3C motif chemokine ligand

CX3CR: CX3C motif chemokine receptor

DARC: Duffy Antigen Receptor for Chemokines

DC: Dendritic cell

DMEM: Dulbecco's modified eagle medium

DOR: Delta opioid receptor

ECL: Extracellular loop

ERK: Extracellular signal-regulated kinase

FBS: Fetal bovine serum

GAG: Glycosaminoglycan

GPCR: G protein-coupled receptor

GRK: G protein receptor kinase

GDP: Guanidine diphosphate

GTP: Guanidine triphosphate

HEK: Human embryonic kidney

HHV: Human herpesvirus

ICL: Intracellular loop

IMD: Intermedin

KOR: Kappa opioid receptor

mAb: Monoclonal antibody

MAPK: Mitogen-activated protein kinase

MFI: Mean fluorescence intensity

MOR: Mu opioid receptor

MRGPRX: Mas-related G protein-coupled receptor X

NK: Natural killer

NOP: Nociceptin opioid peptide receptor

PAMP: Proadrenomedullin N-terminal 20 peptide

qPCR: Reverse transcription quantitative polymerase chain reaction

SD: Standard deviation

SEM: Standard error of the mean

TM: Transmembrane

Th: T helper

WT: Wildtype

XCL: XC Motif chemokine ligand

XCR: XC motif chemokine receptor

Introduction

1. Classification of chemokines

Chemokines are a family of small secreted cytokines with around 50 members, playing a central role in directional cell migration (chemotaxis). Even though they have little sequence identity, they share some common features, such as their size (8-12kDa) and 3D structure, *i.e.* a disordered N terminus, an extended N-loop region followed by three antiparallel β-strands and a C-terminal α-helix (Figure 1A). Based on the arrangement of highly conserved cysteine residues within the chemokine's N terminus, they can be classified into four families designated CC, CXC, CX3C and XC[1] (Figure 1B).

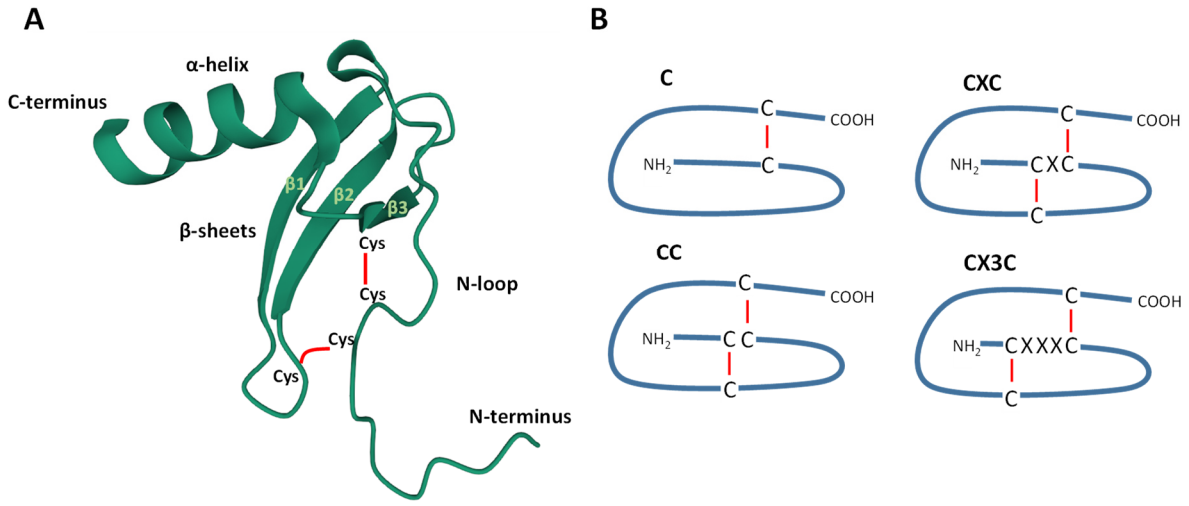

Figure 1. Chemokine structure and schematic representation of the different chemokine families. (**A**) Three-dimensional structure of CXCL12 (PDB ID: 1SDF) represented as ribbon, showing the unstructured N terminus, the conserved cysteine (Cys) residues forming disulphide bridges in red, the chemokine core with the three antiparallel β-sheets and the C-terminal α-helix. (**B**) Relationship between the conserved cysteine residues "C" and the interconnecting disulphide brides (red) they form. Depending on the arrangement of the cysteines, we can distinguish the C-, CC-, CXC-, and CX3C chemokine families.

Besides this structural classification, chemokines can also be categorized functionally as either homeostatic or inflammatory[2]. Expression of homeostatic chemokines, for example in lymphoid organs, is considered as constitutive. These chemokines are mainly involved in mediating homeostatic migration and homing of hematopoietic stem cells, lymphocytes and other cells. A prominent example for such a chemokine is CXCL12, which signals through its receptor CXCR4 without being affected by inflammatory events[3]. Inflammatory chemokines on the other hand become highly upregulated in inflamed tissues and are involved in the recruitment of leukocytes to these regions. Noteworthy, this functional classification is not mutually exclusive, as some chemokines, referred to as "dual-function" participate in immune surveillance and leukocyte development, while also being upregulated under inflammatory conditions and thus contributing to immune defense as well[2]. CXC chemokines can be further divided into ELR+ and ELR- chemokines, based on the presence or absence of a tri-peptide sequence glutamate-leucine-arginine (ELR motif) in the N terminus adjacent to the CXC motif[4]. ELR+

chemokines, which include CXCL1–CXCL8, with the exception of CXCL4, are considered pro-angiogenic[5], promoting vascularization mediated by the receptors CXCR1 and CXCR2[6]. Chemokines CXCL4, CXCL9, CXCL10 and CXCL11, not containing an ELR motif, are described as angiostatic chemokines, having either no or even inhibitory effects on angiogenesis[7]. An exception to this structurally based classification is CXCL12, which was shown to have angiogenic properties in vivo, but lacks the ELR motif nonetheless[8].

2. Chemokine receptors

Chemokines bind to and signal through over 20 different chemokine receptors, which can be divided into CCR, CXCR, CX3CR and XCR according to the type of chemokine they bind[9]. All chemokine receptors belong to the class A (or rhodopsin-like) G protein-coupled receptor (GPCRs) superfamily. They all share a common three-dimensional structure, with seven hydrophobic transmembrane (TM) α-helices connected by alternating three hydrophilic extracellular loops (ECL) and three intracellular loops (ICL). The receptor's unstructured flexible N terminus is situated extracellularly, and, together with the ECLs, interacts with chemokine ligands, conferring specificity to the receptor. The receptor's C-terminal tail is located intracellularly and is involved, together with ICLs, in the recruitment and interaction with signaling and adaptor proteins like G proteins and β-arrestins [10]. TM α-helices of chemokine receptors (as well as of most other GPCRs) are considered to form two distinguishable subpockets or crevices into which ligands can bind: TMs 1, 2, 3 and 7 form the so-called minor pocket and TMs 3, 4, 5, 6 and 7 form the major pocket[11-13] (Figure 2). Although largely simplified, there is a tendency that the major ligand binding pocket is mainly occupied the by N terminus of CXC chemokines, whereas the N terminus of CC chemokines tends to enter the minor binding pocket[14-17].

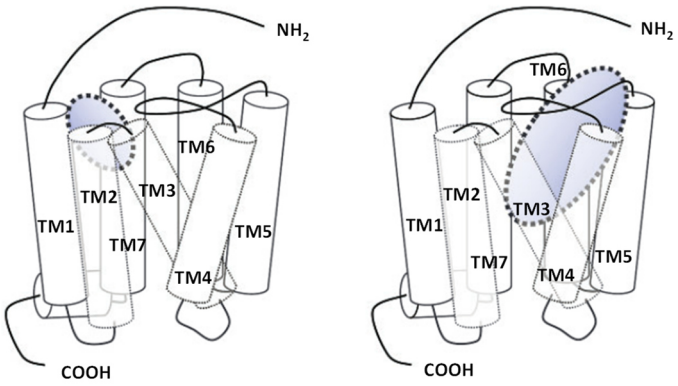

Figure 2. Schematic representation of a chemokine receptor and the two ligand binding pockets. Like all GPCRs, chemokine receptors are composed of seven hydrophobic transmembrane (TM) α-helices connected by three intra- and three extracellular loops. TMs 1,2 ,3 and 7 constitute the minor ligand binding pocket (left) and TMs 3, 4, 5, 6 and 7 form the major ligand binding pocket (right). Adapted from Scholten *et al.* [12]

3. Chemokine-receptor interactions

The complexity of chemokine–receptor interactions is twofold in that many chemokines can activate more than one receptor while many receptors bind multiple ligands. These interactions are often termed as promiscuous. (Figure 3). However, while some receptors like ACKR2 can have over 10 different chemokine ligands, other receptors like CXCR4 or CCR6 can only become activated by a single ligand, CXCL12 or CCL20 respectively. Of note, virus encoded chemokines can also block or activate several human chemokine receptors, one example being the Kaposi's sarcoma-associated herpesvirus (HHV-8) encoded vMIP-II/vCCL2, a CC chemokine that binds over 10 human chemokine receptors of all four classes[18].

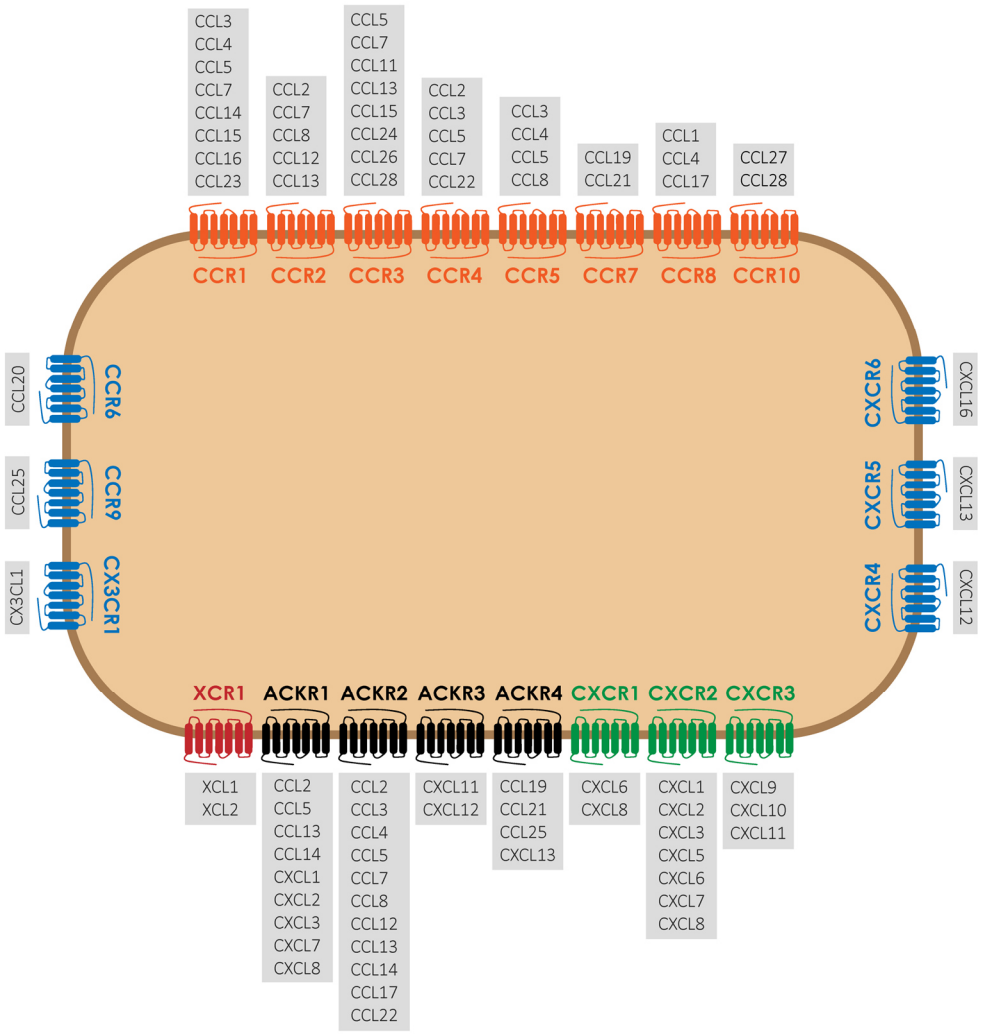

Figure 3. Overview of the chemokine receptor–ligand network. Overview of the different chemokines and chemokine receptors. Many chemokines can bind more than one receptor and most of the receptors have multiple ligands. This is the case for CC receptors (orange), CXC receptors (green), XC receptor (dark red) and atypical chemokine receptors (black). Some receptors from CC and CXC family and CX3CR1 have only one ligand (blue). Based on Lazannec & Richmond [19].

Biochemically, chemokines were previously described to interact with their receptors through a so-called two-site binding model [20-22]. In the first step, the globular core of the chemokine interacts with the flexible, unstructured N terminus of the receptor (also referred to as chemokine recognition site 1, CRS1). Binding to CRS1 is mainly dominated by ionic interactions between positively charged residues of the chemokine and negatively charged residues of the receptor and is enhanced by post-translational sulfatation of tyrosine residues in the receptor N terminus[10, 23, 24]. This interaction is believed to confer receptor–ligand specificity. Furthermore, it promotes correct chemokine orientation, subsequently allowing the second interaction step in which the chemokines' N terminus binds to the transmembrane segments of the receptor (CRS2), triggering receptor activation by inducing conformational changes and stabilization of an active conformation of the latter[25, 26]. This model is supported by several mutational studies, where chemokines with deletions or altered N-termini were no longer able to activate their cognate receptor, while retaining high binding affinity[27-29]. However, although it has been extremely useful for over 20 years, recently resolved structures of chemokine–receptor complexes showed that this model is somewhat oversimplified[17, 30-32].

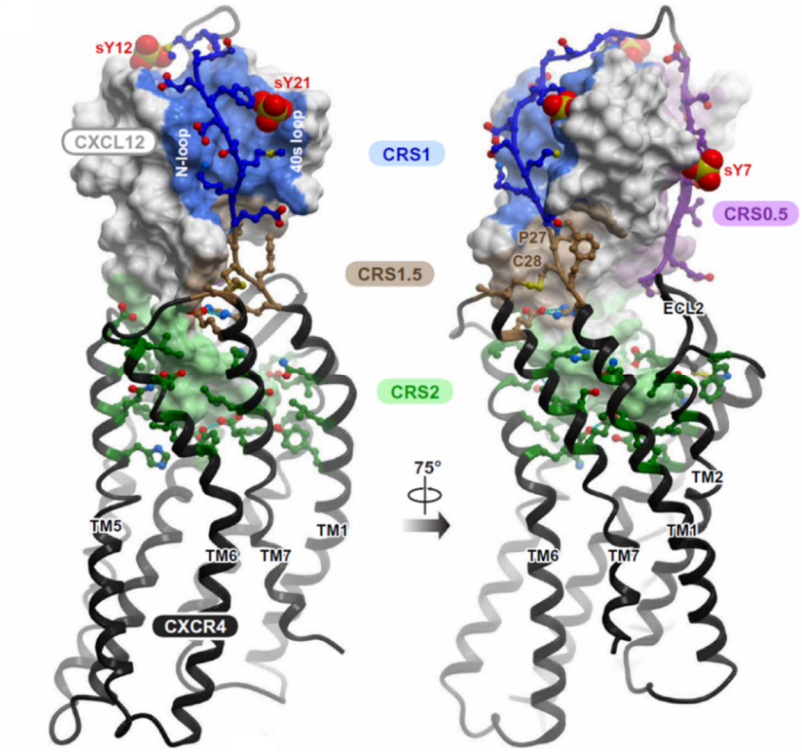

Figure 4. Computational model of the CXCR4-CXCL12 complex. CXCL12 is shown as surface mesh and CXCR4 as ribbon. The model is viewed along the plane of the plasma membrane. Chemokine recognition site (CRS) 1, depicted in blue represents the interaction of N-terminal residues 21–26 of the receptor with the groove of CXCL12, formed by the N-loop and 40s loop. In CRS2, depicted in green, the flexible N terminus of the chemokine reaches into the transmembrane (TM) domain of the receptor. CRS1 and CRS2 make up the classical two-site binding model. In recent years, this model was refined with CRS0.5 (in purple), where N-terminal residues 1-20 "wrap around" the chemokine and CRS1.5 (in brown) in which residues P27 and C28 of CXCR4 pack against the first disulphide bridge (C9-C50) of CXCL12. From *Stephens et al.*[33].

Firstly, interactions between a chemokine and its receptor's extracellular loops were barely taken into account in this model. Furthermore, crystal structures of CXCR4:vCCL2 and US28:CX3CL1 complexes highlighted that the interaction interface between chemokines and their receptors are virtually contiguous, and not limited to two separate sites as initially suggested[17, 30]. By analogy with the existing nomenclature, an intermediate interaction site, CRS1.5, located between CRS1 and CRS2 was proposed, as well as CRS0.5 for the binding of the chemokine to the distal N terminus of the receptor[33, 34] (Figure 4).

Lastly, although chemokines are often considered to interact in a 1:1 stoichiometry with their receptors, numerous studies reported dimerization of chemokine receptors and possible oligomerization of their chemokine ligands[32, 35, 36]. It was shown for example that while CXCR4 recognizes both monomeric and dimeric forms of CXCL12, a constitutively monomeric form of the ligand reproduced the well-characterized β-arrestin recruitment and G protein signaling events[37]. Furthermore, a recent cryo-electron microscopy structure of CXCR2 provided evidence that CXCL8 can bind and activate CXCR2 in either dimeric or monomeric form[38]. An important step for chemokine function is their interaction and immobilization on glycosaminoglycans (GAGs), which allows to locally increase chemokine concentration and to create gradients to direct cell migration[39]. GAG binding can also favor dimer formation for numerous chemokines and likewise, chemokine oligomerization is thought to increase GAG interactions by providing a more extensive surface for interactions[40]. Altogether, ligand and receptor oligomerization adds yet another layer of complexity to our view of chemokine–receptor interactions[41].

4. Chemokine receptor signaling

Chemokine receptors transduce extracellular stimuli, generally the interaction with chemokine ligands, into various intracellular effects, like remodeling of the cytoskeleton, altering gene expression or changing cellular metabolism. In their inactive state, chemokine receptors associate with stable and low-energy heterotrimeric guanidine diphosphate (GDP)-bound G proteins[42]. These trimeric G proteins consist of the subunits G_α, G_β and G_γ. Once activated, the receptors undergo conformational changes, allowing the G_α subunit to exchange GDP for guanidine triphosphate (GTP). This event is followed by dissociation of the trimer I nto GTP-bound G_α, and a stable $G_{\beta\gamma}$ dimer, each triggering downstream signaling events on their own. There are many different classes of Gα subunits[43], but chemokine receptors generally signal through pertussis toxin-sensitive $G_{\alpha i/o}$ subunits, which decrease intracellular cAMP levels by inhibiting the enzyme adenylate cyclase. However, numerous additional downstream signaling events have been described upon chemokine receptor activation, such as increase in intracellular Ca^{2+} levels or phosphorylation of ERK1/2, induced by various effector molecules such as phosphoinositide-3 kinase (PI3K), phospholipase C (PLC) or $G_{\beta\gamma}$, to just name a few[44, 45] (Figure 5).

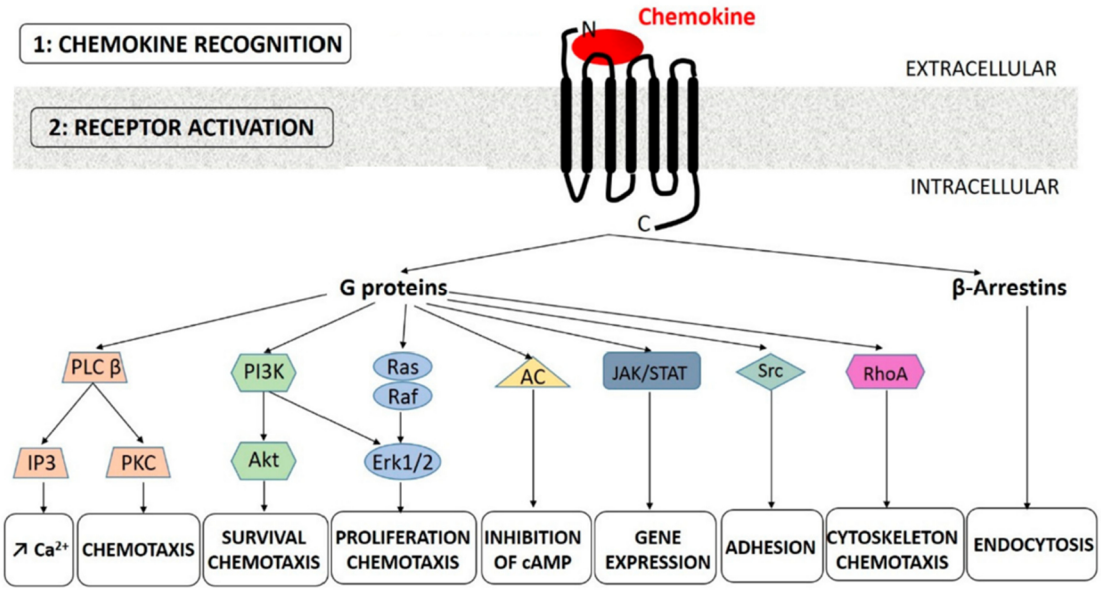

Figure 5: Overview of the different downstream signaling pathways initiated upon agonist-chemokine binding to a chemokine receptor. Once a receptor is activated by a chemokine, it undergoes conformational changes that in turn activate a particular G protein. This G protein then triggers a cascade of signaling events leading to changes in cellular function. In most cases, β-arrestins are recruited to activated chemokine receptors, inducing their endocytosis and possibly further signaling events. AC: Adenylate cyclase, IP3: Inositol trisphosphate, JAK/STAT: Janus kinase/signaling transducer and activator of transcription, Erk: Extracellular signal-regulated kinase, PI3K: Phosphoinositide 3-kinase, PKC, Protein kinase C, PLC: Phosphoinositide-specific phospholipase C. Adapted from Henrot *et al*.[46].

Following dissociation of G protein subunits from the receptor, G protein-coupled receptor kinases (GRKs, seven members) are recruited to the C-terminal tail of the receptor, resulting in the phosphorylation of multiple, now accessible residues, mostly threonine and serine[47, 48]. This leads to the recruitment of the adaptor proteins β-arrestin 1 (also called arrestin 2) and/or β-arrestin 2 (also called arrestin 3), which sterically inhibit new G protein interactions with the receptor's intracellular domains. Ultimately, this leads to internalization of the receptor via clathrin-coated pits where different fates await the receptors: they either dissociate from β-arrestin and their ligand, become dephosphorylated and recycle back to the cell surface, where they can again interact with ligands and trigger signaling events, or they are ubiquitinated and targeted to the lysosomes for degradation[49]. Whether β-arrestins themselves also trigger signaling pathways independently is still contentious after many years and will be discussed in more detail in section 6.4 ACKR3.

5. Biased signalling – functional selectivity

As mentioned above, the chemokine-receptor network is highly complex with many chemokines binding different receptors and vice versa. However, this does not make some chemokine–receptor interactions obsolete, but rather guarantees exquisite fine-tuning of complex biological mechanisms

through different expression and secretion patterns of receptors and ligands in time and space[50]. Chemokines have also different affinities for their individual receptors, as well as for GAGs, which, together with the spatio-temporal differences in expression patterns, define the intricacy of the network. Additionally, GPCRs do not simply undergo conformational changes from an "off" to "on" conformation once activated, but a variety of different active states are stabilized in a ligand-dependent manner[51]. These various active states of GPCRs are able to recruit different signaling partners (i.e. G_i G_s, G_q, $G_{12/13}$) and show a preference or disfavor towards β-arrestin recruitment. This notion for GPCRs in general also perfectly applies to chemokine receptors that were initially considered to signal mainly through $G_{i/o}$ proteins, but are nowadays linked to a plethora of different signaling pathways[52-55]. This concept is known as biased signaling or functional selectivity and seems to largely explain the apparent redundancy in the chemokine ligand-receptor network by greatly diversifying the signaling outcome of a given receptor or ligand. This paradigm of biased signaling is based on three notions: ligand bias, receptor bias and tissue bias[53, 56, 57] (Figure 6).

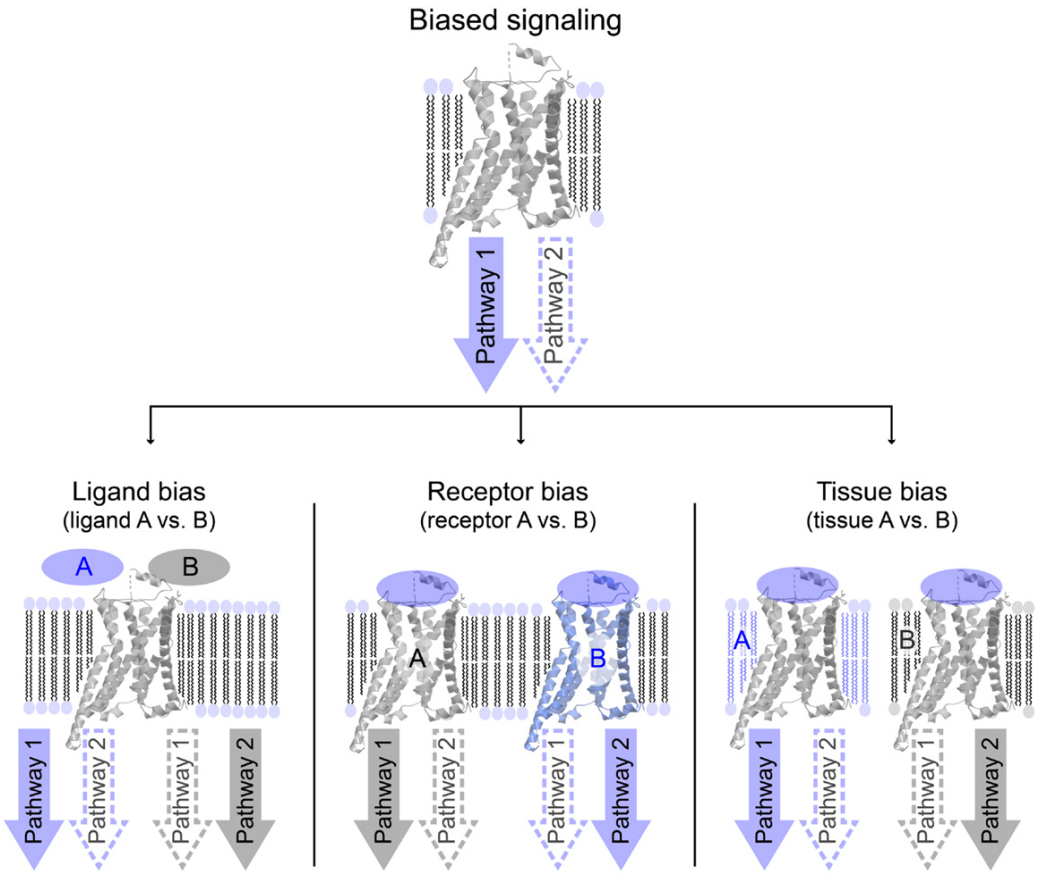

Figure 6. Overview of different forms of biased signaling. One signaling pathway can be preferred over another for different reasons: (Left) Ligand Bias: Two different ligands, binding the same receptor, can trigger different signaling events. (center) Receptor Bias: The same ligand, binding two different receptors, activates different pathways. (right) Tissue Bias: The same ligand-receptor interaction can have different outcomes, depending on the tissue or cell type that express the receptor, due to additional cofactors which are present (or absent) in this cell type. From *Steen et al*. [53].

Ligand bias, as mentioned above, mainly relies on the concept that different chemokines, binding the same receptor, could trigger different downstream effects, based on which active conformation of the receptor they stabilize. Of note, this can even hold true for the same chemokine if differently post-translationally modified, truncated or dimerized[58]. Examples for such bias are monomeric versus dimeric CXCL12[59], or the CCR7 ligands CCL19 and CCL21. While both bind to CCR7 and are able to activate G protein signaling, only CCL19 triggers efficient receptor internalization[60].

Receptor bias, on the other hand, entails the same chemokine ligand inducing different responses on different receptors. This is best described for the family of atypical chemokine receptors (described in more depth in the following section 6), which show different downstream effects when binding a chemokine ligand compared to the classical receptors. A prominent example is CXCL12, which activates CXCR4 and leads to classical G_i signaling[61], but can also bind to ACKR3 without triggering G_i signaling, while conserving the ability to recruit β-arrestin[62].

Lastly, since chemokine receptors are expressed on a tremendous variety of cell types, each expressing their own set of adaptor proteins, cofactors and signaling partners, *i.e.* G proteins, arrestin isoforms, GRKs, receptor activity modifying proteins (RAMPs) etc., it seems quite obvious that depending on the tissue, the same ligand–receptor pair can induce different cellular responses. This form of biased signaling is known as tissue bias or cell bias and can again be found for CXCR4, whose signaling properties can be largely influenced depending on whether ACKR3 is co-expressed on the same cell/tissue or not[63-66] .

The brief description of these three types of signaling bias offers a glimpse of how complex and fine-tuned the chemokine-receptor network is in regard of ligand and receptor expression and promiscuity. However, this paradigm also impressively illustrates the challenges of generating robust pharmacological data for a given ligand–receptor pair. For example, a receptor might induce different downstream signaling events in cell culture than in its natural environment/tissue, due to differentially expressed signaling partners. Similarly, it can be challenging to precisely attribute a functional effect to a specific ligand–receptor pair and to pinpoint a ligand-induced signaling event *in vitro* to one (and only one) specific receptor, since the cell line in which the experiments are performed might endogenously express other receptors, which either directly signal through the same ligand or which dimerize with the receptor, thereby altering its signaling properties. Of note, the concept of biased signaling is not only of interest in the field of chemokine biology. Some evidence suggests that analgesic effects of opioid receptor modulators may rely on G protein signaling, whereas adverse side effects are rather associated with β-arrestin 2 recruitment. This hypothesis recently led to the first FDA approved, biased mu-opioid receptor agonist[67].

6. Atypical chemokine receptors

Of note, not all chemokine receptors undergo classical G protein-related signaling upon chemokine ligand binding. The notion of chemokine-scavenging receptor was first introduced for the chemokine receptor D6 in 2003[68] and in the following years, atypical or complete absence of canonical G protein signaling was also described for the chemokine receptors DARC, CXCR7 and CCRL1 [69-71]. While initially called interceptors, decoys or scavengers, these receptors received the unifying designation of atypical chemokine receptors (ACKRs) in 2014, classifying them as one functionally related group [72, 73]. Noteworthy, these receptors do not cluster phylogenetically, nor do they share particular sets of ligands, their classification being solely based on their atypical signaling behavior and inability to induce classical G protein signaling upon stimulation. In agreement with these altered signaling properties, the conserved DRYLAIV motif within the second intracellular loop, known to play a pivotal role in G protein coupling, is altered or even lacking in ACKRs[74]. However, mutational studies, exchanging intracellular residues of CXCR4 with ACKR3 (including the DRYLAIV motif) could not restore G protein interaction with ACKR3, suggesting a more complex interplay between a G protein and multiple regions of the receptor[75]. Nevertheless, the absence of signaling does not make these receptors obsolete, as ACKRs modulate chemotactic responses, developmental processes and maintain and resolve immune responses. These functions are carried out by efficient internalization and transport of chemokines into degradative compartments, or, in case of polarized cells, to the opposite side of the cell, in order to clear abundant inflammatory responses, to form chemokine gradients or to store chemokines for other signaling receptors[72, 76-78]. Hence, in line with these functions, ACKRs are mainly expressed on epithelial cells of barrier organs, as well as lymphatic and vascular endothelial cells, while some are also expressed on erythrocytes (ACKR1) and leukocytes (ACKR2 and ACKR3). Of note, while ACKR1 and ACKR2 are mainly binding inflammatory chemokines, ACKR3 and ACKR4 are responsive to homeostatic chemokines.

So far, four receptors were officially reclassified as atypical chemokine receptors: DARC, D6, CXCR7 and CCRL1 as ACKR1, ACKR2, ACKR3 and ACKR4 respectively (Figure 7). Two further candidates, the chemerin receptor CCRL2 and the membrane-associated phosphatidylinositol transfer protein 3 PITPNM3, were proposed to be classified as ACKR5 and ACKR6, respectively, as no G protein-related signaling events could be detected for these proteins so far[79-81]. However, their renaming awaits functional confirmation by independent groups. Recently, the so far orphan receptor GPR182 was also suggested as new atypical chemokine receptor for CXCL10, CXCL12 and CXCL13[82]. The authors could show that GPR182 binds and internalizes these chemokines in a β-arrestin independent manner and without inducing downstream signaling events. As for ACKR5 and ACKR6, these observations remain to be confirmed by other research groups before an official reclassification of the receptor.

A concise summary on each atypical chemokine receptor with a focus on ACKR3 will follow in the next section. The interested reader can find a more extensive review on atypical chemokine receptors and their role in cancer in the appendices of this thesis[77], from which the following paragraphs are also adapted.

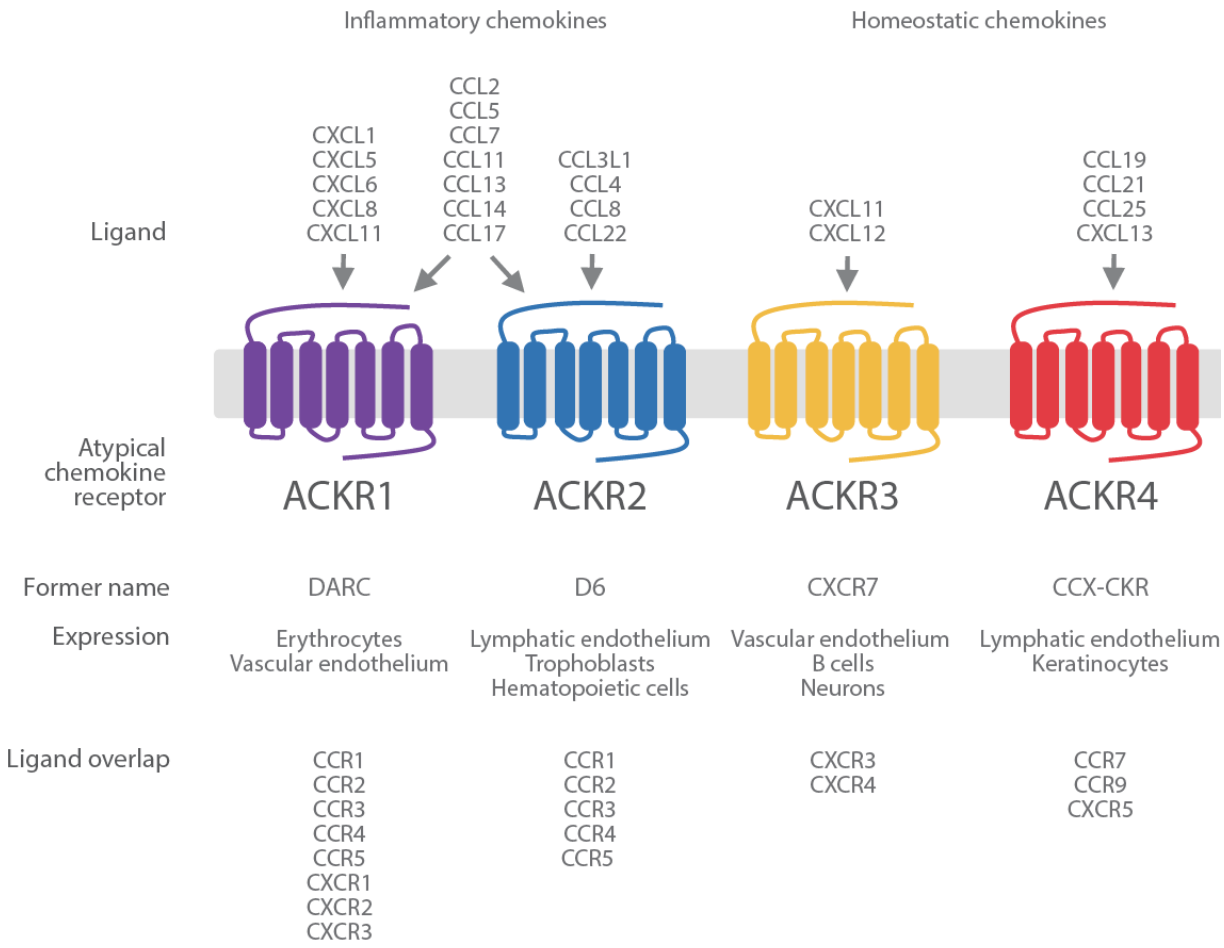

Figure 7 Overview of the atypical chemokine receptors, their ligands and their expression sites. All ACKRs are mainly expressed on epithelial cells of barrier organs. The former name before their reclassification to ACKR in 2014 is indicated. ACKR1 and ACKR2 bind a broad spectrum of inflammatory chemokines that are shared with classical chemokine receptors CCR1-CCR5 and CXCR1-CXCR3. ACKR3 and ACKR4 have a more restricted number of (mostly homeostatic) chemokine ligands, which are shared with CCR7, CCR9 and CXCR3-CXCR5. From *Sjoberg and Meyrath et al.* [77].

6.1 ACKR1

ACKR1 (formerly Duffy Antigen Receptor for Chemokines, DARC) was initially described as a blood group antigen, later as a receptor for the Duffy Binding Proteins (DBP) from *Plasmodium knowlesi* and *Plasmodium vivax* malaria parasites and finally as a promiscuous chemokine receptor [83-85]. Although ACKR1 was the first described receptor for chemokines, it is barely recognizable as one from its primary

amino acid sequence and its phylogenetic association[86, 87]. The receptor is prominently expressed on erythrocytes and venular endothelial cells [88-90]. ACKR1 binds over 10, primarily inflammatory chemokine ligands from CC and CXC chemokine families[88, 91, 92]. Unlike other atypical chemokine receptors, ACKR1 does not scavenge its ligands, but rather internalizes them in polarized cells, mediating transcytosis of intact chemokines (Figure 8A). By doing so, ACKR1 can function as a "presenting" receptor, binding chemokines and increasing their bioavailability for other chemokine receptors in a spatiotemporally well-defined manner[93]. Furthermore, erythrocytic ACKR1 can reduce the bioavailability of its ligands and prevent excessive systemic leukocyte activation but can also release bound ligands in order to avoid fluctuations in circulating chemokine concentrations, acting thus as a "sink" or as a "buffer" [94, 95] (Figure 8B). Lastly, ACKR1 can also influence the signaling properties of other chemokine receptors. Interaction with CCR5 for example can impair CC chemokine-induced responses, like intracellular calcium release and chemotaxis[96].

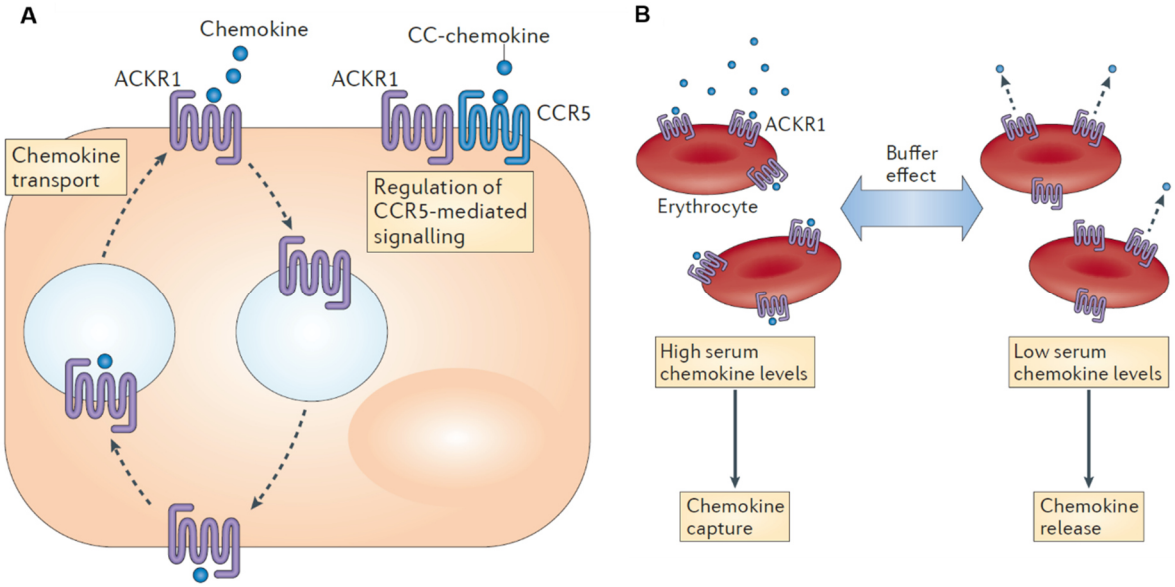

Figure 8 Cellular functions of ACKR1. (A) In polarized cells, ACKR1 transports intact chemokines across the cell and presents them to leukocytes. It can also modulate the signaling properties of co-expressed receptors like CCR5. (B) In erythrocytes, ACKR1 can act as a chemokine buffer, capturing chemokines upon high serum chemokine levels (left panel) and releasing them once the chemokine abundance in the serum drops (right panel). From *Nibbs et al.*[76].

6.2 ACKR2

ACKR2 (formerly D6 or CCBP2), was the first atypical chemokine receptor described as a "scavenger"[68]. It has been long reported to bind inflammatory chemokines exclusively from the CC family (see also chapter 6). ACKR2's main ligands include CCL2-8, CCL11-13, CCL17 and CCL22, which are agonists of the classical receptors CCR1-5[68, 97-99]. ACKR2 constitutively cycles between the cell surface and endosomal compartments. By doing so, it progressively depletes its inflammatory chemokine ligands

by internalizing them and targeting them for lysosomal degradation[100, 101] and hence plays a major role in the regulation and resolution of inflammatory responses (Figure 9).

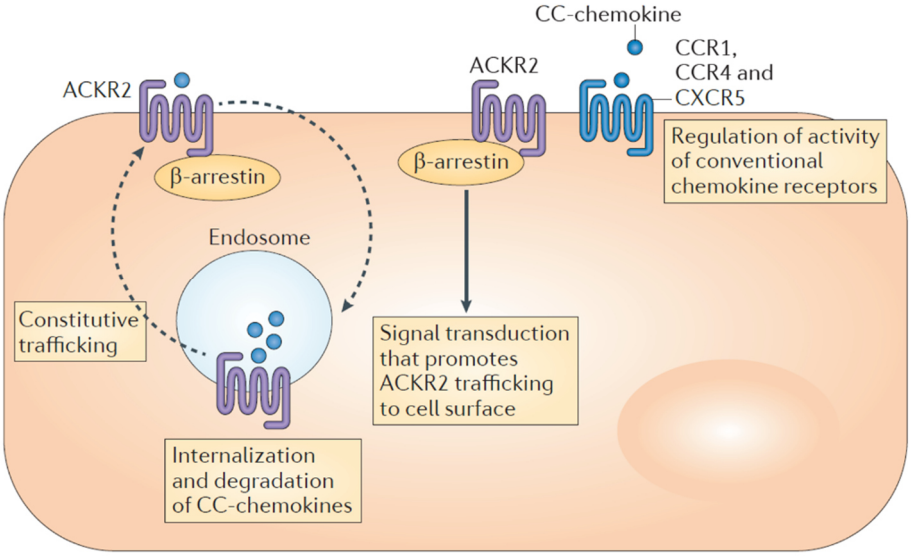

Figure 9 Cellular functions of ACKR2. By constitutively cycling between the cell surface and endosomal compartments, ACKR2 can capture and internalize chemokines, eventually leading to their lysosomal degradation. Interactions with β-arrestin can influence this trafficking behavior. ACKR2 furthermore regulates the activity of co-expressed chemokine receptors like CCR1, CCR4 and CXCR5. From *Nibbs et al.*[76].

ACKR2 is expressed on lymphatic endothelial cells, epithelial cells, trophoblasts in placenta and some subsets of leukocytes, including alveolar macrophages and innate-like B cells[102-104]. Although the majority of studies could convincingly identify ACKR2 as a regulatory, scavenging receptor[68, 101, 105], other studies report signaling events such as Ca^{2+} mobilization or ERK phosphorylation in ACKR2 expressing cells[97, 106]. Moreover, in response to ligand exposure, ACKR2 surface expression is upregulated through β-arrestin-dependent activation of the cofilin signaling pathway[107]. Similarly, by activating a β-arrestin1-dependent signaling pathway, ACKR2 triggers both the actin and the microtubule cytoskeletal networks, which control its trafficking and scavenger properties[108]. In a collaborative effort, we could also recently show that ACKR2 mediates signaling effects of CXCL14, promoting tumor growth and metastasis, but a direct interaction of CXCL14 with ACKR2 could not be demonstrated[106]. Although these different studies do not show that ACKR2 is directly generating these signaling events, it seems that ACKR2 can at least influence cellular signaling, for example by regulating the expression levels or activity of other signaling receptors [103, 105].

6.3 ACKR4

ACKR4 is the homeostatic complement to the inflammatory chemokine scavenger ACKR2 and was initially proposed to bind the chemokines CCL19, CCL21, CCL25 and CXCL13, which are the ligands for CCR7, CCR9 and CXCR5, respectively[109, 110]. Of note, CXCL13 interaction with the mouse ACKR4 could

not be confirmed[69]. The human CXCL13–ACKR4 pairing inferred from binding competition studies[109] was later re-evaluated and the observations were reattributed to co-operative GAG binding rather than direct receptor interactions[111]. The receptor is expressed on keratinocytes, astrocytes, lymphatic endothelial cells and on thymic epithelial cells[69, 112, 113]. Like other atypical chemokine receptors, ACKR4 does not seem to induce any classical G protein-mediated signaling, but was shown to efficiently degrade homeostatic chemokines[69, 110, 113, 114] (Figure 10). By doing so, ACKR4 regulates mainly the trafficking and positioning of T-cells and dendritic cells (DCs)[113, 115].

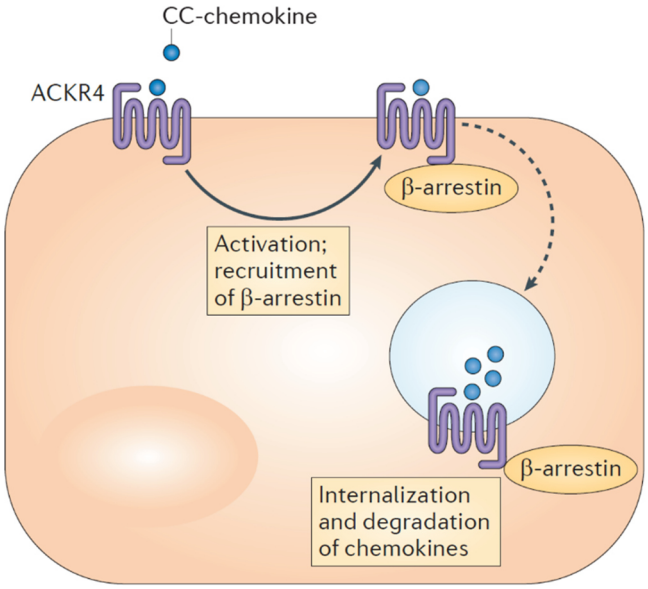

Figure 10 Cellular functions of ACKR4. After activation of ACKR4 upon chemokine binding, β-arrestin is recruited to the receptor, leading to internalization of the chemokine–receptor complex and ultimately to lysosomal degradation of the chemokine. From *Nibbs et al.*[76].

ACKR4 is best known for its role in shaping the gradient of CCL19 and CCL21 for CCR7-expressing DCs in the subcapsular sinuses of lymph nodes during the initiation phase of the adaptive immune response [114, 116]. ACKR4 is also involved in anti-tumour immunity and modulates epithelial-mesenchymal transition and metastasis[77, 117, 118]. Recently, Matti *et al.* described that ACKR4 additionally acts as a scavenger for CCL20, thus regulating the availability of this chemokine for its signalling receptor CCR6 and thereby regulating the positioning of CCR6-positive leukocytes within secondary lymphoid tissues to initiate effective humoral and memory immune responses[119, 120] (see chapter 5). To our knowledge, no study could so far identify any ACKR4-mediated activation of signal transduction.

6.4 ACKR3

ACKR3 was initially isolated from a dog thyroid cDNA library and named receptor dog cDNA, RDC1[121, 122]. Due to its chromosomal proximity and high degree of similarity with CXCR2 and CXCR4, it was then assumed that RDC1 was an orphan CXC chemokine receptor[123]. It took over seven years for CXCL12 and CXCL11 to be identified as chemokine ligands of this receptor, which was consequently renamed

CXCR7[70, 124]. In 2014, due to contradictory reports on CXCR7 signaling, its altered DRYLAIV motif and apparent absence of G protein interaction, CXCR7 was officially integrated in the family of atypical chemokine receptors and renamed ACKR3[72, 73].

ACKR3 is expressed by endothelial cells, mesenchymal cells, cardiomyocytes, in diverse regions of the central nervous system and in the adrenal glands[125-130]. Furthermore, ACKR3 seems to be expressed on B-cells, but its expression profile on other leukocytes is controversial[124, 131-134].

ACKR3 shares its two chemokine ligands CXCL12 and CXCL11 with the receptors CXCR4 and CXCR3, respectively. However, while ACKR3 shows about a 10-fold higher affinity towards CXCL12 than CXCR4, its affinity towards CXCL11 is comparable to that of CXCR3. Besides these endogenous chemokines ligands, more and more reports suggest additional ligands for ACKR3. The Kaposi's sarcoma-associated herpesvirus (HHV-8)-encoded CC-chemokine vMIP-II/vCCL2 was shown to interact with and induce β-arrestin recruitment to ACKR3[18, 135]. The pseudo-chemokine MIF (macrophage migration inhibitory factor), a pro-inflammatory chemotactic cytokine was also proposed to activate ACKR3[136]. The proangiogenic peptide hormone adrenomedullin (ADM) was already described to interact with ACKR3 back in 1995, but could not be confirmed as a ligand in later studies[137, 138]. However, two recent reports by the same research group indicated again that ACKR3 regulates the abundance of ADM by acting as its scavenger[139, 140] (see also chapter 4). A recent report further suggested that the secreted glycoprotein Dkk3 (dickkopf-3), highly expressed on endothelial cells and playing important roles in cardiovascular biology can interact with ACKR3. However, these findings have to be independently confirmed by other research groups and preliminary experiments in our group could not detect any Dkk3 induced β-arrestin recruitment to ACKR3. Last, ACKR3 was shown to interact with proenkephalin derived opioid peptides like BAM22[141] (see chapter 3).

Consistent with its plethora of different ligands, the biological roles of ACKR3 are manifold, although the vast majority of studies focus solely on the role of ACKR3 in CXCL12/CXCR4 biology. ACKR3 knockout mice die perinatally due to semilunar heart valve malformation and ventricular septal defects and show disrupted lymphangiogenesis and cardiomyocyte hyperplasia, while their hematopoiesis remains normal[134, 142]. ACKR3 is primarily found intracellularly and is continuously cycling from endosomal compartments to the cell surface, a typical characteristic of a scavenging receptor[143-145]. Indeed, studies done in zebrafish embryos convincingly show the role of ACKR3 scavenging function in CXCL12 gradient shaping during development [146, 147]. In addition, ACKR3 was shown to play crucial roles in neuronal development and in the migration and homing of hematopoietic stem and progenitor cells[148-150]. Regulation of neuronal migration by ACKR3 seems to be largely independent of β-arrestin but does however require phosphorylation of the receptor[151]. Furthermore, a recent report showed that ACKR3 is required for development of a functional marginal zone and for positioning of marginal

zone B cells in mice[152]. Another recent study by our group further suggests that ACKR3 regulates the abundance of endogenous opioid peptides and might thus be involved in modulating various processes like pain, mood or anxiety[153] (see chapter 3). This concept is supported by the observation that the ACKR3 specific small-molecule compound CCX771 induces anxiolytic-like effects in mice, synergistically with adrenocorticotropic hormone (ACTH)[141]. Last, there are also uncountable reports demonstrating that ACKR3 promotes proliferation, migration and invasion of different types of cancer cells, resulting in enhanced tumor growth and metastasis[154-159].

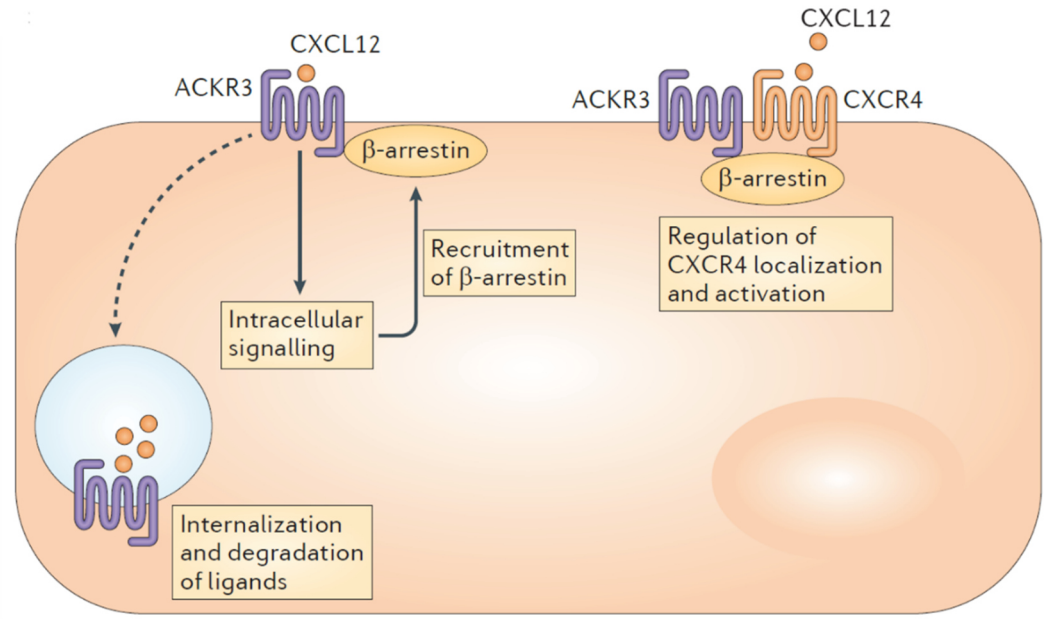

Figure 11 Cellular functions of ACKR3. ACKR3 constitutively interacts with β-arrestin and the interaction is reinforced upon agonist binding. This leads to internalization and subsequent degradation of the ligand. Depending on the cellular context, this interaction may also lead to intracellular signaling events. ACKR3 furthermore plays an important role for co-expressed CXCR4, regulating its expression and activity. From *Nibbs et al.*[76]

It is still unclear how ACKR3 conducts all these different biological roles. Despite its well-established function as a scavenger receptor, ACKR3's signaling potential remains debated and might be cell type-dependent (see also section 5, tissue bias), and may rely on less regarded signaling pathways that might have been overlooked or be indirect by the modulation of other receptors (Figure 11). While numerous studies claim that ACKR3 acts solely as a scavenging receptor[70, 145, 153, 156, 160], others could detect G protein-independent, β-arrestin-dependent signaling, such as ERK or AKT phosphorylation [62, 161-163], and a few studies reported direct G protein-mediated signaling in astrocytes[164, 165]. Noteworthy, a recent mouse study suggested that ACKR3 regulation of neuronal migration is largely independent of β-arrestins, speaking against the notion that ACKR3 mediates direct β-arrestin signaling, at least in this cellular context[151]. The concept of G protein-independent, direct β-arrestin signaling has both been challenged[151, 166-168] and advocated[169, 170] by different groups in the recent years. In any case, as

previously mentioned, there is in the meantime an exhaustive body of literature describing active signaling of ACKR3, mainly thought to depend on arrestin[171]. However, it is also important to note that ACKR3 can interact with various other membrane receptors and alter their subcellular distribution and signaling properties, the most prominent example being the chemokine receptor CXCR4[66, 132, 149], with which ACKR3 shares the ligand CXCL12. It is likely that some signaling properties attributed to ACKR3 itself may rather be associated with a modified expression or signaling profile of CXCR4 by ACKR3. For instance, animal studies showed that both ACKR3 and CXCR4 are required for proper interneuron migration and neuronal development[148, 149]. Overall, the signaling properties of ACKR3 seem to be versatile, cell type-dependent and relying on additional interaction partners like CXCR4[172].

7. Aims of the project and thesis outline

The main scope of this PhD thesis was to carry out an in-depth, multilevel molecular analysis of the atypical chemokine receptor 3 (ACKR3) with its different interaction partners:

First, a ligand-centered approach was applied to unravel crucial determinants of ACKR3 ligands involved in receptor binding and activation. These determinants were then compared with their binding and activation profile towards CXCR4 and CXCR3, two classical receptors with which ACKR3 shares its ligands CXCL12 and CXCL11, respectively (**chapter 1**).

Following this, a receptor-centered approach gave insights into the receptor's structural determinants needed for proper surface expression, as well as for ligand binding and receptor activation. In line with the previous ligand-centered approach, the results were compared between ACKR3 and the ligand-sharing chemokine receptors, with a strong focus on CXCR4 (**chapter 2**).

These two approaches unraveled that the binding and activation mechanisms of chemokines are somewhat different on ACKR3 compared to the classical chemokine receptors and that the receptor has a strong propensity for activation.

A large part of the PhD thesis aimed at characterizing existing and new unconventional, non-chemokine ligands of ACKR3, hence going beyond the concept of ACKR3 as a pure chemokine receptor (**chapter 2, 3 and 4**). This was first done by enlarging the panel of endogenous opioid peptides as ACKR3 ligands, shifting the paradigm of ACKR3 as a regulator of a narrow panel of chemokines to a broad-spectrum scavenger of opioid peptides from at least three different families (**chapter 3**). The study could show that ACKR3 is not modulated by prescription opioids like fentanyl or methadone, but that its scavenging capacity towards endogenous opioid peptides could regulate the availability of these peptides towards classical signaling opioid receptors. Additionally, this study led to the filing of a patent for a new class of small peptide ACKR3 modulators, with the most potent baptized LIH383, which was

shown to restrain ACKR3's negative regulatory function on opioid peptides in rat brain, opening new therapeutic avenues for opioid-related disorders.

Besides opioid peptides, this PhD project uncovered yet another new family of non-chemokine ACKR3 ligands, namely PAMP (Proadrenomedullin N-terminal 20 peptide) and its endogenously processed form, PAMP-12 (**chapter 4**). Similarly to opioids, we could show that ACKR3 has comparable affinity towards PAMP-12 as its classical receptor MrgX2. Furthermore, ACKR3 efficiently internalizes PAMP-12 without inducing detectable downstream G protein or ERK signaling, reminiscent of its scavenging activity observed for chemokines and opioid peptides.

Ultimately, a goal of this PhD thesis was to enlarge the network of atypical chemokine receptor–ligand interactions beyond ACKR3 to two other members of the atypical chemokine receptor family, namely ACKR2 and ACKR4. This led to the discovery of CCL20 and CCL22 as novel ACKR4 chemokine agonist ligands (**chapter 5**), as well as CXCL10 as the first CXC chemokine agonist ligand of the so far CC-exclusive receptor ACKR2 (**chapter 6**). This systematic reassessment of ACKR–chemokine pairings enlarged the panel of ligands scavenged by ACKRs and should encourage the community to extend such reassessments to all chemokine receptors, since important interactions may have been overlooked in the early days of chemokine research.

In the framework of several collaborations, the role of the orphan chemokine CXCL14 in the chemokine receptor network also became of interest during my thesis. Since this was not the focus of this PhD project, the two research articles originating from these collaborations will not be discussed in detail here. The interested reader will however find two paragraphs in the discussion (section 2) about this still enigmatic chemokine and the related publications in the appendices.

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Results

Chapter 1: Unravelling important ACKR3 binding determinants: a ligand-based approach

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The first chapter of this thesis intended to shed light on the question, whether the atypical chemokine receptor ACKR3 has a similar chemokine binding and activation mode compared to classical receptors CXCR4 and CXCR3, with which ACKR3 shares its chemokines. For this, full length chemokines CXCL12, CXCL11 and vCCL2 as well as synthetic peptides derived from the N terminus of these chemokines were analyzed for their capacity to bind and trigger signaling through G proteins and/or recruitment of β-arrestin-2 to the receptors CXCR4, CXCR3 and ACKR3. The peptides, consisting of different lengths of chemokine N terminus and harboring amino acids substitutions, D-stereoisomers or truncations were designed to focus on important features of the chemokine N-terminal portion, like the cysteine motif, the N-loop and the flexible N terminus, which are known to be key determinants of chemokine activity and selectivity.

Abstract

Background and purpose

Chemokines and their receptors form an intricate interaction and signaling network that plays critical roles in various physiological and pathological cellular processes. The high promiscuity and apparent redundancy of this network makes probing individual chemokine/receptor interactions and functional effects, as well as targeting individual receptor axes for therapeutic applications, challenging. Despite poor sequence identity, the N-terminal regions of chemokines, which play a key role in their activity and selectivity, harbor several conserved features. Thus far, little is known regarding the molecular basis of their interactions with conventional vs. atypical chemokine receptors or the conservation of their contributions across chemokine-receptor pairs.

Experimental Approach

In this study, using a broad panel of chemokine variants and modified peptides derived from the N-terminal region of chemokines CXCL12, CXCL11, and vCCL2, we compare the role of various features in binding and activation of their shared receptors, the two canonical G protein-signaling receptors, CXCR4 and CXCR3, as well as the atypical scavenger receptor CXCR7/ACKR3, which shows exclusively arrestin-dependent activity.

Key Results

We provide exhaustive molecular insights into the plasticity of the ligand-binding pockets of these receptors, their chemokine binding modes, and their activation mechanisms. We show that, although the chemokine N-terminal region is a critical determinant, neither the most proximal residues nor the N-loop are essential for ACKR3 binding and activation, as opposed to CXCR4 and CXCR3.

Conclusion and Implications

These results suggest a different interaction mechanism between this atypical receptor and its ligands and illustrates its strong propensity to activation.

1. Introduction

Chemokines are a superfamily of small (7-12 kDa), secreted, chemo-attractant cytokines, that regulate vital cellular mechanisms including migration, adhesion, as well as growth and survival[1]. They play critical roles in many physiological and pathological processes including immune responses and surveillance, development, atherosclerosis, HIV infection, and cancer[2, 3]. Despite their low sequence similarity, all chemokines display a common fold consisting of a flexible N terminus followed by a conserved cysteine motif, an N-loop, three anti-parallel β-strands and a C-terminal α-helix[4, 5] (Fig. 1D). The biological effects of chemokines are mediated through specific interactions with chemokine receptors, which belong to the superfamily of seven transmembrane G protein-coupled receptors (GPCRs). To date, 47 chemokines and 19 chemokine receptors have been identified in humans[6, 7]. The chemokine-receptor network is highly intricate and a given chemokine may bind to several receptors while a single chemokine receptor usually has multiple ligands. Based on the conserved cysteine motifs present in their N termini, chemokines are divided into four subfamilies (XC, CC, CXC, CX3C) and the receptors are named according to the subfamily of chemokines they bind (XCR, CCR, CXCR and CX3CR)[6]. In addition to canonical receptors, four receptors referred to as atypical chemokine receptors (ACKR1-4) can act as scavengers, regulating chemokine availability, or signal through alternative G protein-independent pathways, further contributing to the complexity of the chemokine network[7, 8].

Because of its implication in HIV infection and in many cancers, CXCR4 is one of the most-studied chemokine receptors and is often considered the model for CXC chemokine receptors as a whole[9-13]. It binds a unique endogenous agonist chemokine, CXCL12, as well as the human herpesvirus 8 (HHV-8)-encoded broad-spectrum antagonist chemokine, vCCL2 (vMIP-II) [13-16]. CXCL12 but also vCCL2 are both agonists of ACKR3, formerly designated as CXCR7, one of the most recently deorphanized chemokine receptors[16-18]. In addition, ACKR3 shares one ligand, the chemokine CXCL11, with CXCR3, to which CXCL10 and CXCL9 also bind, albeit with lower affinities than CXCL11[18-20] (Figure. 1). Unlike CXCR4 and CXCR3, which signal via both the canonical G protein pathways, that modulate cAMP production and induce intracellular calcium release, and the arrestin pathways, ACKR3 has been proposed to exclusively trigger arrestin-dependent signaling[13, 21-23]. ACKR3 has also been shown to act as a scavenger receptor for CXCL12, CXCL11 and vCCL2 thus regulating their availability for other chemokine receptors[16, 24-27]. Thus far however, the molecular basis accounting for its atypical functions and signaling remains unclear.

Based in part on the large amount of data regarding CXCL12 and CXCR4, the interaction between chemokines and their receptors is generally described as a multi-step process with extensive contacts between the two partners and 1:1 stoichiometry [12, 28-31]. During the initial step of the interaction, the N terminus of the receptor (chemokine recognition site 1, CRS1) binds the core of the chemokine

including the N-loop region, allowing for optimal orientation with respect to the top of the ligand-binding pocket (CRS1.5) [28]. This enables the insertion of the flexible chemokine N terminus into the receptor transmembrane cavity (CRS2), stabilizing an active state of the receptor, that in turn triggers intracellular signaling [9, 12, 28, 32, 33]. Although chemokine receptors can function as monomeric signaling units, direct evidence suggests that they are able to form both homo- and heterodimers in a ligand-independent manner [10, 34, 35]. Such oligomeric states were also observed for chemokines, possibly adding another level of fine-tuning to the already highly intricate interplay between chemokines and receptors [36-38].

The N-terminal portion of chemokines is a key determinant of their activity and selectivity that harbors several features involved in chemokine-receptor interactions, including the flexible N terminus, the cysteine motif, and the N-loop [4, 5, 32]. In addition, approximately one third of CC and CXC chemokines possess a proline in their proximal N terminus, usually at position 2, proposed to play an essential role not only in receptor activation, but also in the regulation of chemokine availability through their degradation by extracellular proteases [31, 39]. Peptides derived from the N-terminal region of CXCL12 and vCCL2 have been shown to be sufficient to specifically bind to CXCR4, while also conserving the agonist or antagonist activity of the parental chemokine [32, 40-42]. Notably, the introduction of further modifications to these peptides, such as mutations, truncations, dimerization, or D-amino acid replacement, emerged as a means by which to assess the importance of specific residues for receptor binding and activation, the propensity of the receptor to dimerize, or the plasticity of CRS2 [43-45]. Moreover, N-terminal deletions or the P2G mutation convert peptides derived from the N terminus of CXCL12 to CXCR4 antagonists, illustrating their therapeutic potential [32, 33, 42, 46]. However, with the exception of CXCR4, little is known regarding the importance of these proximal features of chemokines in receptor recognition and activation, or the conservation of their contributions across chemokine-receptor pairs. Therefore, CXCR4, ACKR3, and CXCR3 in conjunction with their ligands CXCL12, CXCL11, CXCL10, CXCL9, and vCCL2, of which some are shared or display opposite activities, offer the opportunity to investigate these questions.

In this study, using peptides derived from the N-terminal regions of chemokines and modified full-length chemokines, we compared the role of various N-terminal features in their interactions with canonical receptors, CXCR3 and CXCR4, and the atypical receptor ACKR3. Our data provide insights into the plasticity of receptor ligand-binding pockets and their activation mechanisms. Unlike CXCR4, CXCR3, and other classical chemokine receptors, ACKR3 was relatively insensitive to chemokine N-terminal modifications maintaining a high propensity for activation. These results show that ligand recognition and activation of ACKR3 differs significantly from that of the classical chemokine receptors

CXCR4 and CXCR3, suggesting that the atypical chemokine receptors may employ an interaction mechanism that departs from the well-established two-step, two-site model.

2. Methods

Cells and antibodies

HEK293E cells were obtained from Invitrogen and U87 cells from Dr. Deng and Dr. Littman through the NIH AIDS program[47]. U87.CXCR3, U87.CXCR4 and U87.ACKR3 cell lines were established by Lipofectamine transfection (Life Technologies) of U87 cells with pBABE-puro (Addgene) or pcDNA3.1-hygro (Invitrogen) vectors encoding the different receptors, subsequent puromycin (1 or 0.5 µg/mL) or hygromycin selection (250 µg/mL) and single-cell sorting. For each cell line, receptor surface expression was verified by flow cytometry using mAbs specific for ACKR3 (clones 11G8 (R&D Systems) and 8F11 (BioLegend)), CXCR4 (clones 4G10 (Santa Cruz Biotechnology) and 12G5 (BD Biosciences)) and CXCR3 (clone 1C6 (BD Biosciences)).

Peptides and chemokines

All peptides were purchased from JPT and contain a free amine at the N terminus and an amide group at the C terminus to avoid additional negative charge. Chemokines CXCL12, CXCL11, vCCL2 (vMIP-II), CXCL10 and CXCL9 were purchased from PeproTech. Alexa Fluor 647-labeled CXCL12 (CXCL12-AF647) and CXCL11 (CXCL11-AF647) were purchased from Almac and radiolabeled CXCL12 (^{125}I-CXCL12) and CXCL11 (^{125}I-CXCL11) from PerkinElmer. Peptide and chemokine cytotoxicity was monitored using an amino-reactive cell viability dye (Life Technologies) and an ATP quantification-based cell viability assay (Promega).

Cloning and purification of recombinant modified CXCL11 chemokines

The N-loop-swapped CXCL11 chimeras, N-terminally truncated, and P2G-mutated CXCL11 chemokines were cloned into previously described pQE30 vectors that incorporate an N-terminal His_6 and *Saccharomyces cerevisiae* SUMO protein (Smt3) fusion tag for use in purification[48, 49].

Recombinant wild type ($CXCL11_{1-73}$) and modified CXCL11 ($CXCL11_{Nloop12}$, $CXCL11_{Nloop10}$, $CXCL11_{3-73}$, $CXCL11_{5-73}$ and $CXCL11_{7-73}$) were purified as N-terminal His_6SUMO fusion proteins in *E. coli* as previously described[7, 12, 50]. Cells were grown in Terrific Broth and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) before being harvested and stored at -80 ˚C. Cell pellets were lysed, and lysates clarified by centrifugation (12,000 x g for 20 minutes). The supernatant and solubilized inclusion body pellets were loaded onto Ni-NTA resin and after 1 hour proteins were eluted with 6 M guanidinium chloride, 50 mM Na_2PO_4 (pH 7.4), 300 mM NaCl, 500 mM imidazole, 0.2% sodium azide, and 0.1% β-mercaptoethanol. The eluate was pooled and refolded via dilution overnight before

cleavage of the His_6SUMO fusion tag by Ulp1 protease for 4 hours. The His_6SUMO fusion tag and chemokine were separated using cation-exchange chromatography (SP Sepharose Fast Flow resin GE Healthcare UK Ltd.) and the eluate subjected to reverse-phase high-performance liquid chromatography as a final purification. Proteins were frozen, lyophilized and stored at -20 ˚C. Purification, folding, and homogeneity of recombinant proteins were verified by SDS-PAGE, MALDI-TOF spectroscopy and ^{1}H-^{15}N HSQC NMR spectroscopy.

Binding competition assays with labeled CXCL12 and CXCL11

Binding of full-length chemokines and peptides derived from chemokine N termini to CXCR4 and ACKR3 expressed at the surface of U87 cells was evaluated by competition with Alexa Fluor 647 (AF647)-labeled CXCL12. For ACKR3 binding, U87.ACKR3 cells were incubated with CXCL12-AF647 (40 ng/mL) and chemokines or peptides for 90 min at 4°C. CXCR4 binding was evaluated by incubation of U87.CXCR4 cells with CXCL12-AF647 (100 ng/mL) and chemokines or peptides for 45 minutes at 37°C. All binding experiments were performed in phosphate buffered saline containing 1% BSA and 0.1% NaN_3 (FACS buffer). Nonspecific chemokine binding was evaluated by the addition of 250-fold excess unlabeled CXCL12 or CXCL11. Simultaneous staining with Zombie NIR™ Fixable Viability dye (Biolegend) allowed to determine peptide cytotoxicity. Chemokine binding was quantified as the mean fluorescence intensity on a BD FACS Canto or Fortessa cytometer (BD Biosciences).

Binding of wild-type and modified recombinant CXCL11 chemokines to CXCR3 and ACKR3 was assessed in competition studies using radiolabeled CXCL11 (Perkin Elmer). Membranes from CXCR3- or ACKR3-expressing HEK293 cells were prepared as previously described [51, 52]. Binding was performed using 5 µg of membrane protein per point with either 50 pM ^{125}I-CXCL11 or 50 pM ^{125}I-CXCL12 (PerkinElmer). Samples were equilibrated for 2 hours at 4ºC prior to the separation of unbound radioligand from bound radioligand using filtration and counting.

cAMP modulation assay

U87 cells stably transfected with cAMP GloSensor 22F vector (Promega) (U87.Glo) were selected using hygromycin resistance (10 µg/mL) and the forskolin-induced luminescence response was assessed. U87.Glo cells were then stably transfected with pBABE or pIRES vectors encoding CXCR3, CXCR4, or ACKR3, and selected using puromycin (0.5 or 1 µg/mL) or hygromycin (250 µg/mL) respectively. Single clones were isolated by cell sorting, using the corresponding monoclonal antibodies, and further validated by flow cytometry. For cAMP modulation measurements, cells were incubated for 90 minutes in the dark at 37°C in phenol red-free DMEM containing IBMX (500 µM) and 2% luciferin (GloSensor reagent, Promega). 15×10^4 cells per well were distributed onto white 96-well LumitracTM plates (Greiner) already containing chemokines or peptides at different concentrations in phenol red-

free DMEM containing IBMX (500 µM) and 2% luciferin. Luminescence was recorded at different time points under forskolin-free conditions [53] using a POLARstar Omega.

cAMP modulation induced by CXCL11 variants was assessed as previously described [54]. HEK293E cells transiently expressing the BRET reporter fusion construct protein GFP_{10}-Epac-Rluc3 and either CXCR3 or ACKR3 were seeded onto poly-D-lysine-coated 96-well plates. At 48 hours post-transfection culture medium was replaced with PBS supplemented with 0.1% BSA, 20 µM forskolin (Sigma-Aldrich), and cells stimulated with chemokines. cAMP modulation was measured after 10 minutes by the addition of Coelenterazine 400A at a final concentration of 5 µM (NanoLight Technology). Fluorescence and luminescence readings were collected using a Mithras LB940 plate reader (Berthold Technologies).

Ligand-induced calcium mobilization

Intracellular calcium mobilization, induced by chemokines or chemokine-derived peptides, was evaluated using a calcium-responsive fluorescent probe and a FLIPR Tetra device. U87.CXCR3, U87.CXCR4 and U87.ACKR3 cells were seeded in black-wall, gelatin-coated 96-well plates at 2×10^4 cells per well, and incubated for 12 hours. Cells were then loaded with either the fluorescent calcium probe Fluo-2 acetoxymethyl (AM; TefLabs) at a final concentration of 4 µM in assay buffer (Hanks' balanced salt solution (HBSS) containing 20 mM HEPES buffer and 0.2% bovine serum albumin, pH 7.4) for 45 minutes at 37ºC. The intracellular calcium mobilization induced by the chemokines (2 nM to 1 µM) or chemokine-derived peptides (200 nM to 100 µM) was then measured at room temperature by monitoring the fluorescence as a function of time in all wells simultaneously using a fluorescence imaging plate reader (FLIPR Tetra, Molecular Devices) as previously described [55].

Signaling of chimeric CXCL11 chemokines through CXCR3 was assessed using Ready-to-Assay™ CXCR3-expressing Chem-1 cells (Eurofins Pharma Bioanalytics). Cells were seeded onto 0.3 mL v-bottom 96-well plates (Costar) according to the manufacturer's protocol. The medium was removed 24 hours later and cells were washed with 200 µL Hanks' balanced salt solution (HBSS). Assay buffer (HBSS, 20 mM HEPES, 0.1% BSA pH 7.4) and FLIPR 4 Calcium Flux kit dye (Molecular Devices) were added to each well in a 1:1 ratio. Plates were centrifuged for 15 seconds at 250 x g and subsequently incubated for 1 hour at 37ºC. Fluorescence was measured at 37ºC using a FlexStation2 microplate reader with excitation and emission of 485 nm and 515 nm, respectively.

Arrestin recruitment assays

Chemokine- and peptide-induced β-arrestin-2 recruitment to CXCR3, CXCR4, and ACKR3 was monitored by NanoLuc complementation assay (NanoBit, Promega)[56]. 1.2 x 10^6 U87 cells were plated in 10 cm-culture dishes and 48 hours later transfected with pNBe vectors containing human β-arrestin-2 N-terminally fused to LgBiT and receptors C-terminally fused to SmBiT. 48 hours post-transfection

cells were harvested, incubated 40 minutes at 37°C with 200-fold diluted Nano-Glo Live Cell substrate and distributed into white 96-well plates ($5x10^4$ cells per well). β-arrestin-2 recruitment in response to chemokines (0.1 nM - 1000 nM) or peptides (2 nM to 100 µM) was evaluated with a Mithras LB940 luminometer (Berthold Technologies).

Modified recombinant CXCL11-induced β-arrestin2 recruitment to CXCR3 and ACKR3 was monitored by Bioluminescence Resonance Energy Transfer (BRET) measurements as previously described[57]. Briefly, HEK293E cells were transiently transfected with receptor-YFP fusion constructs and β-arrestin-2-Rluc. Transfected cells were seeded onto poly-D-lysine treated 96-well plates. At 48 hours post-transfection the culture medium was replaced with PBS supplemented with 0.1% BSA. Cells expressing receptor-YFP and β-arrestin-2-Rluc at a ratio resulting in $BRET_{MAX}$ were stimulated with chemokine ligands for 5 minutes at 37ºC followed by the addition of Coelenterazine H to a final concentration of 5 µM (Nanolight technology). Fluorescence and luminescence were measured using a Mithras LB940 plate reader (Berthold Technologies). The Net-BRET signal was calculated by subtracting the background BRET signal from the signal obtained with the expression of β-arrestin-2-Rluc alone. The maximum signal of the mutants is reported as a percentage of WT, with $CXCL11_{WT}$ being set to 100%.

Data and statistical analysis

Concentration-response curves were fitted to the four-parameter Hill equation using an iterative, least-squares method (GraphPad Prism version 7.02) to provide pEC_{50}, pIC_{50}, EC_{50} or IC_{50} values and standard errors of the mean. Unpaired t tests were used to analyze the differences in pEC_{50}/ pIC_{50} obtained from at least five experiments (n=5). Peptides 1-17 (for CXCL12 and CXCL11) and 1-21 (for vCCL2) were considered as reference. Dimeric peptides were compared with their monomeric counterparts. CXCL11 variants were compared with the wild-type chemokine. P value of <0.05 was considered as statistically significant.

3. Results

The ability of synthetic peptides derived from CXCL12, CXCL11, CXCL10, CXCL9, and vCCL2 to interact with CXCR4, CXCR3, and ACKR3 was evaluated in competition studies with fluorescently labeled chemokines and in various G protein signaling or β-arrestin-2 recruitment assays and compared with that of the parental chemokines (Figure 1).

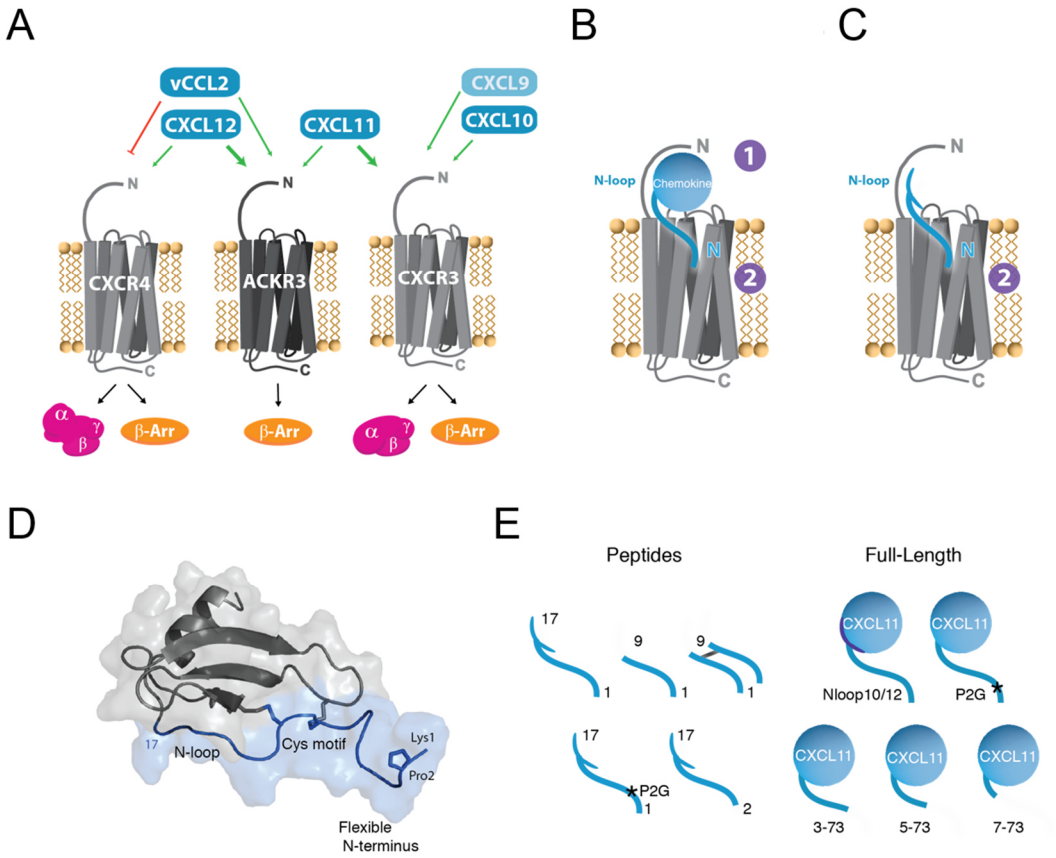

Figure 1. CXCR4-ACKR3-CXCR3 receptor-ligand interaction network and chemokine N-terminal features. (A) Selectivity and crosstalk between the two canonical G protein-signaling chemokine receptors, CXCR4 and CXCR3, the atypical β-arrestin-biased receptor ACKR3 and their shared ligands. CXCL12 is the only endogenous chemokine ligand for CXCR4. It is also the highest affinity chemokine for ACKR3 but does not bind CXCR3. The viral chemokine vCCL2 is a CXCR4 antagonist but acts as an agonist of ACKR3. CXCL11 is the dominant ligand for CXCR3 and binds also to ACKR3. CXCL10 and CXCL9 bind and activate CXCR3, but not ACKR3. (B) Two-site/two-step model for the interactions of full-length chemokines with their cognate receptors. In the first step, the body of the chemokine and the N-loop are specifically recognized by the N terminus of the receptor (CRS1). During the second step the insertion of the chemokine N terminus into the receptor-binding pocket (CRS2) stabilizes its active form triggering downstream signaling. (C) Peptides derived from the N-terminal region of chemokines, which represent useful probes to investigate the interaction between chemokines and receptors. (D) Schematic representation of CXCL12 and location of the N-terminal features investigated in this study (blue). The N-terminal region encompasses the flexible N terminus (1-8), the CXC cysteine motif (9-11) and the N-loop (13-17). (E) Chemokine-derived peptides and CXCL11 variants investigated in this study.

3.1 Binding and activity of chemokine N-terminal peptides towards CXCR4, CXCR3, and ACKR3

First, peptides comprising the flexible N terminus, the cysteine motif, and the N-loop of CXCL12, CXCL11, CXCL10, CXCL9 and vCCL2 chemokines ($CXCL12_{1-17}$, $CXCL11_{1-17}$, $CXCL10_{1-17}$, $CXCL9_{1-17}$, and $vCCL2_{1-21}$) were examined (Figure 1C, D and E).

In the CXCR4 binding assay, peptides $CXCL12_{1-17}$ and $vCCL2_{1-21}$ showed an 8000- and 100-fold weaker interaction, respectively, compared with full-length chemokines (Table 1, Figure 2A and B). $vCCL2_{1-21}$ was approximately 50 times more potent (IC_{50} = 2.0 µM, pIC50 = 5.71 ± 0.24) in displacing the labeled CXCL12 from CXCR4 than $CXCL12_{1-17}$ (IC_{50} ~ 100 µM, pIC_{50} ~4), whereas the parental chemokines of the two peptides interacted with the receptor with similar IC_{50} values (IC_{50} = 13 nM, pIC_{50} = 7.89 ± 0.04 vs IC_{50} = 20 nM, pIC_{50} = 7.70 ± 0.06 for CXCL12 and vCCL2, respectively). In functional assays, CXCL12- and vCCL2-derived peptides retained the activity of the parental chemokine, with $CXCL12_{1-17}$ inducing a decrease in basal cAMP levels (EC_{50} = 2.1 µM, pEC_{50} = 5.67 ± 0.06) and intracellular calcium release (EC_{50} = 2.6 µM, pEC_{50} = 5.58 ± 0.04) (Figure 2C, D) and $vCCL2_{1-21}$ acting as antagonist of CXCL12-induced calcium release (IC_{50} = 7.0 µM, pIC_{50} = 5.15 ± 0.05) (Table 2). Although its capacity to trigger G protein signaling was maintained, the ability of $CXCL12_{1-17}$ to induce β-arrestin-2 recruitment to CXCR4 was markedly reduced, and at the highest concentration tested (100 µM), reached only 18% of the maximal signal observed with CXCL12 (Table 3, Figure 2 E).

Similar behaviors were observed with peptides derived from CXCL11, CXCL10, and CXCL9 towards CXCR3 (Tables 2 and 3, Figure 2F, G and H). However, strong non-specific binding of fluorescently labeled CXCL11, resistant to competition by unlabeled chemokines, made it impossible to evaluate their binding to CXCR3 in competition studies on U87 cells. Moreover, due to peptide cytotoxicity, it was not possible to characterize the activity of $CXCL11_{1-17}$ and its variants as well as $CXCL10_{1-17}$ at concentrations above 30 to 50 µM, depending on the assay. Among the three peptides, $CXCL11_{1-17}$ and $CXCL10_{1-17}$ retained the ability to induce G protein-mediated signaling as shown in calcium release assay, albeit with an over 1500-fold reduction in potency (EC_{50} ≈ 50 µM, pEC_{50} ≈ 4.30 and EC_{50} = 26.0 µM, pEC_{50} = 4.58 ± 0.03) compared with the parental chemokines (EC_{50} = 19 nM, pEC_{50} = 7.72 ± 0.13 for CXCL11 and EC_{50} = 18 nM, pEC_{50} = 7.76 ± 0.19 for CXCL10) (Figure 2G). This decrease of potency was reminiscent of the 1500-fold difference observed between $CXCL12_{1-17}$ and the full-length CXCL12 in G protein signaling through CXCR4 (Tables 2). Although it showed no cytotoxicity, $CXCL9_{1-17}$ was unable to induce G protein signaling even at a concentration as high as 100 µM, likely originating from the already low potency of its parental chemokine (EC_{50} = 190 nM, pEC_{50} = 6.73 ± 0.15) (Table 2). $CXCL11_{1-17}$ induced 45% of the maximum β-arrestin-2 recruitment at a concentration of 10 µM, while $CXCL10_{1-17}$ was surprisingly unable to induce β-arrestin-2 recruitment even at a concentration of 30 µM (Table 3, Figure 2H).

Table 1. Binding properties of full-length chemokines and peptides derived from their N-terminal regions towards CXCR4 and ACKR3.

Name	Sequence	Binding competition	
		CXCR4	ACKR3
		pIC_{50} ± SEM	pIC_{50} ± SEM
CXCL12		**7.89 ± 0.04**	**8.74 ± 0.07**
$CXCL12_{1-17}$	KPVSLSYR**C**P**C**RFFESH	~ 4.00	5.39 ± 0.10
$CXCL12_{1-9}$	KPVSLSYR**S**	< 3.00	5.05 ± 0.13^{ns}
$(CXCL12_{1-9})2$	$(KPVSLSYR\mathbf{C})_2$	5.31 ± 0.16	$5.65 \pm 0.09^{**}$
$CXCL12_{1-17}D$	*KPVSLSYR**C**P**C**RFFESH*	~ 4.00	< 4.00
$CXCL12_{2-17}$	- PVSLSYR**C**P**C**RFFESH	~ 4.00	5.23 ± 0.25^{ns}
$CXCL12_{1-17/P2G}$	K**G**VSLSYR**C**P**C**RFFESH	< 4.00	4.22 ± 1.19^{ns}
vCCL2		**7.70 ± 0.06**	**7.47 ± 0.07**
$vCCL2_{1-21}$	LGASWHRPDK**CC**LGYQKRPLP	5.71 ± 0.24	5.77 ± 0.13
$vCCL2_{1-11}$	LGASWHRPDK**S**	~ 4.00	5.27 ± 0.24^{ns}
$(vCCL2_{1-11})2$	$(LGASWHRPDK\mathbf{C})_2$	6.67 ± 0.06	5.59 ± 0.13^{ns}
$vCCL2_{1-21\,D}$	*LGASWHRPDK**CC**LGYQKRPLP*	6.21 ± 0.21^{ns}	~ 4.00
CXCL11		**< 6.00**	**8.45 ± 0.03**
$CXCL11_{1-17}$	FPMFKRGR**C**L**C**IGPGVK	$< 4.52^{\Psi}$	5.46 ± 0.12
$CXCL11_{1-9}$	FPMFKRGR**S**	ND	5.09 ± 0.13^{ns}
$(CXCL11_{1-9})2$	$(FPMFKRGR\mathbf{C})_2$	ND	$6.03 \pm 0.14^{**}$
$CXCL11_{1-17}D$	*FPMFKRGR**C**L**C**IGPGVK*	ND	$5.06 \pm 0.12^{**}$
$CXCL11_{2-17}$	- PMFKRGR**C**L**C**IGPGVK	ND	< 4.52
$CXCL11_{1-17/P2G}$	F**G**MFKRGR**C**L**C**IGPGVK	ND	$6.72 \pm 0.09^{***}$
CXCL10		**< 6.00**	**< 6.00**
$CXCL10_{1-17}$	VPLSRTVR**C**T**C**ISISNQ	$< 4.52^{\Psi}$	$< 4.52^{\Psi}$

Binding competition studies with fluorescently labeled CXCL12 were performed in U87 cells (n=5). $^{\Psi}$ Highest concentration tested 30 µM. ND: Not determined. ^{ns} $p > 0.05$ * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In ACKR3 binding experiments, the relative rank order of IC_{50} values of the three peptides derived from CXCL12, CXCL11 and vCCL2 was different compared with their parental chemokines (Figure 2I, J and K).

$CXCL12_{1-17}$ and $CXCL11_{1-17}$ showed similar IC_{50} values of 4.1 µM (pIC_{50} = 5.39 ± 0.10) and 3.4 µM (pIC_{50} = 5.46 ± 0.12), corresponding to a 2000- and a 1000-fold loss of binding when compared with their parental chemokines (1.8 nM, pIC_{50} = 8.74 ± 0.07 and 3.5 nM, pIC_{50} = 8.45 ± 0.03) (Table 1) (Figure 2I and J). The reduced potency of these peptides was similar to that seen on CXCR4 and CXCR3, respectively. $vCCL2_{1-21}$ was stronger in binding ACKR3 (IC_{50} = 1.7 µM, pIC_{50} = 5.77 ± 0.13) than peptides derived from the endogenous chemokines, with only a 50-fold reduction compared to the full-length vCCL2 (IC_{50} = 33.6 nM, pIC_{50} = 7.47 ± 0.07) (Figure 2K). Unlike their weak activity towards CXCR4 and CXCR3, $CXCL12_{1-17,}$ $CXCL11_{1-17}$, and $vCCL2_{1-21}$ all strongly induced β-arrestin-2 recruitment to ACKR3, with EC_{50} values of 0.8 µM (pEC_{50} = 6.11 ± 0.05), 0.6 µM (pEC_{50} = 6.22 ± 0.08), and 0.5 µM (pEC_{50} = 6.31 ± 0.10), respectively (Figure 2L, M and N). Interestingly, peptides $CXCL11_{1-17}$, and $vCCL2_{1-21}$, in contrast

to their parental chemokines showing partial agonist profiles (Emax = 75 and 71 % of CXCL12, respectively), were able to induce levels of β-arrestin-2 recruitment respectively higher than (134 %) or comparable to (107 %) the full agonist CXCL12 (Figure 2M and N, Table 3). The maximum efficacy of $CXCL12_{1-17}$ was also comparable to that of the full-length CXCL12.

3.2 Effect of D-stereoisomer replacement on binding and activity of chemokine N-terminal peptides

The properties of peptides $CXCL12_{1-17}$, $CXCL11_{1-17}$ and $vCCL2_{1-21}$ in which each amino acid has been replaced by the corresponding D-stereoisomer ($CXCL12_{1-17}$D, $CXCL11_{1-17}$D, and $vCCL2_{1-21}$D) were then evaluated for their action towards the three receptors in order to assess the plasticity of their ligand-binding pockets and their tolerance to such ligand modifications at the functional level.

D-amino acid replacement had no effect on the binding of the CXCL12-derived peptides to CXCR4 and improved the binding of $vCCL2_{1-21}$ by 3 times (IC_{50} = 0.6 µM, pIC_{50}= 6.21 ± 0.21) (Figure 2A and B, Table 1). Nevertheless, D-isomer replacement turned $CXCL12_{1-17}$ into a CXCR4 antagonist (IC_{50} > 100 µM), while the initial antagonist activity of $vCCL2_{1-21}$ was conserved (IC_{50} = 0.7 µM, pIC_{50}= 5.2 ± 0.04) (Figure 2C, Table 2). A similar agonist-to-antagonist conversion was observed for $CXCL11_{1-17}$D in G protein signaling through CXCR3, (IC_{50}= 7.4 µM, pIC_{50}= 5.13 ± 0.06) (Figure 2G, inset) (Table 2) although the peptide did retain some of the agonist effect in β-arrestin recruitment to the receptor (Figure 2G and H, Table 3).

In contrast, D-isomer replacement significantly impaired the ability of peptides derived from vCCL2 and CXCL12 to bind ACKR3, with a 50-fold increase in IC_{50} for $vCCL2_{1-21}$D and $CXCL12_{1-17}$D compared with the L-stereoisomers (Figure 2I, J and K, Table 1). For the CXCL11-derived peptide this difference was much less marked, with the D-isomer showing only a two-fold decrease in its ability to displace the labeled CXCL12 from ACKR3. Remarkably however, although reduced, all three D-stereoisomer peptides conserved the parental agonist activity towards ACKR3, inducing β-arrestin-2 recruitment with potencies reflecting the effect of the substitution observed in binding competition studies (Figure 2L, M and N, Table 3).

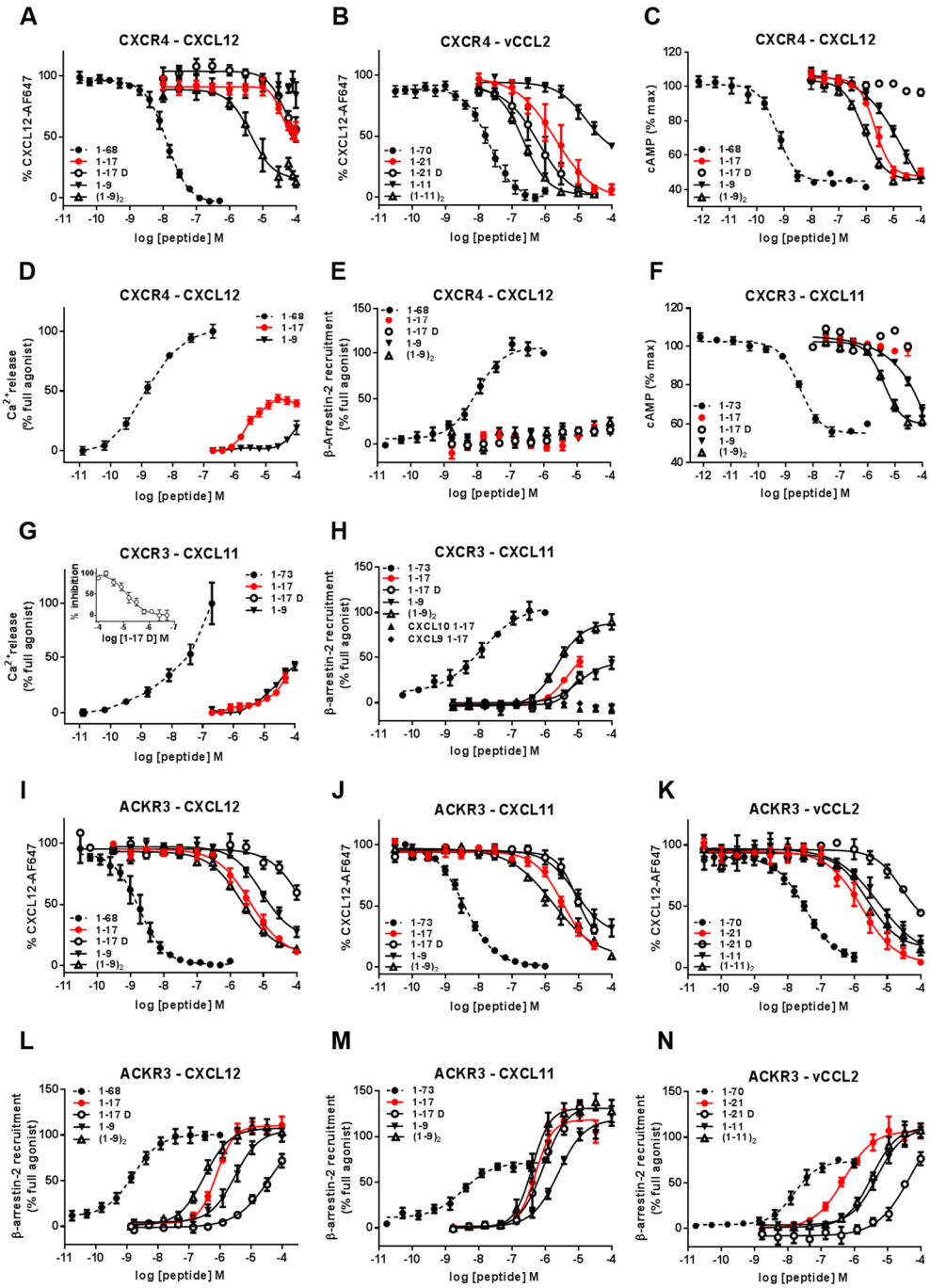

Figure 2. Binding, G protein signaling and β-arrestin-2 recruitment to CXCR4, CXCR3 and ACKR3 induced by full-length chemokines and chemokine N-terminal peptides. Binding and modulation of CXCR4 (A-E), CXCR3 (F-H) and ACKR3 (I-N) by full-length CXCL12, CXCL11, vCCL2 and peptides derived from their N-terminal regions. Binding to CXCR4 (A and B) and ACKR3 (I-K) was assessed by binding competition studies with Alexa Fluor 647-coupled CXCL12 in U87 cells stably expressing the receptors and analyzed by flow cytometry. G protein signaling induced by full-length chemokines and peptides derived from their N-terminal regions towards CXCR4 (C and D) and CXCR3 (F and G) was evaluated by measuring the modulation of the basal intracellular cAMP concentration using Glo-cAMP sensor (C and F) or the release of intracellular calcium using Fluo-2 dye and FLIPR platform (D and G). (G-inset) Antagonist properties of peptide $CXCL11_{1\text{-}17}D$ monitored in calcium assay. β-arrestin-2 recruitment to CXCR4 (E), CXCR3 (H) and ACKR3 (L-N) induced by full-length chemokines and N-terminal peptides was monitored using a Nanoluciferase-based complementation assay (NanoBIT). Each experiment was performed five times and the values correspond to the average ± standard error of the mean.

Table 2. G protein signaling properties of full-length chemokines and peptides derived from their N-terminal regions towards CXCR4 and CXCR3.

Name	Sequence	cAMP				Calcium			
		CXCR4		CXCR3		CXCR4		CXCR3	
		pEC_{50} ± SEM	max (%)	pEC_{50} ± SEM	max (%)	pEC_{50} (pIC_{50}) ± SEM	max (%)	pEC_{50} (pIC_{50}) ± SEM	max (%)
CXCL12		**8.97 ± 0.09**	**100**	**< 6.00**	**2**	**8.84 ± 0.05**	**100**	**< 6.00**	**0**
$CXCL12_{1-17}$	KPVSLSYR**C**P**C**RFFESH	5.67 ± 0.06	103	< 4.00	1	5.58 ± 0.04	44	< 4.00	0
$CXCL12_{1-9}$	KPVSLSYR**S**	5.11 ± 0.09***	101	ND	-	< 4.00	16	ND	-
$(CXCL12_{1-9})2$	$(KPVSLSYR\mathbf{C})_2$	6.07 ± 0.06****	105	ND	-	ND	-	ND	-
$CXCL12_{1-17}D$	*KPVSLSYR**C**P**C**RFFESH*	< 4.00	6	ND	-	(< 4.00)	0	ND	-
$CXCL12_{2-17}$	-PVSLSYR**C**P**C**RFFESH	< 4.00	1	ND	-	(4.50 ± 0.12)	0	ND	-
$CXCL12_{1-17/P2G}$	K**G**VSLSYR**C**P**C**RFFESH	< 4.00	1	ND	-	(4.45 ± 0.06)	0	ND	-
vCCL2		**< 6**	**0**	**< 6.00**	**-**	**(7.67 ± 0.08)**	**0**	**< 6.00**	**0**
$vCCL2_{1-21}$	LGASWHRPDKCCLGYQKRPLP	< 4.00	0	< 4.00	0	(5.15 ± 0.05)	0	< 4.00	0
$vCCL2_{1-11}$	LGASWHRPDK**S**	< 4.00	0	ND	-	(< 4)	0	ND	-
$(vCCL2_{1-11})2$	$(LGASWHRPDK\mathbf{C})_2$	< 4.00	0	ND	-	ND	-	ND	-
$vCCL2_{1-21}D$	*LGASWHRPDK**CC**LGYQKRPLP*	< 4.00	0	ND	-	(5.16 ± 0.04)	0	ND	-
CXCL11		**< 6.00**	**1**	**8.46 ± 0.06**	**100**	**< 6.00**	0	**7.72 ± 0.13**	**100**
$CXCL11_{1-17}$	FPMFKRGR**C**L**C**IGPGVK	< 4.52$^{\Psi}$	0	< 4.52$^{\Psi}$	4	< 4.30$^{\Psi\Psi}$	0	4.32 ± 0.04	78
$CXCL11_{1-9}$	FPMFKRGR**S**	ND	0	4.45 ± 0.08	89	ND	-	4.79 ± 0.05****	37
$(CXCL11_{1-9})2$	$(FPMFKRGR\mathbf{C})_2$	ND	0	5.35 ± 0.07****	95	ND	-	ND	-
$CXCL11_{1-17}D$	*FPMFKRGR**C**L**C**IGPGVK*	ND	0	< 4.52$^{\Psi}$	4	ND	-	(5.13 ± 0.06)	0
$CXCL11_{2-17}$	-PMFKRGR**C**L**C**IGPGVK	ND	0	< 4.52$^{\Psi}$	2	ND	-	(5.32 ± 0.15)	0
$CXCL11_{1-17/P2G}$	F**G**MFKRGR**C**L**C**IGPGVK	ND	0	< 4.52$^{\Psi}$	0	ND	-	(5.73 ± 0.19)	0
CXCL10		**< 6.00**	**0**	**8.36 ± 0.12**	**97**	**< 6.00**	**0**	**7.76 ± 0.19**	**100**
$CXCL10_{1-17}$	VPLSRTVR**C**T**C**ISISNQ	< 4.00	0	< 4.52$^{\Psi}$	0	< 4.30$^{\Psi\Psi}$	0	4.58 ± 0.03	112
CXCL9		**< 6.00**	**0**	**6.47 ± 0.10**	**85**	**< 6.00**	**0**	**6.73 ± 0.15**	**100**
$CXCL9_{1-17}$	TPVVRKGRCSCISTNQG	< 4.00	0	< 4.00	0	< 4.00	0	< 4.00	0

cAMP modulation measurements were performed in U87 cells using GloSensor technology (n=5). $^{\Psi}$ Highest concentration tested 30 µM. Intracellular calcium release was evaluated in U87 cells using a calcium-responsive fluorescent probe and a FLIPR system (n =5). $^{\Psi\Psi}$ Highest concentration tested 50 µM. ND: Not determined. (Data antagonist mode). ^{ns} $p > 0.05$ * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Table 3. β-arrestin-2 recruitment induced by full-length chemokines and peptides derived from their N-terminal regions to CXCR4, CXCR3 and ACKR3

Name	Sequence	Arrestin recruitment					
		CXCR4		CXCR3		ACKR3	
		pEC_{50} ± SEM	max (%)	pEC_{50} ± SEM	max (%)	pEC_{50} ± SEM	max (%)
CXCL12		**-8.03 ± 0.09**	**100**	**< 6.00**	**0 ± 1**	**8.81 ± 0.10**	**100**
$CXCL12_{1-17}$	KPVSLSYR**C**P**C**RFFESH	< 4.00	18 ± 1	< 4.00	1 ± 1	6.11 ± 0.05	111 ± 9
$CXCL12_{1-9}$	KPVSLSYR**S**	< 4.00	12 ± 2	ND	-	5.52 ± 0.09***	97 ± 5
$(CXCL12_{1-9})2$	$(KPVSLSYR\mathbf{C})_2$	< 4.00	25 ± 5	ND	-	6.50 ± 0.09****	103 ± 4
$CXCL12_{1-17}$D	*KPVSLSYR**C**P**C**RFFESH*	< 4.00	16 ± 4	ND	-	4.35 ± 0.43**	72 ± 8
$CXCL12_{2-17}$	- PVSLSYR**C**P**C**RFFESH	< 4.00	3 ± 1	ND	-	4.89 ± 0.22***	100 ± 3
$CXCL12_{1-17/P2G}$	K**G**VSLSYR**C**P**C**RFFESH	< 4.00	2 ± 7	ND	-	4.55 ± 0.47*	63 ±7
vCCL2		**< 6.00**	**10 ± 1**	**< 6.00**	**0 ± 5**	**7.80 ± 0.09**	**71 ± 5**
$vCCL2_{1-21}$	LGASWHRPDKCCLGYQKRPLP	< 4.00	18 ± 2	< 4.00	0 ± 2	6.31 ± 0.10	107 ± 8
$vCCL2_{1-11}$	LGASWHRPDK**S**	< 4.00	0 ± 2	ND	-	5.31 ± 0.09****	105 ± 6
$(vCCL2_{1-11})2$	$(LGASWHRPDK\mathbf{C})_2$	< 4.00	0 ± 2	ND	-	5.48 ± 0.08^{ns}	107 ± 8
$vCCL2_{1-21}$D	*LGASWHRPDK**CC**LGYQKRPLP*	< 4.00	0 ± 6	ND	-	4.27 ± 0.66*	77 ± 7
CXCL11		**< 6.00**	**8 ± 4**	**7.91 ± 0.16**	**100**	**8.47 ± 0.12**	**75 ± 3**
$CXCL11_{1-17}$	FPMFKRGR**C**L**C**IGPGVK	< 4.52$^{\Psi}$	13 ± 7	< 4.52$^{\Psi}$	45 ± 8	6.22 ± 0.08	134 ± 6
$CXCL11_{1-9}$	FPMFKRGR**S**	ND	-	< 4.00	35 ± 9	5.57 ± 0.12**	102 ± 8
$(CXCL11_{1-9})2$	$(FPMFKRGR\mathbf{C})_2$	ND	-	5.64 ± 0.08	89 ± 9	6.40 ± 0.07***	132 ± 12
$CXCL11_{1-17D}$	*FPMFKRGR**C**L**C**IGPGVK*	ND	-	< 4.52$^{\Psi}$	28 ± 6	6.05 ± 0.10^{ns}	136 ± 8
$CXCL11_{2-17}$	- PMFKRGR**C**L**C**IGPGVK	ND	-	< 4.52$^{\Psi}$	2 ± 3	5.81 ± 0.17^{ns}	59 ±15
$CXCL11_{1-17/P2G}$	F**G**MFKRGR**C**L**C**IGPGVK	ND	-	< 4.52$^{\Psi}$	5 ± 5	6.78 ± 0.09**	126 ± 10
CXCL10		**ND**	**-**	**7.59 ± 1.17**	**45 ± 6**	**< 6.00**	**21 ± 1**
$CXCL10_{1-17}$	VPLSRTVR**C**T**C**ISISNQ	ND	-	< 4.00	0 ± 3	< 4.00	2 ± 2
CXCL9		**ND**	**-**	**7.03 ± 0.21**	**46 ± 7**	**ND**	**-**
$CXCL9_{1-17}$	TPVVRKGRCSCISTNQG	ND	-	< 4.00	1 ± 2	ND	-

β-arrestin-2 recruitment was monitored in U87 cells using split Nanoluciferase complementation assay (n =5). $^{\Psi}$ Highest concentration tested 30 µM. ND: Not determined. ^{ns} $p > 0.05$ * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3 Binding and activity of N-loop- and cysteine motif-truncated chemokine N-terminal peptides

Next, truncated peptides devoid of the N-loop and the cysteine motif, and comprising only the flexible N termini of chemokines CXCL12, CXCL11, and vCCL2 ($CXCL12_{1-9}$, $CXCL11_{1-9}$, $vCCL2_{1-11}$) were investigated to assess the importance of these different N-terminal regions in the binding and activity towards the receptors (Figure 1E).

Truncation of $CXCL12_{1-17}$ after the first cysteine ($CXCL12_{1-9}$) resulted in a loss of binding to CXCR4 (IC_{50} >1000 µM, pIC_{50} < 3.00), a marked decrease of calcium mobilization and a reduced ability to modulate cAMP production (EC_{50} = 7.4 µM, pEC_{50} = 5.11 ± 0.09) (Figure 2A, C and D, Tables 1 and 2). The similar truncation of $vCCL2_{1-21}$ to $vCCL2_{1-11}$ affected the peptide's ability to compete with labeled CXCL12 and resulted in an approximately 50-fold reduction in CXCR4 binding (IC_{50} ~ 100 µM, pIC_{50} ~ 4.00) and antagonist properties (Fig. 2B, Table 1 and 2). $CXCL11_{1-9}$ retained its ability to induce cAMP modulation and showed a reduced potency in calcium mobilization through CXCR3 as well as β-arrestin-2

recruitment to the receptor (Figure 2F, G and H, Tables 2 and 3). The effect of the truncation, however, could not be precisely evaluated and compared due to the cytotoxicity of the parental peptide $CXCL11_{1-17}$ at concentrations above 30 µM.

$CXCL12_{1-9}$ and $CXCL11_{1-9}$ showed only a slightly reduced binding to ACKR3 (IC_{50} = 8.9 µM, pIC50 = 5.05 ± 0.13 and 8.1 µM pIC_{50} = 5.09 ± 0.13) as compared with $CXCL12_{1-17}$ and $CXCL11_{1-17}$, suggesting that the first nine residues of the chemokines support a large part of the binding of CXCL12 and CXCL11 N-terminal region to the receptor (Fig. 2I and J, Table 1). In accordance with this assumption, a peptide in which the residues following the first cysteine where permutated randomly showed an IC_{50} comparable with that of $CXCL12_{1-17}$ and $CXCL12_{1-9}$ (data not shown). Similarly, for vCCL2, truncation to $vCCL2_{1-11}$ resulted in a 3 times weaker binding to ACKR3 (IC_{50} = 1.7 µM, pIC_{50} = 5.27 ± 0.24) than $vCCL2_{1-21}$ (IC_{50} = 5.4 µM, pIC_{50} = 5.77 ± 0.13) (Figure 2K, Table 1). In β-arrestin-2 recruitment experiments, truncation resulted only in a 4-fold reduction of the potency of $CXCL12_{1-9}$ and $CXCL11_{1-9}$ compared with $CXCL12_{1-17}$ and $CXCL11_{1-17}$, whereas it decreased the potency of $vCCL2_{1-11}$ by tenfold (Figure 2L, M, and N, Table 3).

In conclusion, N-loop truncation drastically affected the interactions of peptides derived from CXCL12 and vCCL2 with CXCR4, whereas it only moderately reduced peptide ability to bind and activate ACKR3, suggesting that the two receptors have different chemokine recognition modes.

3.4 Effect of dimerization of the flexible chemokine N-terminal peptides on their binding and activity

Dimerization of the C-terminally truncated peptides ($(CXCL12_{1-9})_2$, $(CXCL11_{1-9})_2$, and $(vCCL2_{1-11})_2$) via a disulfide bridge between the terminal cysteine residues had varying impacts on the different peptide-receptor pairs (Figure 1E).

For CXCR4, dimerization of $CXCL12_{1-9}$ and $vCCL2_{1-11}$ substantially enhanced their binding and signaling potencies, as shown by an improvement of their IC_{50} of over 100-fold observed in binding competition and their EC_{50} of over 3 to 10-fold in G protein signaling assays (Figure 2A, B and C, Tables 1 and 2). Similar 10 times higher potency in cAMP modulation and markedly enhanced β-arrestin-2 recruitment were observed for CXCR3 interaction with the dimeric $CXCL11_{1-9}$ (Figure 2F and H, Tables 2 and 3).

For ACKR3, dimerization had different effects on CXCL12 and CXCL11 peptides compared to the vCCL2 peptide. Both $(CXCL12_{1-9})_2$ and $(CXCL11_{1-9})_2$ showed an overall 5 to 10-fold improvement in their binding and β-arrestin-2 recruitment properties compared with their monomeric counterparts (Figure 2I, J, L and M), whereas dimerization had no effect for the vCCL2-derived peptide (Figure 2K and N), suggesting that its binding mode slightly differs from that of the CXCL12- and CXCL11-derived peptides.

Altogether, these data showed that dimerization strongly enhanced the binding and activity of N-terminal peptides not only to CXCR4 but also CXCR3 and ACKR3. Moreover, the N-terminal domain of vCCL2 seems to interact with CXCR4 and ACKR3 according to different binding modes.

3.5 Impact of N-loop replacement on binding and activity of CXCL11 towards CXCR3 and ACKR3

To circumvent CXCL11 peptide cytotoxicity problems described above and to further assess the importance of the N-loop for CXCR3 and ACKR3 binding and activation, we generated variants of full-length CXCL11, in which the N-loop residues were replaced with those of CXCL10 ($CXCL11_{Nloop10}$) or CXCL12 ($CXCL11_{Nloop12}$), and evaluated their binding in competition studies with radiolabeled CXCL11 as well as receptor activation (Figure 1E).

$CXCL11_{Nloop10}$ and $CXCL11_{Nloop12}$ bound to CXCR3 with IC_{50} values of 38.2 nM (pEC_{50} = 7.41 ± 0.11) and 120.5 nM (pEC_{50} = 6.91 ± 0.08) respectively, compared with 3.2 nM (pEC_{50} = 8.48 ± 0.09) for $CXCL11_{WT}$ (Table 4). Compared with $CXCL11_{WT}$, $CXCL11_{Nloop12}$ also showed reduced potency and efficacy in the induction of β-arrestin-2 recruitment to CXCR3, whereas the activity of $CXCL11_{Nloop10}$ (EC_{50} = 37.3 nM, pEC_{50} = 7.42 ± 0.08, Emax = 41 %) was equivalent to that of $CXCL11_{WT}$ (26.8 nM, pEC_{50} = 7.57 ± 0.10)) (Figure 3A, B and C, Table 4). $CXCL11_{Nloop12}$ showed a ~20-fold loss of potency in cAMP modulation relative to $CXCL11_{WT}$, while $CXCL11_{Nloop10}$ was almost unaffected by the substitution of N-loop residues from CXCL10 (Figure 3B, Table 4). These data demonstrate that exchanging the N-loops of CXCL10 and CXCL11, which both bind to CXCR3, does not significantly impair chemokine activity whereas replacement of the CXCL11 N-loop with residues from a non-cognate chemokine ligand (i.e. CXCL12) significantly diminishes its CXCR3 binding and activation.

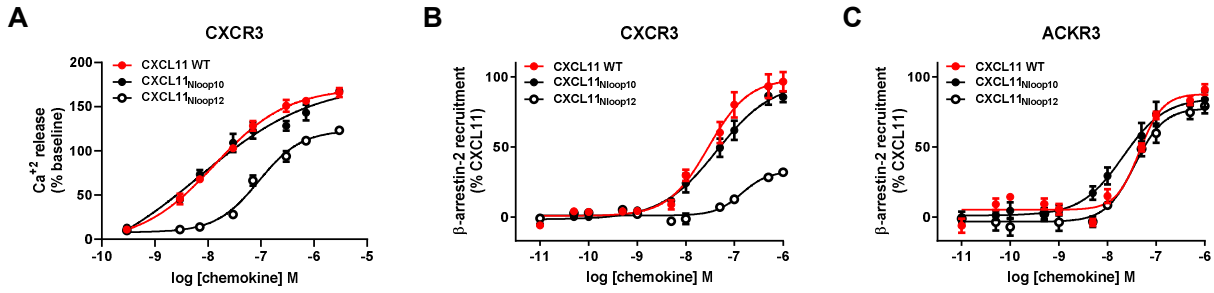

Figure 3. ACKR3 and CXCR3 activation by CXCL11 N-loop variants. (A) CXCR3 activation by CXCL11 WT and N-loop variants monitored in HEK293 cells through intracellular calcium release using FLIPR 4 Calcium Flux kit dye. (B and C) Comparison of β-arrestin-2 recruitment to CXCR3 (B) and ACKR3 (C) induced by $CXCL11_{WT}$ and N-loop variants monitored in HEK293 cells by BRET using receptor-YFP fusion constructs and β-arrestin-2-Rluc. Each experiment was performed five times and the values correspond to the average ± standard error of the mean.

Table 4. Binding and signaling properties of CXCL11 variants towards CXCR3 and ACKR3.

Name	Sequence	Binding competition		cAMP		Calcium		Arrestin recruitment			
		CXCR3	ACKR3	CXCR3		CXCR3		CXCR3		ACKR3	
		$pIC_{50} \pm SEM$	$pIC_{50} \pm SEM$	$pEC_{50} \pm SEM$	max (%)	$pEC_{50} \pm SEM$	max (%)	$pEC_{50} \pm SEM$	max (%)	$pEC_{50} \pm SEM$	max (%)
CXCL11 WT	**1-FPMFKRGRCLCIGPGVKAVK-73**	**8.48 ± 0.09**	**6.52 ± 0.05**	**8.21 ± 0.11**	**100**	**7.80 ± 0.07**	**100**	**7.57 ± 0.07**	**100**	**7.46 ± 0.09**	**100**
$CXCL11_{Nloop10}$	1-FPMFKRGRCLCISISNQAVK-73	$7.41 \pm 0.11^{***}$	$6.11 \pm 0.07^{**}$	7.93 ± 0.13^{ns}	81	7.98 ± 0.11^{ns}	92	7.42 ± 0.08^{ns}	89	7.68 ± 0.10^{ns}	85
$CXCL11_{Nloop12}$	1-FPMFKRGRCLCREFESHAVK-73	$6.91 \pm 0.08^{****}$	$6.29 \pm 0.04^{**}$	$6.85 \pm 0.14^{****}$	102	$7.02 \pm 0.06^{****}$	79	< 6.00	41	7.32 ± 0.09^{ns}	84
$CXCL11_{3-73}$	3-MFKRGRCLCIGPGVKAVK-73	$6.90 \pm 0.09^{****}$	$5.78 \pm 0.07^{****}$	8.39 ± 0.21^{ns}	54	< 6.00	37	< 6.00	22	$7.15 \pm 0.08^{*}$	81
$CXCL11_{5-73}$	5-KRGRCLCIGPGVKAVK-73	$6.18 \pm 0.05^{****}$	$5.29 \pm 0.01^{****}$	< 6.00	-	< 6.00	-	< 6.00	16	< 6.00	30
$CXCL11_{7-73}$	7-RCLCIGPGVKAVK-73	< 5.00	< 5.00	< 6.00	-	< 6.00	-	< 6.00	15	< 6.00	22
$CXCL11_{P2G}$	1-FGMFKRGRCLCIGPGVKAVK-73	$9.63 \pm 0.09^{****}$	$8.76 \pm 0.05^{****}$	7.86 ± 0.15^{ns}	100	$7.40 \pm 0.04^{**}$	83	7.22 ± 0.14^{ns}	61	$7.99 \pm 0.07^{**}$	136

Binding competition studies with radiolabeled CXCL11 (CXCR3) or CXCL12 (ACKR3) were performed in HEK293T cells (n=5). β-arrestin-2 recruitment was monitored by BRET in HEK293T cells expressing YFP-fused receptors and Rluc-fused β-arrestin-2. ^{ns} $p > 0.05$ * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In contrast to CXCR3, only modest differences were observed for the binding of the N-loop variants to ACKR3 as well as for their ability to induce the recruitment of β-arrestin-2 (Figure 3, Table 4). Indeed, ACKR3 activation by $CXCL11_{WT}$ and $CXCL11_{Nloop12}$ were indistinguishable. Surprisingly, N-loop substitution from the non-cognate chemokine (i.e. CXCL10) also had no significant impact on ACKR3 β-arrestin-2 recruitment (Figure 3C, Table 4), which is consistent with data generated using the N-loop-truncated peptides. This suggests that specific N-loop contacts are not required for ACKR3 binding or activation by CXCL11 and CXCL12. Therefore, unlike CXCR3 and CXCR4, ACKR3 appears relatively insensitive to the N-loop composition of its CXC chemokine ligands.

3.6 Effect of P2G mutation on binding and activity of chemokine-derived peptides and modified CXCL11

The proline at position 2 in CXCL12 is critical for CXCR4 activation[33]. Since this proline is also conserved in CXCL11, P2G mutation was introduced in peptides derived from both CXCL12 and CXCL11 and the binding and activity towards their cognate receptors was evaluated (Figure 1D and E).

For CXCR4, P2G mutation ($CXCL12_{1-17/P2G}$) considerably affected the agonist properties of the peptide and turned it into antagonist (IC_{50} = 35.5 μM, pIC_{50} = 4.45 ± 0.06), while it slightly reduced its binding compared with $CXCL12_{1-17}$ (Table 1 and 2). A similar shift from CXCR3 agonist to antagonist was observed in calcium assay with $CXCL11_{1-17}$ bearing the P2G mutation ($CXCL11_{1-17/P2G}$) (IC_{50} = 1.9 μM, pIC_{50} = 5.73 ± 0.19) (Figure 4F-inset, Tables 2).

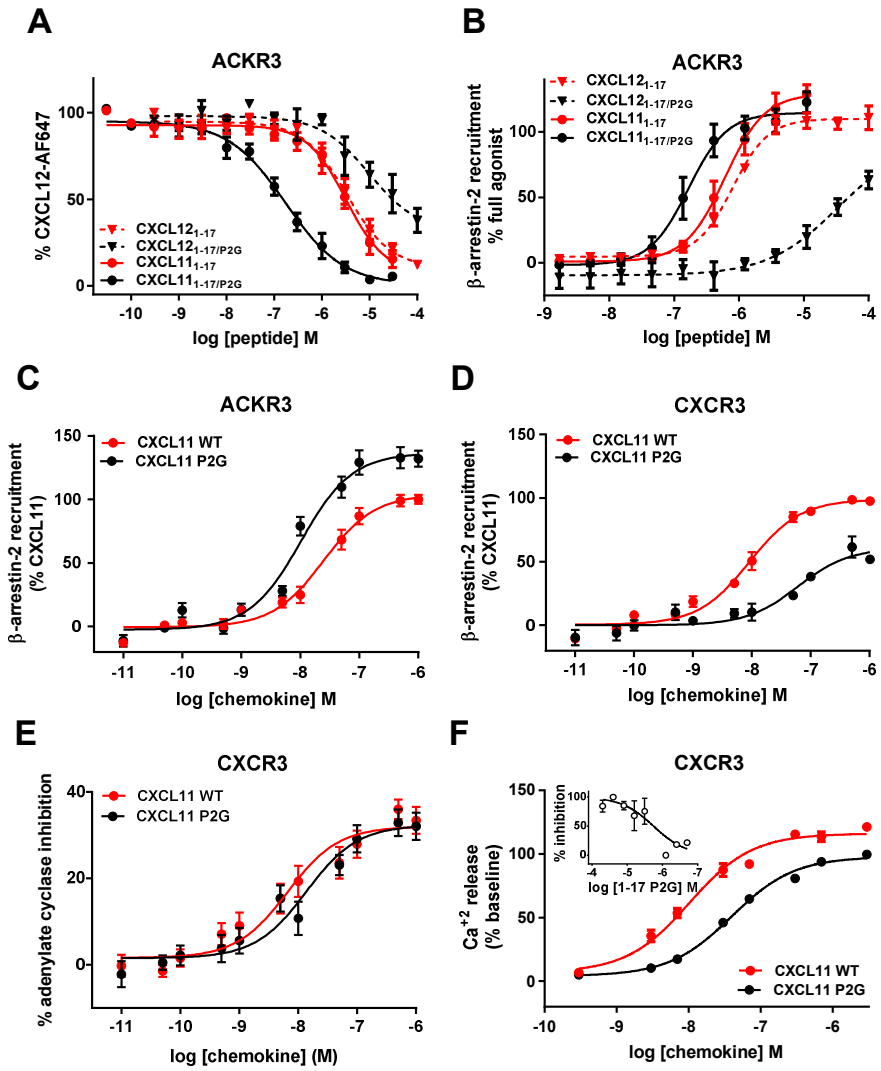

Figure 4. Binding and activation of ACKR3 and CXCR3 by N-terminal peptides and full-length CXCL11 bearing the P2G mutation. (A and B) Comparison of the impact of P2G mutation in CXCL12- and CXCL11-derived peptides on (A) binding to ACKR3 assessed by competition studies with Alexa Fluor 647-coupled CXCL12 and (B) β-arrestin-2 recruitment to ACKR3 monitored by NanoLuc complementation in U87 cells. (C and D) Comparison of β-arrestin-2 recruitment to ACKR3 (C) and CXCR3 (D) induced by full length $CXCL11_{WT}$ and P2G mutant in HEK293 cells monitored by BRET using receptor-YFP fusion constructs and β-arrestin-2-Rluc. (E and F) CXCR3 activation by $CXCL11_{WT}$ and P2G mutant monitored in HEK293 cells through (E) adenylate cyclase inhibition using BRET reporter GFP10-Epac- Rluc3 and (F) intracellular calcium release using FLIPR 4 Calcium Flux kit dye. (F-inset) Antagonist properties of peptide $CXCL11_{1\text{-}17/P2G}$ monitored in calcium assay. Each experiment was performed five times and the values correspond to the average ± standard error of the mean.

The P2G mutation had opposite effect on the interaction of the two peptides with ACKR3. $CXCL12_{1\text{-}17/P2G}$ exhibited a 150-fold reduction of binding (IC_{50} = 60.9 µM, pIC_{50} = 4.22 ± 1.19), while the same substitution in the CXCL11 peptide ($CXCL11_{1\text{-}17/P2G}$) resulted in a surprising 20-fold increase in its binding with an IC_{50} in the nanomolar range (IC_{50} = 190 nM, pIC_{50} = 6.72 ± 0.09) (Figure 4A, Table 1)). Moreover, in contrast to what was observed for CXCR4 and CXCR3, P2G mutation in CXCL12 and CXCL11 peptides

did not convert them into ACKR3 antagonists but rather improved the potency of $CXCL11_{1-17/P2G}$ while it reduced that of $CXCL12_{1-17/P2G}$ (Figure 4B, Table 3).

In agreement with the data obtained with the N-terminal peptides, the replacement of the proline at position 2 in full-length CXCL11 substantially enhanced its IC_{50} in ACKR3 binding competition (170-fold improvement), as well as its potency and efficacy (136%) in recruiting β-arrestin-2 to the receptor (Figure 4C, Table 4). $CXCL11_{P2G}$ also showed a 15-fold improved binding to CXCR3 compared with $CXCL11_{WT}$. However, in contrast to ACKR3, this chemokine showed a drastic reduction in its ability to induce β-arrestin-2 recruitment to CXCR3 (Figure 4D). More surprisingly, the introduction of the P2G mutation in CXCL11 only led to modest reduction of its agonist activity (Figure 4E and F, Table 4), which may be related to its higher affinity.

3.7 Effect of amino-terminal truncation on binding and activity of chemokine-derived peptides and modified CXCL11

N-terminal truncations were also introduced in peptides derived from both CXCL12 and CXCL11 and their binding and activity towards the receptors was compared. For CXCR4, N-terminal residue truncation ($CXCL12_{2-17}$) drastically affected the agonist properties of the peptide, turning it into antagonist in calcium assay (IC_{50} = 31.5 µM, pIC_{50} = 4.50 ± 0.12), while the binding was only slightly reduced compared with $CXCL12_{1-17}$. A similar change of activity towards CXCR3 was observed with $CXCL11_{2-17}$ lacking the amino-terminal phenylalanine (IC_{50} = 7.36 µM, pIC_{50} = 5.32 ± 0.15) (Figures 5E-inset, Tables 1 and 2).

Interestingly, truncation of the first residue of the CXCL12 and CXCL11 peptides (Lys1 and Phe1, respectively) had different effects on their binding to ACKR3, with no impact on the IC_{50} for $CXCL12_{2-17}$ and an over 10-fold increase for that of $CXCL11_{2-17}$ (Figures 5A). Although it substantially affected the potency in CXCL11, the N-terminal deletion in CXCL12 and CXCL11 peptides did not convert them to ACKR3 antagonists as observed for CXCR4 and CXCR3 (Figures 5B).

The Effect of N-terminal truncation was further investigated using full-length chemokines. A series of CXCL11 variants lacking 2, 4 or 6 N-terminal residues were generated and their ability to bind and activate CXCR3 and ACKR3 was measured (Figure 1E, Table 4). Truncated CXCL11 proteins ($CXCL11_{3-73}$, $CXCL11_{5-73}$, and $CXCL11_{7-73}$) all showed diminished maximal β-arrestin-2 recruitment compared with $CXCL11_{WT}$ for both CXCR3 and ACKR3 (Figure 5C). However, $CXCL11_{3-73}$ retained substantially more agonist efficacy at ACKR3 (Emax = 81%) than CXCR3 (Emax = 22%) (Figure 5D and F, Table 4). The EC_{50} value for $CXCL11_{3-73}$-induced β-arrestin-2 recruitment to ACKR3 was also only 2-fold higher than that of $CXCL11_{WT}$ (Figure 5D, Table 4). This result indicates that neither ACKR3 agonist potency nor efficacy is substantially altered by removal of the two N-terminal amino acids of CXCL11.

The analysis of the $CXCL11_{3-73}$-CXCR3 interaction showed that N-terminal truncation has a significant effect on receptor binding and activation. $CXCL11_{3-73}$ bound to CXCR3 with an IC_{50} of 123.3 nM (pIC_{50} = 6.90 ± 0.09) as compared with the 3.2 nM (pIC_{50} = 8.48 ± 0.09) for $CXCL11_{WT}$ (Table 4), which is indicative of a substantial loss of binding affinity. In addition, its potency and efficacy in G protein activation and β-arrestin-2 recruitment were drastically reduced (Figure 5E and F, Table 4).

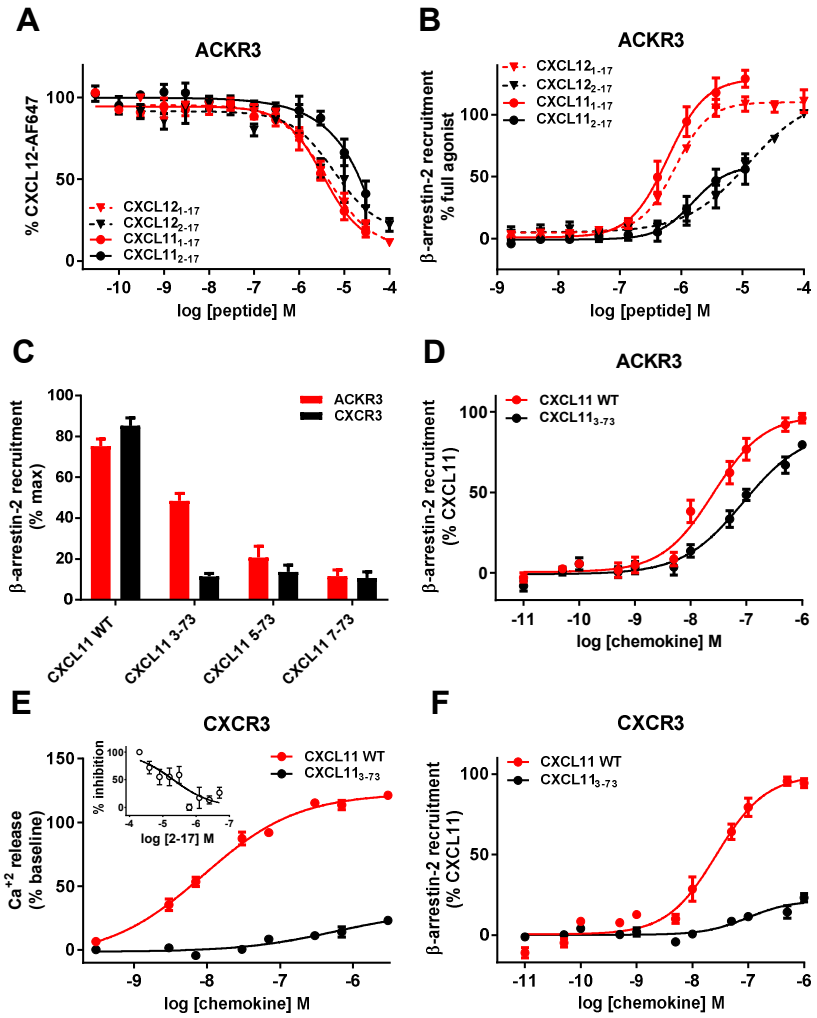

Figure 5. β-arrestin-2 recruitment and G protein signaling induced by N-terminally truncated CXCL11 variants towards ACKR3 and CXCR3. (A and B) Comparison of the effect of N-terminal residue truncation in CXCL12- and CXCL11-derived peptides on (A) binding to ACKR3 assessed by competition studies with Alexa Fluor 647-coupled CXCL12 and (B) β-arrestin-2 recruitment to ACKR3 monitored by NanoLuc complementation in U87 cells. (C) Impact of progressive N-terminal truncation on the ability of CXCL11 (100 nM) to recruit β-arrestin-2 to ACKR3 (red) and CXCR3 (black). Values are expressed as percentage of the maximum β-arrestin-2 recruitment monitored with saturating concentrations of $CXCL11_{WT}$. (D and F) Comparison of β-arrestin-2 recruitment to ACKR3 (D) and CXCR3 (F) induced by $CXCL11_{WT}$ and variants lacking the first two residues ($CXCL11_{3-73}$) monitored by BRET. (E) Comparison of maximum CXCR3 mediated intracellular calcium release induced by $CXCL11_{WT}$ and $CXCL11_{3-73}$ variant in HEK cells using FLIPR 4 Calcium Flux kit dye. (E-inset) Antagonist properties of peptide $CXCL11_{2-17}$ monitored in calcium assay. Each experiment was performed five times and the values correspond to the average ± standard error of the mean.

For the two receptors, the longer N-terminal deletions ($CXCL11_{5-73}$ and $CXCL11_{7-73}$) resulted in a drastic reduction of both binding and β-arrestin-2 recruitment efficacy and potency, however, the shorter truncation ($CXCL11_{3-73}$) maintained a higher portion of ACKR3 activity than CXCR3 activity, suggesting a higher tolerance for N-terminal processing.

Taken together, these results confirm the previously documented role of chemokine proximal N-terminal residues in CXCR3 and CXCR4 for both binding and activation and show for the first time that ACKR3 ligand binding and activation is less dependent on the presence of the first amino acids and N loop residues (Figure 6). Moreover, this study suggests that ACKR3 could also act as a scavenger receptor for N-terminally processed chemokines.

4. Discussion

Chemokine-receptor selectivity is dictated by numerous interactions along the receptor extracellular surface and transmembrane segments[28, 58, 59]. Recent structural and mechanistic breakthroughs demonstrated that chemokines make extensive contacts with their receptors in a 1:1 stoichiometry through at least three major recognition sites[12, 28-30, 58-60]. Despite these advances, and in the absence of comparative structural data, the roles of the conserved features present in chemokines, and especially in their N-terminal regions, remain obscure. Although a growing body of evidence indicates that the two-site/two-step binding mode proposed for chemokines and their receptors is oversimplified, the essential role of chemokine N termini in mediating receptor affinity and activity is well-demonstrated, as their truncation or modification drastically influences chemokine function[28, 33, 38]. While studies have documented the role of chemokine N-terminal interactions with CXCR4 for CXCL12 and vCCL2 [32, 40-42], none has compared the role of chemokine N termini between multiple receptor targets, limiting our understanding of the complex regulatory principles in promiscuous chemokine systems.

In this study, we examined the importance of different features present in the N-terminal regions of CXCL12, CXCL11, and vCCL2 for binding and activation of a trio of receptors: two conventional receptors, CXCR4 and CXCR3, and the atypical chemokine receptor ACKR3. Using this subset of interconnected receptors, we also investigated how widespread the binding capacity of D-stereoisomers and the improved binding of dimeric ligands are, these properties being commonly recognized for CXCR4[43-45].

The results from binding and activity analyses of peptides derived from CXCL12 and vCCL2 re-examined in this study were consistent with previous reports[32, 33, 40-42, 44, 46]. The investigation of a larger set of modifications of chemokine-derived peptides, not only on CXCR4 but also on CXCR3 and ACKR3,

provided new insights into the importance of different chemokine N-terminal features for receptor binding and activation. Notably, our study showed that the use of peptides derived from chemokine N termini to probe the ligand-binding pocket or to evaluate the importance of specific modifications on binding and signaling is not restricted to CXCR4, but can also be extended to other receptors in the CXC and ACKR subfamilies.

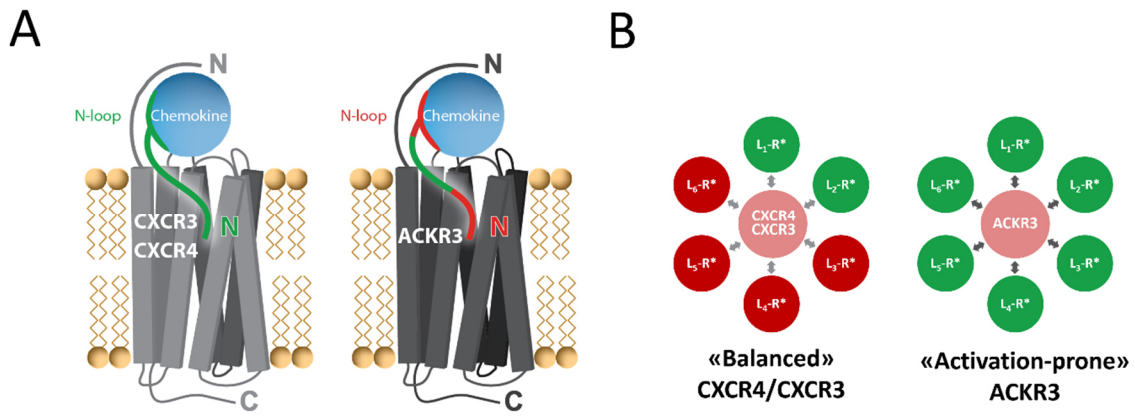

Figure 6. **Differential contribution of chemokine N-terminal features to interactions with CXCR3/CXCR4 and ACKR3 and distribution between receptor active/inactive states.** (A) The entire chemokine N-terminal region (green) is critical for the binding and activation of the canonical receptors CXCR3 and CXCR4, whereas the most N-terminal residues (red) as well as the N-loop (red) do not appear important for activation of the atypical receptor ACKR3. (B) Comparison of the distribution of active (R* green) and inactive (R* red) conformations of CXCR4/CXCR3 and ACKR3 stabilized by ligands (L) targeting the receptor transmembrane ligand-binding pocket (CRS2). CXCR4 and CXCR3 have low basal activity (light red) and are "balanced" receptors as ligand binding to CRS2 can stabilize either the active state (green) or inactive state (red). ACKR3 is an "activation-prone" receptor as ligand binding preferentially stabilizes the active state (green) of the receptor leading to β-arrestin-2 recruitment. Indeed, so far, no ligand targeting the transmembrane pocket of ACKR3 without displaying agonist activity have been reported.

4.1 Different role of chemokine N-loop and proximal N-terminal residues in CXCR3, CXCR4 vs ACKR3 binding and activation

The importance of the chemokine N-loop for tight receptor binding initially observed for the CXCR4-CXCL12 pair was conserved in CXCR3 (Figure 7 A and B). N-loop truncation in peptides derived from CXCL12 and CXCL11 reduced drastically their binding to CXCR4 and CXCR3. Moreover, exchange of the N-loop of CXCL11 for that of CXCL10 had only a minimal effect on CXCR3 activation, consistent with the ability of CXCL10 to bind CXCR3 with a somewhat lower affinity compared to CXCL11[61, 62]. In contrast, substitution with the N-loop of CXCL12 decreased CXCR3 binding and reduced the potency of both G protein and β-arrestin-2 signaling by approximately 10-fold. This finding was expected, since CXCL12 is not a ligand for CXCR3, thus confirming the important role of the N-loop for chemokine binding to CRS1 of canonical chemokine receptors, CXCR3 and CXCR4.

In contrast, N-loop truncation in peptides derived from CXCL12 or CXCL11 only slightly affects ACKR3 binding and signaling. ACKR3 binding and activation by full-length CXCL11 was also insensitive to N-loop substitutions with those of CXCL10 or CXCL12. Taken together, the combined results from the N-loop chimeras and truncated peptides suggest that N-loop contacts, which are important for CXCR4 and CXCR3 recognition, are not essential for ACKR3-chemokine interactions (Figure 7A)

Similar to what has previously been reported for CXCR4, dimeric peptides derived from CXCL11 were also more potent in CXCR3 binding and activation than their monomeric counterparts (Figure 7B). This improvement in affinity and consequentially in potency may result from the presence of the cysteine bridge linking the two monomers possibly mimicking the first disulfide bridge of the parental chemokines, which, as suggested by the first crystal structures of chemokine-receptor complexes, could be key for tight receptor binding[28, 59]. Alternatively, better binding of bivalent peptides may arise from an avidity effect through binding of receptor homodimers. Indeed, as described for CXCR4, CXCR3 may also form homodimers, possibly through a TM5 and TM6 dimer interface, bringing the two chemokine N terminus-binding pockets (CRS2) into close proximity[9, 10]. Finally, it cannot be excluded that the improvement in binding and potency results from allosteric contacts of the second protomer with extracellular non CRS2 determinants of the same receptor.

In our system, dimeric peptides derived from the flexible N termini of CXCL12 and CXCL11 bound to ACKR3 with higher affinities and induced β-arrestin-2 recruitment to the receptor with increased potency. These data indicate that the first disulfide bridge of chemokines may also be an important factor in ACKR3 recognition. Interestingly, dimerization of the vCCL2-derived peptide had little effect on ACKR3 binding suggesting that the vCCL2 N terminus may occupy the receptor's sub-pockets differently or bind more deeply in the TM region of ACKR3 than the N termini of either CXCL12 or CXCL11. Furthermore, although the full-length chemokines CXCL12 and CXCL11 display a higher affinity for ACKR3 than does vCCL2, the N-terminal vCCL2 peptide retained a higher proportion of the parental chemokine binding capacity than the peptides derived from the endogenous chemokines. A similar observation was made for CXCR4 (Figure 7C). This higher affinity may simply result from the larger size of vCCL2 peptide (21 residues versus 17 for the CXC chemokines) or may be related to the ability of vCCL2 to bind a broad spectrum of receptors of all the four classes (XC, CC, CXC and CX3C), implying that its core may have evolved less tight and more promiscuous binding capacity, while the N terminus plays a more important role in specific binding and modulation of downstream activity[15, 46].

Furthermore, peptides bearing simple modifications such as amino-terminal residue deletion, P2G mutation or D-amino acid replacement retained their ability to bind to both CXCR3 and CXCR4 but their agonist activity was drastically reduced or changed to antagonist. These results illustrate the crucial

role of the proximal N-terminal region of chemokines and the stringency of the contacts at CRS2 that are required for classical CXC receptor activation (Figure 7).

Strikingly, although some modifications introduced in the proximal part of the peptides derived from the three chemokines affected their binding to ACKR3, none of them resulted in a loss of receptor activation. For instance, in contrast to the results with CXCR4 and CXCR3, proximal N-terminal truncation, P2G mutation, and even complete D-amino acid replacement in CXCL12- and CXCL11-derived peptides did not change their activity towards ACKR3 (Figure 7).

Collectively, these data suggest that the molecular interaction network within the binding pocket required for ACKR3 activation is different and less stringent than for CXCR4 and CXCR3, and relies less on the proximal residues of the chemokine (Figure 6 and 7D).

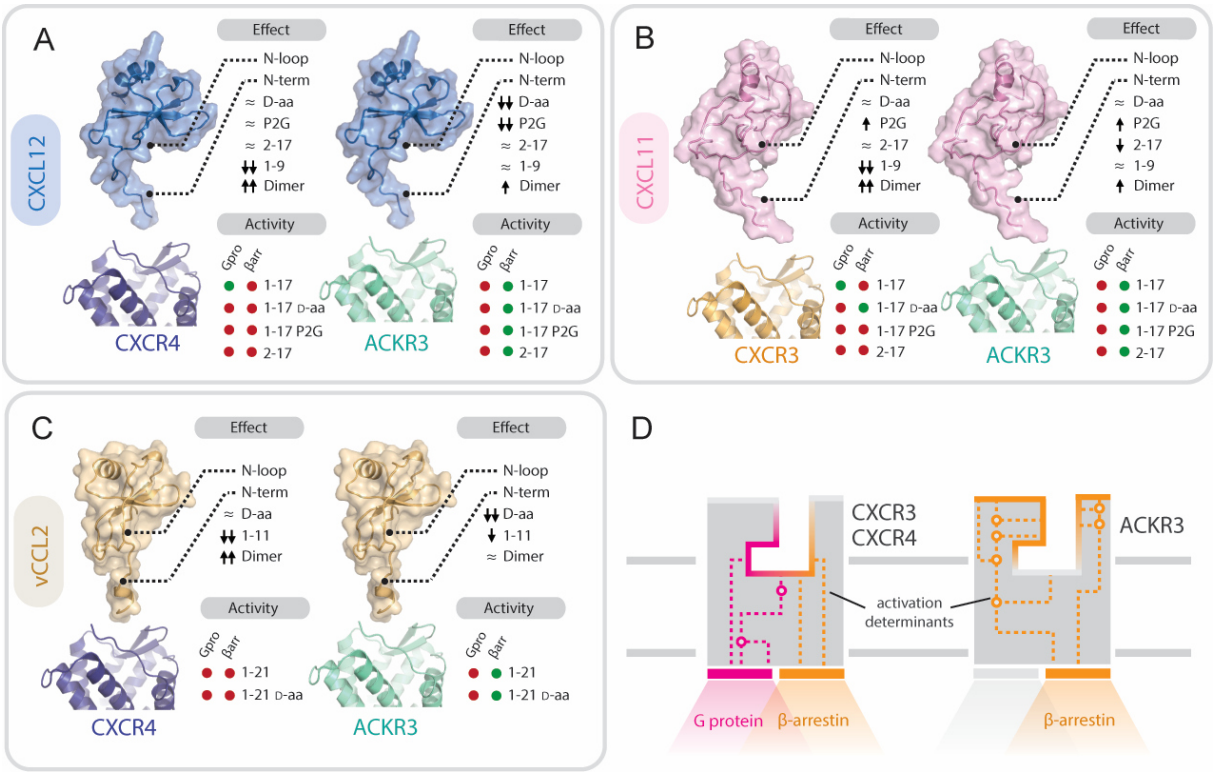

Figure 7. **Contributions of chemokine and receptor regions to binding and activation of ACKR3, CXCR3 and CXCR4** (A-C) Importance of structural determinants of chemokines CXCL12 (A), CXCL11 (B), and vCCL2 (C) for binding and activity towards the receptors **Top**: Impact of the different modifications of chemokine N-terminal domains on receptor binding and/or activity: ≈ no effect or < 5-fold change, ↑ or ↓ increase or decrease by > 5 fold, ↑↑ or ↓↓ increase or decrease by > 50 fold. **Bottom**: Activity in G protein signaling (Gpro) and β-arrestin recruitment (βarr) of peptides comprising the flexible N terminus and the N-loop (1-17/21), their D stereoisomers and proximally modified variants (P2G, 2-17): ● activity, ● no activity. (D) Comparison of ACKR3 and CXCR3/4 binding pockets. The activation determinants of ACKR3 are localized closer to the surface compared with CXCR3 and CXCR4. Ligand binding to ACKR3 pocket triggers a limited signaling repertoire (i.e. β-arrestin-2 recruitment). In contrast, CXCR4 and CXCR3 activation determinants are localized deeper in the ligand binding pocket with chemokine triggering G protein signaling and subsequently arrestin recruitment.

4.2 Mechanistic interpretation of ACKR3 permissiveness to chemokine N-terminal modifications

Our results as well as numerous previous studies conducted with non-chemokine ligands demonstrated that in contrast to classical chemokine receptors, such as CXCR4 or CXCR3, ACKR3 is an "activation-prone" receptor (Figure 6B) and is able to accommodate a diverse set of ligands including small molecules, peptides, and several chemokines, in each case resulting in receptor activation and ultimately arrestin recruitment[18, 52, 63-68]. Numerous CXCR4 antagonists, including AMD3100, TC14012 and vCCL2, act as agonists towards ACKR3. Recent screening by phage display of N-terminally-randomized CXCL12 also led to the selection of variants all displaying ACKR3 agonist properties[69]. Jointly, these results suggest that ACKR3 has a highly plastic binding pocket which funnels the diverse binding modes and unique contacts of its many ligands at the extracellular half of the receptor into a limited signaling repertoire (i.e. arrestin recruitment) arising from its intracellular half (Figure 7D). As such, it is tempting to speculate that the extracellular half of ACKR3 and its intracellular half may operate semi-autonomously, save for the ability of all ACKR3 ligands to set off an easily triggered 'tripwire' leading to arrestin recruitment[70].

ACKR3 was the only receptor studied for which activation was insensitive to changes in the N-loop, D-stereoisomer replacement, mutations and truncations at the proximal N termini of its chemokine ligands. Although recent structural data suggest that ACKR3 activation relies on a mechanism similar to classical receptors[66], the higher propensity of ACKR3 towards activation appears to depend on a less sophisticated mechanism, involving a simple, highly accessible molecular switch leading to the observed "agonism bias". Indeed, our findings suggest that the principal determinants for chemokine affinity and activation of ACKR3 are located between residues 2 to 9 of CXCL12 and CXCL11 with no or little contribution of the N-loop and the N-terminal residues. Therefore, different sub-pocket occupancy or structural changes may be required for ACKR3 as compared with CXCR4 and CXCR3, with activation determinants located not only at the bottom but also more on the outside of the orthosteric ligand binding pocket (Figure 7)[52]. To support this hypothesis, it has been demonstrated that charged residues located at the top of TM4, TM6, and in ECL2 (e.g., R197^{ECL2}, D179$^{4.60}$, and D275$^{6.58}$; Ballesteros-Weinstein nomenclature in superscript[71]) are crucial for activation by TC14012 and derivatives. Due to the localization of these residues to the upper part of ACKR3's TM domains and the roots of the ECLs, it was speculated that ACKR3-mediated β-arrestin-2 recruitment may not rely on a classical CRS2, characterized by activation toggle interactions located exclusively at the bottom of the ligand binding pocket[52]. Recent studies on ACKR3 further corroborate this hypothesis. Firstly, it was shown that CXCL11 and CXCL12 depend on many of the same residues as TC14012 in and adjacent to ACKR3's ECLs for binding and/or activation, as well as some newly described sites (e.g., E114$^{3.22}$ and K206^{ECL2}). Surprisingly, a mutation at the top of TM3 adjacent to ECL2 (K118$^{3.26}$A) caused constitutive β-arrestin

recruitment giving credence to the notion that arrestin signaling could also be triggered by the top of the ligand binding pocket[65]. Conversely, mutation of a tryptophan at the top of the cavity at the ECL2-TM5 junction (W208$^{5.34}$A) caused a decrease in β-arrestin recruitment for CXCL12 and a small molecule partial agonist binding at the bottom of ACKR3 ligand binding pocket[66].

These mechanistic considerations aside, the tendency of ACKR3 towards activation poses challenges to the discovery of efficient and specific antagonists. So far, no small molecules have been shown to decrease arrestin recruitment at ACKR3, illustrating the importance of understanding the interactions and mechanisms underlying its activation. Encouragingly, however, the recent discovery of two intracellular domain-targeting chemokine receptor antagonists at CCR2 and CCR9[72, 73] supports the feasibility of developing analogous antagonist at ACKR3.

4.3 Implications of N-terminal modifications on chemokine scavenging function of ACKR3

The higher permissivity of ACKR3 to ligand modifications, which at first sight may appear surprising, may be of functional importance with respect to its scavenging functions. Indeed, in addition to scavenging native chemokines to limit their agonist activity on CXCR4 and CXCR3, our results suggest that ACKR3 may also bind and clear N-terminally processed chemokine species, such as those resulting from the action of proteases, including dipeptidyl peptidase 4 (DPP4 or CD26). Cleavage of CXCL11 and CXCL12 is an efficient post-translational processing event that inactivates agonist activity towards CXCR3 and CXCR4, and leads to detectable serum levels of $CXCL11_{3\text{-}73}$ and $CXCL12_{3\text{-}73}$ [74, 75]. Although $CXCL11_{3\text{-}73}$ showed ~5-fold reduction in binding to ACKR3 compared to $CXCL11_{WT}$, the deletion of the first two residues of CXCL11 decreased binding to CXCR3 to a larger extent (~40 fold) and, more importantly, had only a modest effect on ACKR3 activation [74, 76]. Our results are in agreement with data from a recent study on proteolyzed CXCL12 and its interactions with CXCR4 and ACKR3[77].

4.4 Conclusion

This study represents the first comparative in-depth structure-function analysis of the importance of the chemokine N-terminal features for the binding to and activation of conventional and atypical chemokine receptors. Our results revealed unexpected contrasts between the structural motifs required for conventional and atypical receptors with CRS1 (binding to the N-loop) or CRS2 (binding to the chemokine N terminus) interactions important for CXCR3 and CXCR4 binding and activation, but dispensable for ACKR3. These data demonstrate that in addition to distinct functional roles, ACKR3 also presents an activation mechanism different from that of CXCR4 and CXCR3, with which it shares

chemokine ligands, supporting its recent classification as an ACKR[8]. However, whether the observations reported in this study are hallmarks of all ACKRs and CKRs remains to be investigated.

5. Highlights

- Chemokine N-loop contacts, which are crucial for CXCR4 and CXCR3 recognition, are not essential for ACKR3, suggesting that ACKR3 has a different chemokine recognition mode. This conclusion was based on the fact that contrarily to what was observed for classical receptors, $CXCL12_{1-9}$, $CXCL11_{1-9}$ and $vCCL2_{1-11}$ showed only slightly reduced potency in binding and activation assays to ACKR3 than the longer $CXCL12_{1-17}$ $CXCL11_{1-17}$ $vCCL2_{1-21}$ analogs, indicating that the first nine residues of chemokines foster the largest part of N-terminal binding and activation capacity to ACKR3.
- In line with these data, full length CXCL11 with substituted N-loop from the non ACKR3 ligand CXCL10 still almost retained $CXCL11_{wt}$ activity, whereas a $CXCL11_{N\ loop\ CXCL12}$ chimera showed significantly reduced activity towards CXCR3, again pointing towards a different chemokine recognition mode and a less crucial impact of chemokine N loop towards ACKR3, compared to classical receptors.
- While the first two N-terminal residues are crucial for chemokine binding to and activation of CXCR3 and CXCR4, they are less important for chemokine activity towards ACKR3, suggesting that ACKR3 could act as well as a scavenger for N-terminally inactivated chemokines generated following proteolysis by the Dipeptidyl peptidase 4 (DPP IV or CD26). This observation is further confirmed in **chapter 3**, where we show that ACKR3 is also able to become activated by N-terminally processed opioid peptides.
- Chemokine peptides with D-isomer replacements turn into antagonists for CXCR4 and CXCR3, but retain agonistic properties for ACKR3, highlighting the high propensity for activation of ACKR3.

Collectively, our data suggest that ACKR3 has a different interaction mode with chemokines than CXCR3 and CXCR4 and is much more activation prone. The molecular determinants of ACKR3 required for activation are less stringent and mostly depend on chemokine residues 3-9 with little contribution of the N-loop.

These results were recently confirmed by another group using a very similar approach, showing that activation of ACKR3 is much more tolerant for chemokine N-terminal substitutions or truncations and confirming ACKR3's high propensity for activation[78]. This trend to become activated upon ligand binding is undoubtedly also one of the reasons, why the development of small molecule ACKR3

antagonists is extremely challenging. However, the recent description of a first selective, potent and orally available ACKR3 antagonist shows that these challenges can be surmounted[79-81]. Development of such potent and specific antagonist are definitively of great interest, considering the various diseases and cancers where ACKR3 is implicated[82, 83].

This study, showing that ACKR3 in general has a high affinity and propensity for small peptides derived from chemokines prompted us to also focus on other peptide ligands of ACKR3, especially BAM22 and adrenomedullin, which share a similar length and show some degrees of sequence similarities with the chemokine N-terminal peptides that were used in this study.

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Chapter 2: Unravelling important structural features of ACKR3 for ligand binding: A receptor-based approach

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Adapted from: Szpakowska, M*., **Meyrath, M***., Reynders, N., Counson, M., Hanson, J., Steyaert, J., and Chevigne, A. (2018) "*Mutational analysis of the extracellular disulphide bridges of the atypical chemokine receptor ACKR3/CXCR7 uncovers multiple binding and activation modes for its chemokine and endogenous non-chemokine agonists"*, **Biochem Pharmacol** *153*, 299-309., This manuscript was selected to be part of the 60th anniversary Diamond Jubilee special Issue. * equal contribution.

After having uncovered that ACKR3 has a high propensity for activation compared to classical receptors CXCR3 and CXCR4, we wanted to analyze in a next step whether ACKR3 behaves also differently in regard of stability, ligand binding and activation when different structural determinants of the receptor were disrupted. The typical chemokine receptor architecture reveals a flexible N terminus followed by seven hydrophobic transmembrane-spanning α-helices (TM1-TM7), connected by three extracellular loops (ECL1-ECL3) and three intracellular loops (ICL1-ICL3). In this study, we were interested in the role of the highly conserved disulphide bridges formed by C117-C196 of ACKR3 (between the top of TM3/end of ECL1 and ECL2, present in a large majority of class A GPCRs) and by C34-C287 (between the N terminus and the top of TM7/end of ECL3, present in so far all tridimensional structures of chemokine receptors). Moreover, we were particularly intrigued by the role of a third disulphide bridge, formed by C21-C26 in the receptor's N terminus, creating a four-residue intra-N-terminal loop (TPA, for TetraPeptidyl Arch) which is unique to ACKR3 and with so far unknown functions. In order to analyze the influence of these different disulphide bridges on ACKR3 in regard of receptor stability and activity, we created cysteine-to-serine double mutants, disrupting each of the different disulphide bridges individually. Additionally, to get deeper insights into the function of the unique N-terminal disulphide bridge of ACKR3, we generated a mutant where the amino acid residues 22-25, comprising the TPA of ACKR3 were substituted to glycine.

Abstract

The atypical chemokine receptor ACKR3/CXCR7 plays crucial roles in numerous physiological processes but also in viral infection and cancer. ACKR3 shows strong propensity for activation and, unlike classical chemokine receptors, can respond to chemokines from both the CXC and CC families as well as to the endogenous peptides BAM22 and adrenomedullin. Moreover, despite belonging to the G protein-coupled receptor family, its function appears to be mainly dependent on β-arrestin. ACKR3 has also been shown to continuously cycle between the plasma membrane and the endosomal compartments, suggesting a possible role as a scavenging receptor. So far, the molecular basis accounting for these atypical binding and signaling properties remains elusive. Noteworthy, ACKR3 extracellular domains bear three disulphide bridges. Two of them lie on top of the two main binding subpockets and are conserved among chemokine receptors, and one, specific to ACKR3, forms an intra-N terminus four-residue-loop of so far unknown function. Here, by mutational and functional studies, we examined the impact of the different disulphide bridges for ACKR3 folding, ligand binding and activation. We showed that, in contrast to most classical chemokine receptors, none of the extracellular disulphide bridges was essential for ACKR3 function. However, the disruption of the unique ACKR3 N-terminal loop drastically reduced the binding of CC chemokines whereas it only had a mild impact on CXC chemokine binding. Mutagenesis also uncovered that chemokine and endogenous non-chemokine ligands interact and activate ACKR3 according to distinct binding modes characterized by different transmembrane domain subpocket occupancy and N-terminal loop contribution, with BAM22 mimicking the binding mode of CC chemokine N terminus.

1. Introduction

Chemokine receptors are class A G protein-coupled receptors (GPCRs) present at the surface of various cell types. By interacting with their cognate chemokines, they regulate vital cellular mechanisms, including cell trafficking, development, immune-modulation and adhesion as well as growth and survival[1]. They are also involved in pathological processes such as inflammation, cancer and HIV-1 infection[2, 3].

ACKR3, the atypical chemokine receptor 3, formerly known as CXCR7, is one of the most recently deorphanized chemokine receptors[4]. It is expressed in various cells such as B and T lymphocytes, neurons and endothelial cells and plays a crucial role in many processes including cardiovascular and neuronal development as well as in migration and homing of hematopoietic stem/progenitor cells[5-11]. ACKR3 is also present in many cancer cell types and on tumor-associated vasculature and accumulating evidence demonstrates its involvement in metastasis development[12-15]. ACKR3 binds to two endogenous chemokines, CXCL12 and CXCL11, which are also the ligands for CXCR4 and CXCR3,

respectively[10, 16-19]. In addition, ACKR3 and CXCR4 can interact with vCCL2, the human herpesvirus 8 (HHV-8)-encoded chemokine, which is an antagonist for a broad spectrum of chemokine receptors, including CXCR4, but acts as agonist towards ACKR3[19-22]. Unlike CXCR4 and CXCR3, which signal via G protein pathways, ACKR3 activity relies mainly on β-arrestin recruitment, although its ability to trigger signaling may be cell context-dependent[22-25]. In addition, ACKR3 is proposed to act as a scavenger or "sink" receptor for CXCL12, CXCL11 and vCCL2, regulating their availability for other chemokine receptors [19, 26-29]. Recently, it has also been shown that ACKR3 is a high-affinity receptor for BAM22, a non-chemokine peptide derived from the proenkephalin A family, which plays a role in the modulation of circadian glucocorticoid oscillation[30]. Similarly, based on phenotypic analysis of knock-out mice, ACKR3 was also proposed as a scavenger receptor for adrenomedullin (ADM), a pro-angiogenic peptide involved in the regulation of vascularisation[31].

Over the last few years, the tridimensional structures of several chemokine receptors, including CXCR4, have been resolved in complexes with small molecules or chemokines[32-38]. These structures revealed the typical receptor architecture comprising a flexible extracellular N terminus followed by a bundle of seven hydrophobic membrane-spanning α-helices (TM1-TM7) connected by three extracellular (ECL1-3) and three intracellular (ICL1-3) hydrophilic loops. In addition to the conserved disulphide bond linking the top of TM3 (end of ECL1) to the middle of ECL2, present in a large majority of class A GPCRs, all currently available tridimensional structures of chemokine receptors reveal a second disulphide bridge between the N terminus and the top of TM7 (end of ECL3)[39, 40]. As a consequence, the last residues of the receptor N terminus form an additional extracellular loop ("ECL4") connecting TM1 and TM7 and closing the receptor into a ring [39, 40] (Figure 1A and B). These structures together with recent mutational and functional studies were also key to refining our current understanding of chemokine-receptor interactions, moving from a simple two-step mechanism to a more continuous one characterized by extensive contacts between the two partners and 1:1 stoichiometry [33, 41-44]. These interactions involve the core of the chemokine, including the N-loop region, with the flexible N terminus of the receptor (chemokine recognition site 1, CRS1), a key determinant for selectivity [41, 43, 45] ensuring optimal chemokine orientation with respect to the top of the ligand-binding pocket and ECL4 (CRS1.5) [33, 39]. This enables the insertion of the flexible chemokine N terminus into the receptor transmembrane cavity (CRS2), made of the transmembrane segments and the extracellular loops, stabilizing an active state of the receptor and leading to intracellular signaling [33, 43, 46-48]. These tridimensional structures also showed that the orientation relative to the receptor varies for the different classes of chemokines and confirmed the presence of two distinguishable binding pockets - the major ligand binding pocket formed by the TM3, 4, 5, 6 proposed to be mainly occupied by the N

terminus of CXC chemokines, and the minor pocket delineated by TM1, 2, 3, 7 suggested to accommodate the N terminus of CC chemokines (Figure 1D) [33, 34, 48-50].

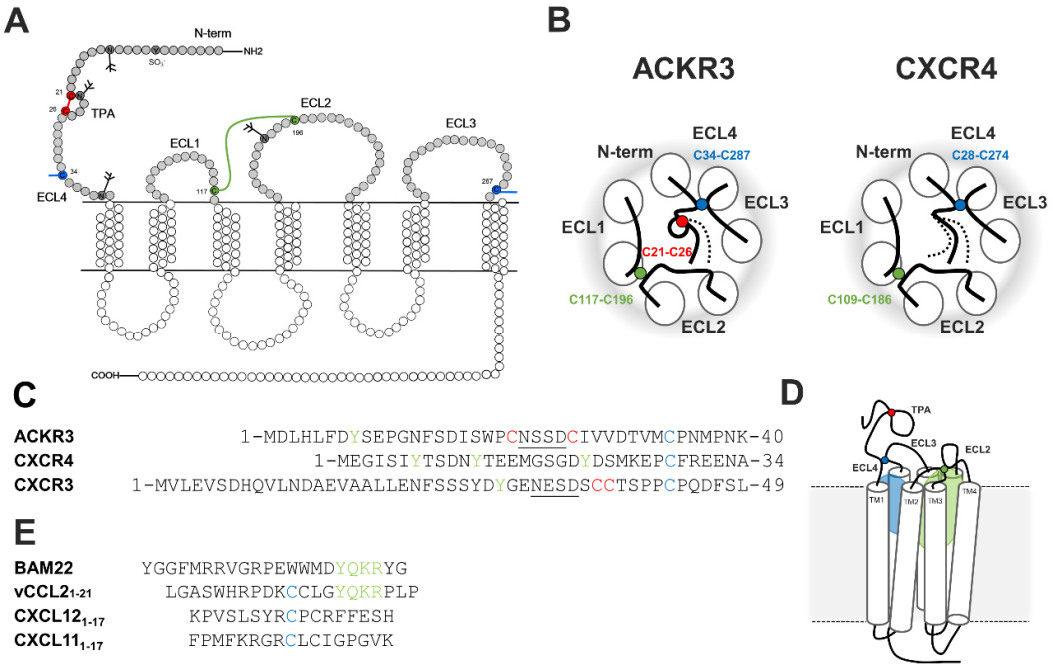

Figure 1. Localization and pairing of the extracellular disulphide bridges of ACKR3 and CXCR4. (**A**) Snake diagram of ACKR3. The putative post-translational modification sites identified in the N terminus and the extracellular loops are shown. Three potential disulphide bridges: within the N terminus (C21-C26), between the top of TM7 and the distal part of the N terminus (C34-C287) and between the top of TM3 and ECL2 (C117-C196) are colored in red, blue and green, respectively. Putative N-glycosylation sites (Ψ) and a sulfotyrosine (SO_3^-) are indicated in dark grey. (**B**) Representations in top-down view of the extracellular disulphides bridges in ACKR3 and CXCR4. The seven transmembrane segments are represented as white circles. The disulphide bridges connecting TM3 to ECL2 (highly conserved in class A GPCRs), the N terminus to TM7 (specific to chemokine receptors), or forming a unique ring within the ACKR3 N terminus are colored in green, blue and red, respectively. (**C**) Sequence comparison between the extracellular domains of ACKR3, CXCR4 and CXCR3. The sequences were aligned with respect to the cysteine residues predicted to be involved in the disulphide bridge with the top of TM7 (ECL4). (**D**) ACKR3 architecture showing the position of the different disulphide bridges on top of the major (green) and minor (blue) ligand binding pockets. (**E**) Sequence comparison between BAM22 and the N termini of chemokines. The sequences of BAM22 and vCCL2$_{1-17}$ were aligned with respect to the shared YQKR motif (green) at their C terminus. The sequences of chemokines N terminus were aligned with respect to their first cysteine residue (blue).

We have recently shown that ligand binding and activation of ACKR3 is in some measure different compared with CXCR4 and CXCR3[51]. Indeed, ACKR3 has a strong propensity to activation, possibly linked to its scavenging role, and as opposed to CXCR4 and CXCR3, the most N-terminal residues of the chemokine ligands as well as the N-loop have a minor importance for ACKR3 binding and activation. Moreover, vCCL2 seems to stabilize a different conformation of ACKR3 than do its endogenous CXC chemokine ligands, whereas very little is known on the binding mode of BAM22 and adrenomedullin to ACKR3[19, 30].

An intriguing particularity of ACKR3, which may contribute to its atypical nature, is the presence of two additional cysteine residues at positions 21 and 26, which have recently been shown to be linked by a disulphide bridge, giving rise to an intra-N terminus four-residue loop (Figure 1)[32, 39, 52]. With the exception of CXCR3 that bears two additional successive cysteines at positions 37 and 38, other chemokine receptors do not have analogous cysteine residues. Given the crucial role of the flexible receptor N terminus in chemokine recognition, the intra-N terminus loop in ACKR3 could account for the existence of either a unique receptor structure or an unusual mode of interaction with its ligands[39, 41].

Here, by targeted mutagenesis, we examined the role of the extracellular disulphide bridges in chemokine receptors CXCR4 and ACKR3. Using various binding and functional assays, we investigated the role of the unique ACKR3 intra-N terminus disulphide bridge for receptor-ligand interactions and compared the importance of the ECL4-forming disulphide bridge as well as the one linking TM3 and ECL2 for chemokine binding, receptor integrity and activation. We demonstrated that, in contrast to classical chemokine receptors, the other conserved extracellular disulphide bridges are not essential for ACKR3 surface expression, ligand binding and activation. In addition, we showed that the unique N-terminal loop of ACKR3 is important for activation by CC but not CXC chemokines and that BAM22 mimics the binding mode of CC chemokine N terminus.

2. Material and methods

Peptides and chemokines

Chemokines CXCL12, CXCL11, vCCL2, CXCL10 and CXCL9 were purchased from PeproTech. Alexa Fluor 647-labelled CXCL12 (CXCL12-AF647) was purchased from Almac. BAM22 and adrenomedullin (ADM) were purchased from Bachem. Peptides derived from the N terminus of chemokines and peptide TPA (21-CNSSDC-26, with C21-C26 cyclization) were purchased from JPT. Peptides CCS (encompassing residues 1-MDLHLFDYSEPGNFSDISWPCNSSDCIVVDTVMSPNMPNKS-40, with a disulphide bridge between cysteines 21 and 26 and a cysteine-to-serine mutation at position 34), SSS (encompassing residues 1-MDLHLFDYSEPGNFSDISWPSNSSDSIVVDTVMSPNMPNKS-40, in which the three cysteine residues were mutated to serines) and SSS_{scrbld} (1-PPYDVISSMLKDSISNENFVSLPNGPSDTVHMSWDNFMDS-40, in which all residues were randomly permuted) were purchased from ChinaPeptides. All peptides contain a free amine at the N terminus and an amide group at the C terminus to avoid additional negative charge.

Generation of U87 cell lines expressing wild-type and mutant ACKR3 and CXCR4

pBABE.puromycin vectors (Addgene) encoding wild type ACKR3, variants bearing double cysteine-to-serine mutations (C21S-C26S, C34S-C287S and C117S-C186S) or glycine-substituted variant (C21-G_4-C26), and wild-type CXCR4 or C28S-C274S or C109S-C186S mutants were transfected into U87 cells[51]. This cellular background was chosen for its absence of endogenous CXCR4, ACKR3 and CXCR3 as previously demonstrated[19]. Cells stably expressing the modified receptors were obtained following puromycin selection and subsequent single-cell sorting using BD FACSAria II cell sorter (BD Biosciences). The presence of the mutations and the surface expression level of the mutated receptors were verified by genomic DNA sequencing and flow cytometry using antibodies recognizing the proximal N-terminal part of CXCR4 (clone 4G10, Santa Cruz Biotechnology) or ACKR3 (clones 11G8, R&D Systems and 9C4, MBL Life Science). CXCR3 in transiently transfected U87 cells was detected using the mAb 1C6 (BD Pharmingen).

Chemokine and peptide binding

U87 cells expressing wild-type or mutant ACKR3 or CXCR4 were distributed into 96-well plates (15 × 10^4 cells per well) and incubated with Alexa Fluor 647-labelled CXCL12 at concentrations ranging from 35 pM to 115 nM for 90 minutes on ice or for 45 minutes at 37°C, respectively. Non-specific binding of CXCL12-AF647 was evaluated on ACKR3- and CXCR4-negative U87 cells and subtracted. For binding competition studies, U87 cells expressing wild-type or mutant ACKR3 or CXCR4 were incubated with CXCL12-AF647 at concentrations equivalent to twice the EC_{50} values determined for each ACKR3 variant in saturation binding experiments (Table 1) or 11.5 nM for CXCR4, mixed with unlabeled CXCL12, CXCL11, vCCL2, CXCL10, BAM22 or ADM at concentrations ranging from 6 pM to 1 μM. All binding experiments were performed in PBS containing 1 % BSA and 0.1 % NaN_3 (FACS buffer). Nonspecific chemokine binding was evaluated by the addition of 250-fold excess of unlabeled CXCL12 and the signal obtained was used to define 0 % specific binding. The signal obtained for CXCL12-AF647 in the absence of unlabeled chemokines was used to define 100 % binding. Chemokine binding was quantified by mean fluorescence intensity on a BD FACS Fortessa cytometer (BD Biosciences).

β-arrestin recruitment

β-arrestin-2 recruitment to wild-type and mutant ACKR3, CXCR4 or CXCR3 induced by chemokines, chemokine N terminus-derived peptides or BAM22 was monitored by NanoLuc complementation assay (NanoBit, Promega)[51, 53, 54]. 1.2 × 10^6 U87 cells were plated in 10 cm-culture dishes and 48 hours later transfected with pNBe vectors containing human β-arrestin-2 N-terminally fused to LgBiT and receptors C-terminally fused to SmBiT. 48 hours post-transfection cells were harvested, incubated 40 minutes at 37°C with 200-fold diluted Nano-Glo Live Cell substrate and distributed into white 96-well

plates (5×10^4 cells per well). β-arrestin-2 recruitment in response to chemokines, N terminus derived peptides or BAM22 was evaluated after 10-minute incubation with a Mithras LB940 luminometer (Berthold Technologies). For each receptor and each experiments the maximum signal recorded with a saturating concentration (200 nM) of full agonist (i.e CXCL12 for ACXCR3 and CXCR4) was set as 100%.

Neutralisation of mAb 9C4 by ACKR3 N-terminal peptides

The mAb 9C4 (3.3 µg/ml) was first incubated for 30 minutes at room temperature with peptides CCS, SSS (100 nM) or TPA (100 µM) derived from the N terminus of ACKR3. Peptide SSS_{scrbld} (100 nM) was used as negative control. The antibody-peptide mix was then incubated for 60 minutes at 4°C with U87 cells expressing wild-type ACKR3 (15×10^4 cells/well in a 96-well plate). The binding of 9C4 to ACKR3 was revealed with an allophycocyanin-conjugated goat anti-mouse IgG $F(ab')_2$ (Jackson ImmunoResearch) and quantified by mean fluorescence intensity on a BD FACS Fortessa cytometer (BD Biosciences).

Immunocytochemistry and fluorescent imaging

For microscopic analysis of CXCR4 and ACKR3 distribution, 5×10^4 U87 cells were plated on sterile coverslips in a 24-well plate and cultured overnight. Cells were transiently transfected with equal amounts of pBABE plasmid encoding wild type or mutated CXCR4 or ACKR3 using X-tremeGENE 9. 48 hours later, cells were washed with PBS and fixed for 20 minutes on ice with 4% (w/v) paraformaldehyde. After one washing step with PBS supplemented with 50mM NH_4Cl and two washes with PBS, cells were permeabilised with 0.1 % Triton X-100 for 20 minutes at room temperature. Subsequently, cells were blocked with 10% (w/v) normal goat serum for 1 hour and incubated overnight at 4°C with the mAb 4G10 (Santa Cruz Biotechnology) diluted 1:75 for CXCR4 or the mAb 11G8 (R&D Systems) diluted 1:100 for ACKR3 staining in 1 % BSA PBS, respectively. After three washing steps, cells were incubated for 1 hour at room temperature with a goat anti-mouse Alexa Fluor 647-conjugated secondary antibody (abcam) diluted 1:1200 in PBS complemented with 5 % normal goat serum. Cells were washed twice, stained with Hoechst 33342 dye (Sigma) for 10 minutes at 4°C, washed twice and mounted on glass slides with 5 µl ProLong Diamond anti-fade mounting medium (Molecular Probes). Images were acquired using a 63× oil-immersion objective on a Zeiss Axio Observer Z1 microscope equipped with an Apotome.2 and a Colibri LED illumination system. Representative images of two independent experiments are shown.

Data and statistical analysis

Concentration-response curves were fitted to the four-parameter Hill equation using an iterative, least-squares method (GraphPad Prism version 7.02) to provide pEC_{50}, pIC_{50}, EC_{50} or IC_{50} values, standard errors of the mean (SEM) and the Hill coefficient. Fitting was performed on data from at least

three independent experiments (n=3). Unpaired t tests were used to analyze the differences in pEC_{50}/ pIC_{50} for each ligand using the wild-type receptor as reference. p value of <0.05 was considered as statistically significant.

3. Results

3.1 Extracellular disulphide bridges are dispensable for ACKR3 surface expression and folding

The conserved disulphide bridges:

First, the importance for ACKR3 conformational fidelity of the disulphide bridge between the N terminus and the top of TM7 (C34-C287) as well as the one between TM3 and ECL2 (C117-C196) was evaluated and compared to CXCR4 (Figure 1A and B). The effect of cysteine pair mutations on the receptor cellular distribution was monitored by flow cytometry and fluorescence microscopy using antibodies detecting linear epitopes located in the receptor N terminus (11G8 for ACKR3 and 4G10 for CXCR4) or recognizing more complex epitope spread over different extracellular domains (8F11-M16 for ACKR3 and 12G5 for CXCR4).

In line with previous reports, a higher proportion of the wild-type ACKR3 was present intracellularly compared to the cell surface, while CXCR4 was localized at the plasma membrane (Figure 2E). Mutation of the cysteine pair engaged in the disulphide bridge between the N terminus and top of TM7 (the ECL4-forming bridge), typical of chemokine receptors, had little effect on ACKR3 (C34S-C287S) and CXCR4 (C28S-C274S) surface expression but had strong consequences for CXCR4 architecture, as shown by the inability of the conformational mAb 12G5 to recognize the receptor (Figure 2 A and B). In contrast, recognition of the ACKR3.C34S-C287S variant by the conformational mAb 8F11-M16 was equivalent to that of wild-type receptor. The disruption of the TM3-ECL2 bridge (C117S-C196S) only moderately affected the ACKR3 distribution (Figure 2E), in stark contrast to CXCR4, for which the equivalent mutation (C109S-C186S) abolished the receptor surface expression (Figure 2B) and resulted in a strong intracellular retention (Figure 2E). Nonetheless, a 75% reduction of ACKR3 surface expression was observed with the conformational mAb 8F11-M16 compared to the wild-type receptor (Figure 2A), indicating that the TM3-ECL2 bridge does play a role in ACKR3 structural integrity, although it is not critical for receptor export.

Overall, these results show that the receptor surface expression and architecture in the absence of disulphide bridges was much less affected for ACKR3 than for CXCR4.

The atypical disulphide bridge of ACKR3:

Next, the potential role played by the unique four-residue intra-N-terminal loop of ACKR3 formed by the disulphide bridge between residues C21 and C26 (TPA for TetraPeptidyl Arch) in receptor

expression, localization and conformation was assessed. No difference in ACKR3 surface expression or folding was observed when each of the four residues within the loop (NSSD) was substituted with a glycine (C21-G_4-C26) (Figure 2A and E). The disruption of the loop-forming cysteine bridge (C21S-C26S) did however have an effect on the receptor cellular distribution, resulting in an apparent 40 % increase in surface expression compared with wild-type ACKR3 (Figure 2A and E). Similar tendency was observed for CXCR3 variant in which the two N-terminal cysteines were mutated to serines (C37S-C38S) (Figure 2C).

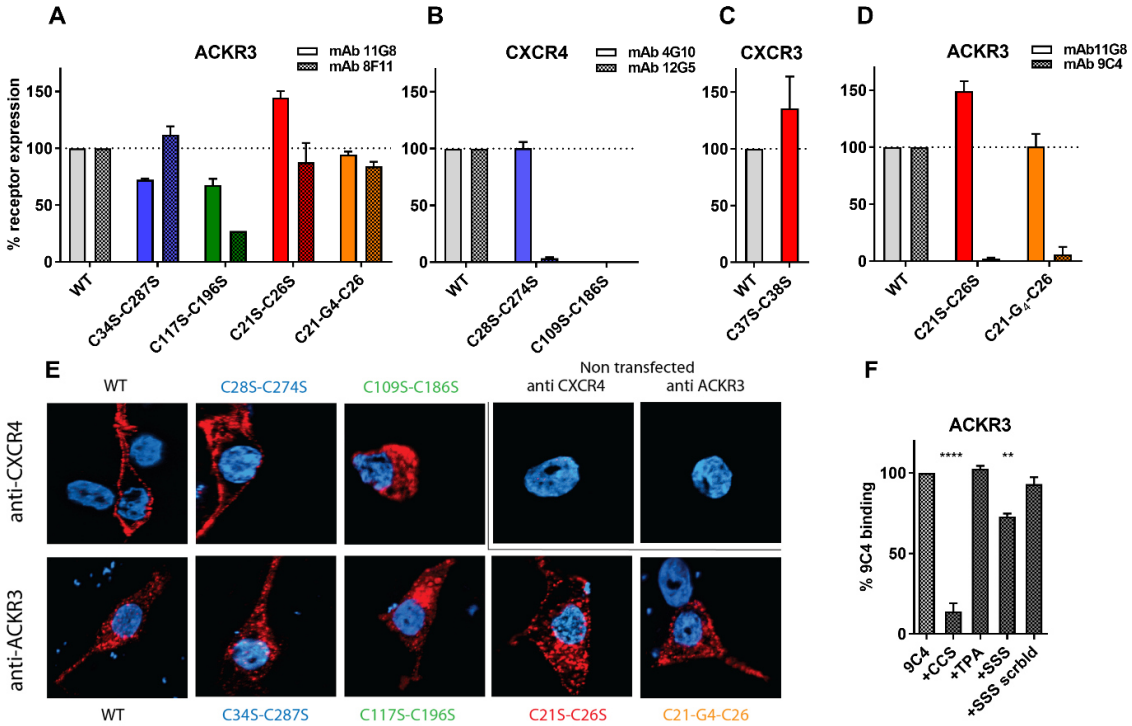

Figure 2. Surface expression and folding of receptor cysteine variants and 9C4 recognition of ACKR3. (A and **B)** Receptor surface expression in U87 cells transiently transfected with pBABE vectors encoding wild-type and mutant ACKR3 (A) and CXCR4 (B) revealed with monoclonal antibodies recognizing linear N terminus (11G8 and 4G10) or conformational (8F11-M16 and 12G5) epitopes and quantified by flow cytometry. **(C)** Receptor surface expression in U87 cells transiently transfected with a vector encoding wild-type and C37S-C38S variant of CXCR3 revealed with the mAb 1C6 and quantified by flow cytometry. Values represent the mean ± standard error of the mean (SEM) of at least three independent experiments. **(E)** Cellular localization of the wild-type and cysteine variants of CXCR4 (top) and ACKR3 (bottom) monitored by fluorescent microscopy using mAb 4G10 or 11G8 and a secondary Alexa Fluor 647-conjugated antibody (red). The nuclear DNA was stained with Hoechst (blue). A representative picture of at least 10 acquired images from two independent experiments is shown. **(D** and **F)** mAb 9C4 recognition of ACKR3. (**D**) Receptor surface expression in U87 cells transiently transfected with pBABE vectors encoding wild-type and TPA-mutated ACKR3 revealed with mAbs 11G8 and 9C4 recognizing the receptor N terminus. (**F**) 9C4 neutralization by peptides derived from the N terminus of ACKR3. 9C4 (3.3 µg/ml) binding in the presence of peptides CCS, SSS, SSS_{scrbld} (100 nM) and TPA (100 uM) was monitored in U87.ACKR3 cells by flow cytometry. Values represent the mean ± standard error of the mean (SEM) of at least three independent experiments. ** $p < 0.01$,**** $p < 0.0001$, NS: not significant.

Surprisingly, although the mutants C21S-C26S and C21-G_4-C26 were detected at the cell surface using the mAb 11G8, they were not recognized by the mAb 9C4, also raised against the N-terminal domain of ACKR3 (Figure 2D)[55]. In line with this observation, 9C4 binding to the wild-type ACKR3 was reduced by 86 % in the presence of the cyclic peptide CCS, derived from the N terminus of ACKR3 and bearing the TPA, whereas the equivalent linear SSS peptide caused only a 25 % reduction (Figure 2F). Peptide SSS_{scbld} had no effect on 9C4 binding to the wild-type receptor. These results show that the four-residue intra-N-terminal loop of ACKR3 (TPA) is a crucial contributor of the 9C4 binding, although it is not the sole determinant of its epitope as demonstrated by the inability of the shorter cyclic peptide comprising only the TPA (C21-NSSD-C26) to neutralize the antibody binding to the wild-type receptor, even at a concentration as high as 100 µM. In addition, these data reveal that the TPA is well exposed and accessible to the extracellular ligands and makes mAb 9C4 a useful probe to evaluate its presence on the receptor in different cellular contexts.

3.2 CXC and CC chemokines have different binding and activation modes on ACKR3

The binding of Alexa Fluor 647-labelled CXCL12 to cysteine-mutated ACKR3 and CXCR4 was first evaluated and compared in U87 cell lines stably expressing wild-type or mutated receptor.

In contrast to CXCR4, all cysteine mutants of ACKR3 retained their ability to bind CXCL12-AF647, although the maximal binding and potency were affected to different extents. The mutation of the TM3-ECL2 cysteine bridge (C117S-C196S) had the most pronounced effect, resulting in a marked reduction of potency because of which no EC_{50} nor maximum binding could be determined (Figure 3B). The mutation of the ECL4-forming cysteine bridge linking the N terminus and TM7 of ACKR3 had less effect, but still led to a 60 %-decrease of the maximum CXCL12-AF647 binding and a significant 4-fold increase in EC_{50} as compared to the wild-type receptor (Figure 3B, Table 1). Interestingly, the analogous mutant of CXCR4 (C28S-C274S) was no longer capable of binding CXCL12 (Figure 3A and Table 1), further confirming the stronger tolerance of ACKR3 to alterations in TM-linking disulphide bridges. The C21-G_4-C26 mutant showed an EC_{50} value equivalent to that of wild-type ACKR3, while the C21S-C26S mutation led to an approximately two-fold increase in EC_{50} (Figure 3B, Table 1) indicating that it is the constraint brought by the intra-N terminus loop rather than its decorating residues that plays a role in CXCL12 binding.

The effect of cysteine mutations on the ACKR3 interactions with its other chemokine ligands was then evaluated in binding competition studies with CXCL12-AF647. CXCL11 binding to mutant ACKR3.C34S-C287S was reduced by approximately two-fold compared to the wild-type receptor, while vCCL2 binding was improved by nearly 4-fold (Figure 3C and D, Table 1). Both CXCL11 and vCCL2 were less potent in displacing CXCL12-AF647 from ACKR3.C21S-C26S where the binding of the viral chemokine

was the most impacted (2.4- and 14.3-fold increase in IC_{50} values respectively). Binding of CXCL11 and vCCL2 to the mutant C21-G_4-C26 was affected to a similar extent (3-fold increase in IC_{50} values), despite the absence of effect of the mutation on CXCL12-AF647 binding. These results suggest that the presence of the TPA influences the binding of all chemokines with a significantly higher effect on vCCL2 binding while the residues of the TPA themselves appear to be involved in interactions with vCCL2 and CXCL11 but not CXCL12.

The effect of disulphide bridge disruption on ACKR3 activation was then monitored in a β-arrestin-2 recruitment assay. Overall two clearly contrasting trends could be observed in how disrupting the disulphide bridges affected the ability of chemokines to activate ACKR3, one - for the two CXC chemokines, CXCL12 and CXCL11, second - for the CC chemokine vCCL2. Firstly, β-arrestin-2 recruitment induced by CXCL12 and CXCL11 was only negatively affected by the mutation disrupting the TM3-ECL2 disulphide bridge located on top of the major ligand-binding pocket (6.5- and 6.0-fold increase in EC_{50} values, respectively) (Figure 3F and G, Table 2). This mutation however had no effect on vCCL2 potency to induce β-arrestin-2 recruitment (Figure 3H, Table 2). Interestingly, whereas the TM3-ECL2 mutation resulted in a non-functional CXCR4, in case of ACKR3 it allowed CXCL11 and vCCL2 to achieve efficacies equivalent to CXCL12, as opposed to their partial response (75 %) observed with the wild-type receptor and all the other cysteine mutants. The mutation of ECL4 (C34S-C287S) did not affect β-arrestin-2 recruitment to ACKR3 for any of the ligands (Figure 3F-H), but had a strongly negative effect on CXCL12-induced recruitment to the equivalent mutant (C28S-C274S) of CXCR4 (Figure 3E). β-arrestin-2 recruitment to the TPA-modified ACKR3 variants in response to CXCL12 and CXCL11 was comparable to the wild-type receptor, whereas it was considerably impaired in response to vCCL2 (4.6- and 6.6- fold reduction for the C21S-C26S and C21-G_4-C26 mutants, respectively), showing the importance of the TPA in the vCCL2-induced ACKR3 activation. By comparison, mutagenesis of cysteine residues at position 37 and 38 of the CXCR3 N terminus had no significant impact on the receptor activation by CXCL11, CXCL10 but decreased the potency of CXCL9 by more than 2 fold (Table 4).

Overall, these data reveal that whereas all cysteine-mutated ACKR3 variants were able to bind and efficiently respond to chemokine ligands, the disruption of the disulphide bridges in CXCR4, without exception, led to severe impairment of the receptor functionality. Furthermore, the differences in the impact of cysteine pair mutations for the CXC chemokines on one side and vCCL2 on the other clearly point to distinct binding and activation modes for ACKR3, either largely relying on the TM3-ECL2 disulphide bridge or on the N-terminal TPA (Figure 5).

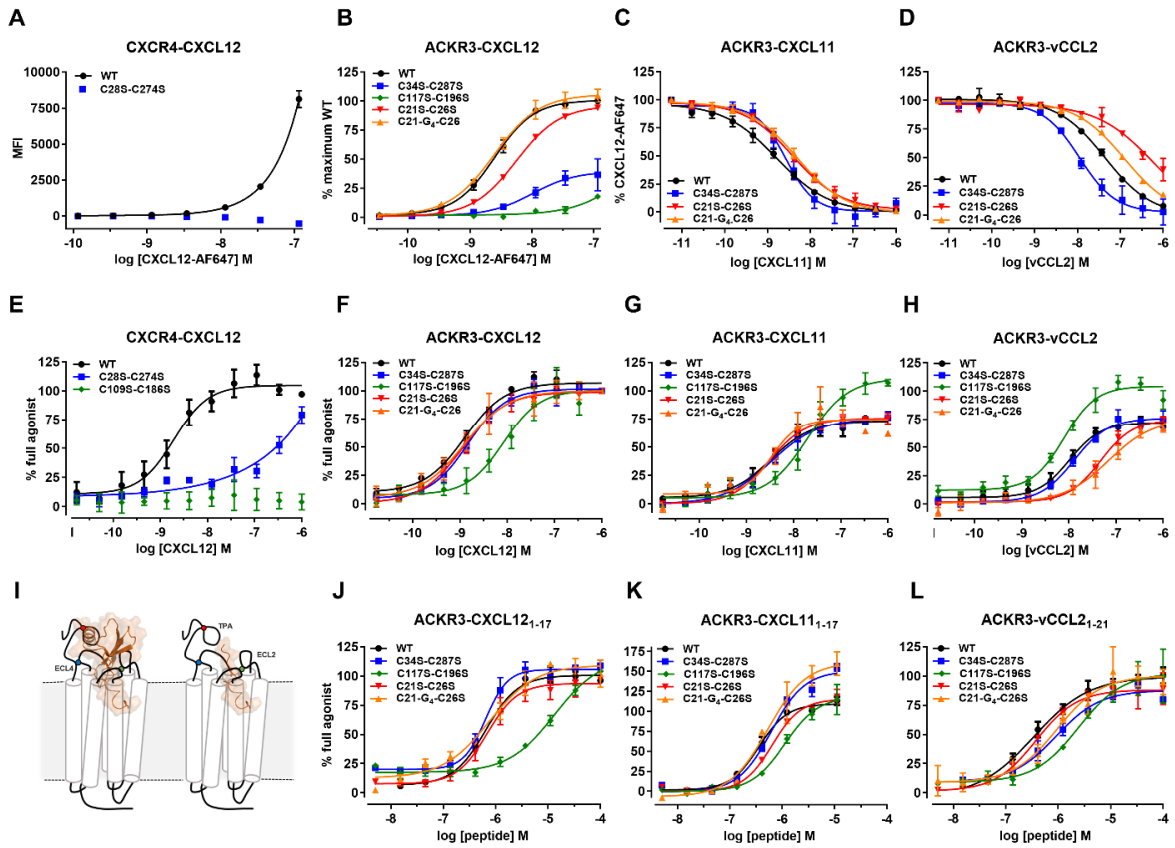

Figure 3. Binding and activation of cysteine-mutated receptors by chemokine ligands. (A-B) Binding of Alexa Fluor 647-labelled CXCL12 to wild-type and cysteine mutants of CXCR4 (A) and ACKR3 (B) monitored by flow cytometry. CXCL12-AF647 binding to ACKR3 was normalized to receptor cell surface expression based on staining with mAb 8F11-M16. **(C-D)** Binding of CXCL11 (C) and vCCL2 (D) to wild-type and mutated ACKR3 assessed by binding competition with CXCL12-AF647 and analyzed by flow cytometry. Binding of CXCL12-AF647 in the absence of unlabeled chemokines was considered as 100 %, whereas binding in the presence of a 250-fold excess of unlabeled CXCL12 was used to define 0 %. **(E-H and J-L)** β-arrestin-2 recruitment to CXCR4 (E) and ACKR3 (F-L) variants induced by CXCL12 (E and F), CXCL11 (G) vCCL2 (H), $CXCL12_{1-17}$ (J), $CXCL11_{1-17}$ (K) and $vCCL2_{1-21}$ (L). **(I)** Schematic representation of the interaction between ACKR3 and a full-length chemokine (left) or a peptide derived from its N terminus (right). For all panels, values represent the mean ± standard error of the mean (SEM) of at least three independent experiments.

To gain further insight into the determinants involved in the recognition and activation of ACKR3, peptides derived from the N-terminal region of CXCL12, CXCL11 and vCCL2 were tested for their ability to induce β-arrestin-2 recruitment to the wild-type and mutated receptors. Similarly to what has previously been shown for the wild-type ACKR3[51], CXCL12-, CXCL11- and vCCL2-derived peptides covering the flexible N terminus, the cysteine motif and the N loop ($CXCL12_{1-17}$, $CXCL11_{1-17}$ and $vCCL2_{1-21}$) were able to trigger β-arrestin-2 recruitment to the cysteine-mutated receptors, although differences were observed between the peptides. The mutation of the cysteine bridge between TM3 and ECL2, covering the major binding pocket, strongly reduced the activity of $CXCL12_{1-17}$ and $vCCL2_{1-21}$ (EC_{50} increase by >20 and 6.5 fold, respectively) and to a lesser extent that of $CXCL11_{1-17}$ (3 fold),

suggesting that small peptides directly targeting the transmembrane pocket require this structure for efficient receptor activation. In contrast, the disruption of the disulphide bridge between the N terminus and TM7 stabilizing the minor binding pocket had no effect on $CXCL12_{1-17}$ activity but slightly decreased that of $CXCL11_{1-17}$ and $vCCL2_{1-21}$ (1.7 and 2.3 fold respectively) (Figure 3J-L).

Finally, the mutation of cysteine residues forming the TPA (C21S-C26S) had no effect on the activity of $CXCL12_{1-17}$ and slightly reduced that of $CXCL11_{1-17}$ (1.8 fold). Surprisingly however, this mutation had little impact on $vCCL2_{1-21}$, in contrast to what was observed for full-length vCCL2, indicating that the core of the viral chemokine is the main contributor in the interactions with TPA (Figure 3H and L).

3.3 The endogenous peptide BAM22 reveals a vCCL2-like binding and activation mode towards ACKR3

Among the different non-chemokine ligands described to bind to ACKR3, only BAM22, a 22-amino acid peptide showing sequence similarities with chemokine N termini (Figure 1E), was able to compete with the binding of labelled CXCL12 to wild-type ACKR3 (IC_{50} = 43.4 nM, pIC_{50}= 7.36 ± 0.05). No displacement was observed with adrenomedullin (ADM) in the concentration range tested (Figure 4A).

BAM22 was then tested towards the cysteine mutants to provide the first information about its binding and activation modes. In line with what has previously been reported, fluorescently labeled CXCL12 was displaced by nanomolar concentrations of unlabeled BAM22[30]. Interestingly, reminiscent of what was observed for vCCL2, the mutation C34S-C287S disrupting ECL4, reduced the IC_{50} of BAM22 binding by nearly two-fold compared to the wild-type receptor (Figure 4B).

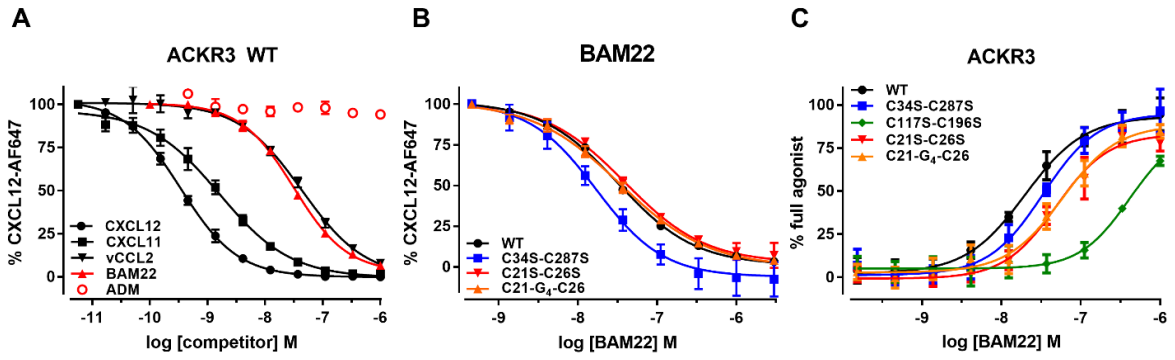

Figure 4. Binding and activation of non-chemokine ligands to the wild-type and cysteine-mutated ACKR3. (A) Binding of CXCL12, CXCL11, vCCL2, BAM22 and adrenomedullin (ADM) to wild-type ACKR3 assessed by binding competition with CXCL12-AF647 and analyzed by flow cytometry. **(B)** Binding of BAM22 to WT and mutated ACKR3 assessed by binding competition with CXCL12-AF647 and analyzed by flow cytometry. **(C)** β-arrestin-2 recruitment to wild-type ACKR3 and cysteine-mutated variants induced by BAM22. Values represent the mean ± standard error of the mean (SEM) of at least three independent experiments.

Table 1. Binding properties of full-length chemokines and BAM22 towards ACKR3.

ACKR3	Binding												
	CXCL12-AF647				CXCL11			vCCL2			BAM22		
	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)*	pIC_{50} ± SEM	IC_{50} (nM)	Fold WT	pIC_{50} ± SEM	IC_{50} (nM)	Fold WT	pIC_{50} ± SEM	IC_{50} (nM)	Fold WT
WT	8.60 ± 0.04	2.5	1.0	100	8.79 ± 0.07	1.6	1.0	7.36 ± 0.05	43.4	1.0	7.49 ± 0.03	32.2	1.0
C34S-C287S	7.99 ± 0.18	10.2	4.1	40 ± 7	8.58 ± 0.07	2.7	1.7	7.95 ± 0.08	11.2	0.3	7.77 ± 0.11	17.1	0.5
C117S-C196S	< 6.3	>500	> 200	ND	ND	ND	-	ND	ND	-	ND	ND	-
C21S-C26S	8.25 ± 0.02	5.6	2.2	96 ± 1	8.41 ± 0.06	3.9	2.4	6.21 ± 0.15	622.5	14.3	7.40 ± 0.06	39.4	1.2
C21-G_4-C26	8.61 ± 0.06	2.5	1.0	106 ± 4	8.32 ± 0.05	4.8	3.0	6.90 ± 0.03	124.6	2.9	7.46 ± 0.05	34.4	1.1

CXCL12-AF647 binding to ACKR3 mutants was normalised to receptor cell surface expression based on staining with mAb 8F11-M16 considering WT receptor as 100%.

Table 2. β-arrestin-2 recruitment induced by full-length chemokines and BAM22 to ACKR3.

ACKR3	β-arrestin-2 recruitment															
	CXCL12				CXCL11				vCCL2				BAM22			
	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)
WT	8.89 ± 0.07	1.27	1.0	100	8.48 ± 0.11	3.35	1.0	73 ± 4	7.99 ± 0.08	10.35	1.0	72 ± 3	7.70 ± 0.10	20	1.0	93 ± 5
C34S-C287S	8.83 ± 0.07	1.44	1.1	100	8.47 ± 0.10	3.41	1.0	75 ± 3	7.88 ± 0.07	13.08	1.3	76 ± 3	7.48 ± 0.10	33	1.7	95 ± 6
C117S-C196S	8.07 ± 0.13	8.45	6.5	100	7.70 ± 0.11	20.01	6.0	111 ± 7	8.10 ± 0.08	7.96	0.8	104 ± 4	>6.40	>400	>20	ND
C21S-C26S	8.93 ± 0.09	1.17	0.9	100	8.51 ± 0.05	3.07	0.9	75 ± 2	7.33 ± 0.05	47.31	4.6	75 ± 3	7.31 ± 0.09	49	2.5	83 ± 6
C21-G_4-C26	8.90 ± 0.16	1.25	1.0	100	8.47 ± 0.15	3.42	1.0	74 ± 5	7.16 ± 0.19	68.79	6.6	75 ± 7	7.24 ± 0.11	57	2.9	89 ± 7

β-arrestin-2 recruitment was monitored in U87 cells using split Nanoluciferase complementation assay (n =3).

Table 3. β-arrestin-2 recruitment induced by peptides derived from chemokine N-terminal regions to ACKR3.

ACKR3	β-arrestin-2 recruitment											
	$CXCL12_{1-17}$				$CXCL11_{1-17}$				$vCCL2_{1-21}$			
	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)
WT	6.22 ± 0.04	610	1.0	101 ± 2	6.45 ± 0.04	360	1.0	110 ± 3	6.45 ± 0.09	360	1.0	99 ± 4
C34S-C287S	6.23 ± 0.7	600	1.0	106 ± 3	6.21 ± 0.05	620	1.7	151 ± 6	6.10 ± 0.09	790	2.2	89 ± 4
C117S-C196	4.85 ± 0.18	14270	23.4	104 ± 4	5.95 ± 0.09	1120	3.1	113 ± 10	5.63 ± 0.16	2340	6.5	104 ± 9
C21S-C26S	6.18 ± 0.09	660	1.1	94 ± 4	6.19 ± 0.04	650	1.8	115 ± 4	6.43 ± 0.10	370	1.0	88 ± 4
C21-G_4-C26	6.20 ± 0.12	630	1.0	106 ± 10	6.27 ± 0.10	540	1.5	160 ± 12	6.04 ± 0.14	920	2.6	100 ± 7

β-arrestin-2 recruitment was monitored in U87 cells using split Nanoluciferase complementation assay (n =3).

Table 4. β-arrestin-2 recruitment induced by full-length chemokines to CXCR3.

CXCR3	β-arrestin-2 recruitment											
	CXCL11				CXCL10				CXCL9			
	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)
WT	8.03 ± 0.19	9.4	1.0	100	7.60 ± 1.17	25.7	1.0	45 ± 5	7.03 ± 0.2	92.9	1.0	46 ± 7
C37S-C38S	8.68 ± 0.10	8.7	0.9	100	7.41 ± 0.18	38.5	1.5	65 ± 3	6.60 ± 0.15	226.4	2.5	54 ± 4

β-arrestin 2 recruitment was monitored in U87 cells using split Nanoluciferase complementation assay (n=3)

However, in contrast to vCCL2, mutations of the TPA had no effect on BAM22 binding to ACKR3. BAM22 was also able to induce β-arrestin-2 recruitment towards all ACKR3 mutants (Figure 4C). The strongest impairment was observed with the C117-C196 mutant, just like for the CXC chemokines, all small chemokine N-terminal peptides but not for vCCL2. While the mutation in ECL4 had a modest effect on ACKR3 functionality, the two mutations affecting the TPA significantly influenced the potency of BAM22 to induce β-arrestin-2 recruitment (2.5 fold for C21S-C26S and 2.9 fold for C21-G_4-C26, respectively), comparable to what was observed for vCCL2. These data suggest that the endogenous peptide BAM22 has a vCCL2-like ACKR3 binding and activation modes, consistent with its sequence homology with the N-terminal region of vCCL2 (Figure 1E, Figure 5).

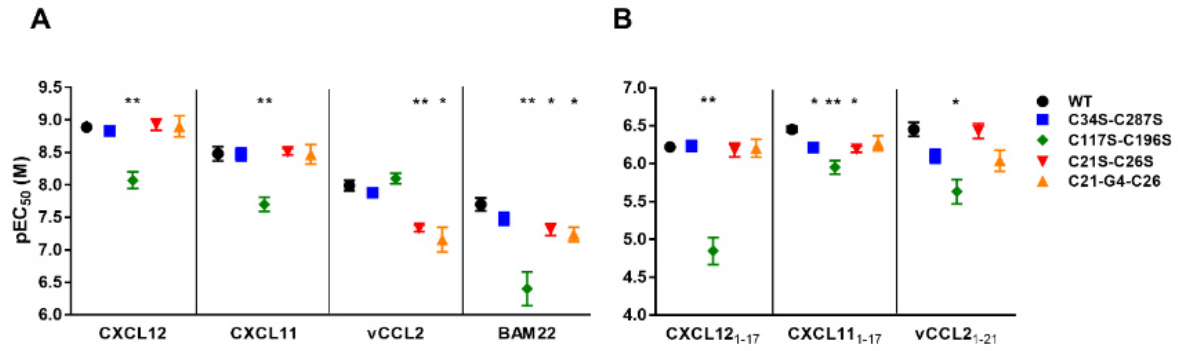

Figure 5. Overview of the effects of cysteine mutation on ACKR3 activity. ACKR3 activation in response to full-length chemokines and BAM22 **(A)** and chemokine N-terminal peptides **(B)** monitored in β-arrestin recruitment assay. pEC_{50} values represent the mean ± standard error of the mean (SEM) of at least three independent experiments. Unpaired t tests were used to compare the differences in pEC_{50}/ pIC_{50} for each mutant using the WT receptor as reference. * $p < 0.05$, ** $p < 0.01$

4. Discussion

ACKR3 is involved in different vital physiological but also pathological processes and has emerged as a highly relevant drug target, especially for cancer therapy. Although many efforts have been undertaken over the last years, no small molecule antagonist of ACKR3 has been identified so far. ACKR3 shows several distinctive functional characteristics some of which have been suggested to be linked to its chemokine scavenging function. The most remarkable ones are its lack of G protein coupling, its continuous cycling, predominantly intracellular localization, responsiveness to both CXC and CC chemokines as well as to endogenous non-chemokine peptides, and its high propensity for activation. In addition to these functional particularities, an uncommon structural feature of ACKR3 is the disulphide bridge within its N terminus, which creates a small loop whose function and impact on the receptor biology remain unknown[32, 41, 52]. In this study, by disrupting this additional disulphide bridge, as well as the two conserved disulphide bridges, we uncovered new functional properties of ACKR3 and demonstrated that chemokine and endogenous non-chemokine ligands interact and activate ACKR3 following different binding modes characterized by distinct transmembrane domain subpocket occupancy and intra-N terminus loop contribution.

The roles of the conserved disulphide bridges for chemokine receptor functions have previously been shown to reflect unique traits of a single receptor rather than being shared between receptors, even within the same subfamily[40]. Their impact was also shown to vary depending on the ligand, thus offering an interesting means to explore different binding and/or activation modes for a specific receptor. Indeed, the two conserved disulphide bridges are positioned at opposing critical regions of the receptor extracellular surface and shape the entrance of the ligand-binding subpockets (Figure 1D).

Our data showed that ACKR3 folding and export is largely independent of the presence of its extracellular disulphide bridges. Only the disruption of the TM3-ECL2 disulphide reduced the presence of the receptor at the plasma membrane and moderately impaired the structure of the receptor. These results markedly contrast with the observations for TM3-ECL2 disulphide mutants in this and previous studies for CXCR4[56] or CCR6[57], which were both fully retained within the cell. However, maintenance of folding and export were reported for CXCR2 and CCR1[40, 58].

The substitution of the two N-terminal cysteine residues engaged in the formation of the intra-N terminus loop (TPA) resulted in a slight increase of surface expression levels compared to the wild-type receptor. Similar results were observed for the CXCR3 variant bearing the C37S-C38S mutation, suggesting that this apparent higher surface expression in N-terminal cysteine mutants may either reflect a facilitated transfer through the cell secretory pathway or simply a better accessibility of the epitope to the probe. A recent mutagenesis study of ACKR3 focusing on its N-terminal features

reported corroborating results and found that the disruption of the N terminus-TM7 or the TPA-forming disulphide bridges did not significantly affect ACKR3 surface expression [52].

The maintenance of cell surface expression for all ACKR3 mutants allowed us to further probe the role of the disulphide bridges in ligand binding and receptor activation, providing new insights into the importance of the different extracellular regions and subpockets of ACKR3. The absence of the disulphide bridge linking TM3 to ECL2, lying on top of the major subpocket, reduced the ability of ACKR3 to bind CXCL12 and respond to CXC chemokines, but not the CC chemokine. Noteworthy, the disruption of this disulphide turned CXCL11 and vCCL2 from partial to full agonists, suggesting that this structural constraint regulates the maximal responsiveness of the receptor to certain chemokines. Conversely, the disruption of the disulphide bridge linking the N-terminal domain to TM7, surrounding the minor ligand binding pocket, enhanced the apparent binding of vCCL2, which was likely linked to the weaker interaction of the fluorescent tracer and may point to differential ACKR3 binding pocket occupancy by CXC and CC chemokines. Strikingly, the disruption of or substitution within the N-terminal loop (TPA) markedly impaired the recognition and activation by CC and to a lesser extent by CXC chemokines. Altogether these results provide strong evidence for a different binding modes for CXC versus CC chemokines to ACKR3 characterized by different roles of the N-terminal arch and different ligand subpocket occupancies. Our data suggest that CXC chemokines mainly occupy the major subpocket, whereas vCCL2 interacts with structural determinants located in the vicinity of the minor subpocket, including the TPA. These observations are supported by structural data and comparative models of the binding mode of CXCL12 and vCCL2 to CXCR4, which shares 29 % identity with ACKR3, that indicate that although the tip of the N-termini of the two chemokines reach similar depths in the transmembrane region, they occupy the extracellular surface and the receptor subpockets differently. While vCCL2 lies mainly above TM1 and TM7 with its N terminus spanning the minor binding pocket, CXCL12 shows a rotation of approximately 80° relative to vCCL2, consequently positioning it more on top of the major binding pocket (TM5-TM6) [33, 59].

In this study, no interaction between adrenomedullin and ACKR3 was detected in the concentration range for which all other ligands were highly potent. This observation may be due the requirement of additional partners for adrenomedullin binding to occur, which are absent in the U87 cells. The binding and activation by BAM22, showed an apparent mixed CXC/CC profile for the effect of disulphide bridge disruptions. BAM22 interactions was negatively impacted by the disruption of the disulphide bridge TM3-ECL2 similarly to what was observed for CXC chemokines, suggesting that it also activates the receptor by occupying mainly the major binding pocket. However, its binding and activity were also significantly affected by the disruption or the substitution of the TPA indicating that its C terminus is most likely positioned more on top of the minor ligand binding pocket reminiscent of vCCL2 binding

mode. These binding similarities between vCCL2 and BAM22 may be partially explained by the sequence identity between the N-loop of vCCL2 and the C terminus of BAM22, both regions bearing the YQKR motif. We recently demonstrated that the N-loop of vCCL2, but not that of CXCL12 and CXCL11, is important for ACKR3 binding and activation further supporting the observation that this stretch could interact with the TPA by for instance forming electrostatic interactions with negatively charged residues (D25) located within the TPA[51]. Sequence alignment revealed that D25 occupies a position similar to that of the sulfotyrosine sY21 in the CXCR4 N terminus, a residue known to be crucial for CXCR4-chemokine interactions[42]. Furthermore, the shortening of CRS1 resulting from the formation of the TPA would also bring the potential sY8 of ACKR3 at a position equivalent to that of sY7 in CXCR4. Moreover, although it is highly speculative, the oxidation state of the two TPA-forming cysteines, by influencing the strength of ACKR3 interactions with internalized ligands, may regulate their handling or release during the trafficking through the different intracellular compartments. Finally, it cannot be excluded that this arch could also support the binding of other yet unknown ligands or interacting partners.

5. Highlights

- ACKR3 folding and surface export are very tolerant in regard of disulphide bridge breakage, since all ACKR3 mutants were still detectable by antibodies recognizing linear or conformational N-terminal epitopes of ACKR3. This was in stark contrast to CXCR4, where C28-C274 breakage led to conformational impairment and C109-C186 breakage completely blocked receptor surface export.

- Along these lines, we discovered that the epitope of ACKR3 antibody clone 9C4 specifically includes the TPA, since mutants with a TPA disruption were no longer recognized by this antibody. On top, peptides, derived from ACKR3 N terminus including this TPA could efficiently neutralize the 9C4 antibody, whereas linear peptides only had mild neutralization capacity.

- Both CXCR4 disulphide bridges play critical roles for the receptor function, as both cysteine-mutated receptor variants were severely affected in regard of binding and activation capacity. This again was significantly different for ACKR3, where all variants were still able to bind chemokines and recruit β-arrestin, although to a different extend, with the strongest effect observed for the disruption of the highly conserved C117-C196 bridge.

- Mutational analysis also revealed two different binding and activation modes towards ACKR3, one for classical CXC chemokines CXCL12 and CXCL11 mainly occupying the major subpocket

of the receptor, and one for CC-chemokine vCCL2, interacting with structural determinants around the minor binding pocket, including the TPA .

- The non-chemokine ligand adrenomedullin could not be confirmed in a binding competition assay as a potent ACKR3 ligand. However, we could detect strong binding of BAM22 opioid peptide to ACKR3 with a mixed CXC/CC binding and activation profile.

While we could already show in the first chapter that ACKR3 has a different interaction mode with chemokines and a high propensity for activation, i.e. for β-arrestin recruitment compared to classical receptors CXCR4 and CXCR3, mutational analysis of the receptor in this chapter deepens this concept even further. Here, we show that none of the three disulphide bridges are essential for ACKR3 activity, in contrast to classical chemokine receptor CXCR4, where both conserved disulphide bridges play essential role for receptor integrity and activity. Furthermore, disruption of disulphide bridges had either consequences for the activity towards CXCL12 and CXCl11, or towards vCCL2, pointing towards clearly two distinct activation mechanisms of the receptor.

However, despite the fact that we could show that the unique N-terminal TPA plays an important role for vCCL2 binding to the minor subpocket and activation of ACKR3, the exact function of this arch remains to be fully elucidated. Indeed, even though we could observe a strong impact of the C21S-C26S mutations on vCCL2 binding and ACKR3 activation, it is unlikely that such a unique structure was evolutionary conserved to ensure binding of a virus encoded chemokine to the receptor. The only other GPCR that we found that shares a similar four-residue TPA is the kappa-opioid receptor (KOR), which shares several other ligands with ACKR3, as will be discussed in the next chapter in more detail.

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Chapter 3: Beyond a chemokine receptor: Characterization of novel unconventional ACKR3 ligands

Part1: ACKR3 as an opioid peptide scavenger

Adapted from: **Meyrath, M.*,** Szpakowska*, M., Zeiner, J., Massotte, L., Merz, M. P., Benkel, T., Simon, K., Ohnmacht, J., Turner, J. D., Kruger, R., Seutin, V., Ollert, M., Kostenis, E., and Chevigne, A. (2020) *"The atypical chemokine receptor ACKR3/CXCR7 is a broad-spectrum scavenger for opioid peptides"*, **Nature Communications** *11*, 3033. * equal contribution.

In the recent years, increasing evidence rose that ACKR3 is more than "just" a scavenger for the chemokines CXCL12 and CXCL11. Within the last few years, several papers reported multiple other, non-classical ligands of the receptor. These included proenkephalin-derived BAM22 and related peptides in the adrenal gland[1], the pseudo-chemokine macrophage migration inhibitory factor MIF[2], the human herpesvirus-8-encoded chemokine vCCL2[3], the angiogenesis and vasodilation promoting peptide adrenomedullin (ADM)[4] and recently, the atheroprotective cytokine Dkk3 (dickkopf-3)[5]. However so far, most of these new interactions were only described in one publication and require cross-confirmation by other groups. Noteworthy, while different signaling events were attributed to ACKR3 after stimulation with Dkk3, MIF and BAM22, ACKR3 was described as scavenger for vCCL2 and ADM.

While in the first chapter, we could show that ACKR3 is activated by various small chemokine-derived peptides which have roughly the same size than opioid peptides, we confirmed in the second chapter that BAM22 is a potent ACKR3 ligand sharing some sequence similarities with the N-terminal parts of other chemokine ligands of ACKR3. These indications, together with the fact that opioid peptides share high sequence homology, including many positive charged residues and an N-terminal YGGFM/L motif, prompted us to screen a larger array of opioid peptides to investigate whether BAM22 is the only opioid peptide ligand of ACKR3.

Abstract

Endogenous opioid peptides and prescription opioid drugs modulate pain, anxiety and stress by activating opioid receptors, currently classified into four subtypes. Here we demonstrate that ACKR3/CXCR7, hitherto known as an atypical scavenger receptor for chemokines, is a broad-spectrum scavenger of opioid peptides. Phylogenetically, ACKR3 is intermediate between chemokine and opioid receptors and is present in various brain regions together with classical opioid receptors. Functionally, ACKR3 is a scavenger receptor for a wide variety of opioid peptides, especially enkephalins and dynorphins, reducing their availability for the classical opioid receptors. ACKR3 is not modulated by prescription opioids, but we show that an ACKR3-selective subnanomolar competitor peptide, LIH383, can restrain ACKR3's negative regulatory function on opioid peptides in rat brain and potentiate their activity towards classical receptors, which may open alternative therapeutic avenues for opioid-related disorders. Altogether, our results reveal that ACKR3 is an atypical opioid receptor (AOR) with cross-family ligand selectivity.

1. Introduction

Opioid receptors are G protein-coupled receptors (GPCR) that play a central role in reward processing, euphoria, analgesia, stress, anxiety and depression. The family consists of three classical receptors: mu (μ or MOR), delta (δ or DOR) and kappa *(*κ or KOR) and a fourth, non-classical nociceptin receptor (NOP, also known as orphanin FQ receptor)[6,7]. The classical receptors are activated by three major families of opioid peptides, namely endorphins, enkephalins and dynorphins, each showing a preference for one or two families, while the non-classical NOP receptor shows a high affinity and selectivity towards nociceptin[8]. All endogenous opioid peptides derive from proteolytic cleavage of large protein precursors and are mainly produced in the central nervous system (CNS), but also in the adrenal and pituitary gland and by several types of immune cells[9, 10]. With some exceptions, these ligands trigger downstream receptor signaling via G proteins, which is followed by arrestin recruitment, leading to receptor desensitization and internalization[11, 12]. Opioid receptors are established drug targets for non-peptide opioids such as morphine, fentanyl or naloxone. These opioid receptor modulators are the most widely used analgesics in the clinic but their use is associated with severe drawbacks like tolerance, dependence or respiratory depression, which were proposed to be linked to ligand and receptor bias towards arrestin recruitment[13-15]. Thus, a better understanding of opioid receptor signaling regulation and bias as well as new strategies to modulate opioid receptors with less adverse effects are not only timely but also urgently needed, especially considering the current opioid crisis.

Opioid receptor expression, signaling and desensitization are furthermore influenced by their interactions with other GPCRs, notably chemokine receptors[16-18]. Chemokine receptors bind to

chemokines, which are small (8-14 kDa) secreted chemo-attractant cytokines, regulating cellular processes like migration, adhesion and growth and thereby playing a crucial role in inflammatory and developmental processes[19, 20]. To date, nearly 50 chemokines and 20 classical chemokine receptors have been identified in humans[21, 22]. Similar to the opioid receptor-ligand network, many chemokine receptors recognize multiple chemokines, and, vice versa, many chemokines activate more than one receptor. Notably, within this network, a small subfamily of receptors, called atypical chemokine receptors (ACKRs), plays essential regulatory roles. ACKRs bind chemokines without triggering G protein signaling but instead participate in chemotactic events by transporting or capturing the chemokines or internalizing and degrading the ligands in order to resolve inflammatory processes or to shape chemokine gradients[23-25].

One such atypical chemokine receptor, ACKR3 (formerly CXCR7), is expressed in numerous regions of the CNS and in the adrenal glands but also on endothelial cells and diverse immune cells[26-28]. It plays crucial roles in neuronal and cardiovascular development and in the migration of hematopoietic stem cells[29, 30]. The activity of ACKR3 is proposed to mainly rely on arrestin recruitment, which is indicative of ACKR3 activation, while its signaling capacity remains highly debated and may be cell-context-dependent[31-34]. ACKR3 binds two endogenous chemokines, CXCL12 and CXCL11, which also activate the classical chemokine receptors CXCR4 and CXCR3[35], respectively, as well as the virus-encoded CC chemokine vMIP-II/vCCL2[36]. Moreover, ACKR3 was described as the receptor for the non-chemokine ligand adrenomedullin[4] and MIF[2]. ACKR3 was also shown to bind to BAM22, a small proenkephalin-derived peptide in the adrenal glands, inducing direct anxiolytic-like behavior in mice[1]. Recently, ACKR3 was proposed to have a distinctive ligand-binding mode and activation mechanism, showing a particularly high propensity for arrestin recruitment and ligand internalization[37, 38].

In this study, we show that ACKR3 is abundantly expressed in the same brain regions as the classical opioid receptors and that, besides BAM22, ACKR3 is activated by a large array of endogenous opioid peptides found in the CNS and immune cells, including those from the enkephalin, dynorphin and nociceptin families. However, contrary to the other four opioid receptors but in keeping with its atypical receptor features, ACKR3 is unable to activate canonical G protein signaling. Instead, it exclusively recruits arrestins in response to opioid peptides. We show that ACKR3 acts as a scavenger towards this family of neuromodulators, thus regulating their availability for signaling through the established opioid receptors, similarly to its role in chemokine gradient modulation. Hence, we propose ACKR3 as a promiscuous atypical opioid receptor (AOR) that functions as a scavenger receptor to regulate not only the abundance of chemokines but also of opioids.

2. Material and Methods

Peptides and chemokines

Non-labeled chemokines CXCL12, CXCL11 and vCCL2 were purchased from PeproTech. Alexa Fluor 647-labeled CXCL12 (CXCL12-AF647) was purchased from Almac. The opioid peptide library and all opioid peptides as well as FAM-labeled Dynorphin A (1-13) and FAM-labeled Nociceptin were acquired from Phoenix Pharmaceuticals. BAM22 and Big Dynorphin labeled with Cy5 were generated using an Amersham QuickStain Cy5 kit for proteins according to manufacturer's protocol. Adrenorphin-derived peptides were synthesized by JPT. These peptides contain a free amine at the N terminus and an amide group at the C terminus to avoid additional negative charge. Besides Levallorphan, which was purchased from Sigma, all non-peptide opioids were obtained from Tocris.

Cell culture

U87 cells derived from human brain glioblastoma were obtained through the NIH AIDS Reagent Program from Dr. Deng and Dr. Littman[39, 40] and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 15 % fetal bovine serum and penicillin/streptomycin (100 Units per ml and 100 µg per ml). U87-ACKR3 and U87-CXCR4 cells[38] were maintained under puromycin selective pressure (1 µg per ml). HEK293T and CHO-K1 (ATCC) cells were grown in DMEM supplemented with 10 % fetal bovine serum and penicillin/streptomycin (100 Units per ml and 100 µg per ml). smNPC cells (small molecule neural precursor cells)[41] derived from a healthy donor (C1-1), whose informed consent was obtained[42], were grown on Geltrex™-coated surface in N2B27 medium supplemented with 0.5 µM Purmorphamine, 3 µM CHIR 99021 and 150 µM ascorbic acid. N2B27 medium consisted of DMEM/F12 and NeuroBasal medium 50:50 with 0.5 % N_2 supplement, 1 % B27 supplement lacking vitamin A, 1 % GlutaMAX and 1 % penicilin/streptomycin. Medium was renewed every other day.

Binding competition assays

U87-ACKR3 cells were distributed into 96-well plates (1.5×10^5 cells per well) and incubated with a mixture of 5 nM CXCL12-AF647 and unlabeled chemokines or opioid peptides at indicated concentrations for 90 minutes on ice, then washed twice with FACS buffer (PBS, 1 % BSA, 0.1 % NaN_3) at 4 °C. Dead cells were excluded using Zombie Green viability dye (BioLegend). ACKR3-negative U87 cells were used to evaluate non-specific binding of CXCL12-AF647. 0 % receptor binding of CXCL12-AF647 was defined as the signal obtained after addition of 1 µM of unlabeled CXCL12. The signal obtained for CXCL12-AF647 in the absence of unlabeled chemokines was used to define 100 % binding. Ligand binding was quantified by mean fluorescence intensity on a BD FACS Fortessa cytometer (BD Biosciences) using FACS Diva 8.01 (BD Biosciences).

Nanoluciferase complementation-based assays

Ligand-induced β-arrestin recruitment to chemokine and opioid receptors was monitored by NanoLuc complementation assay (NanoBiT, Promega)[37, 43]. In brief, 1.2×10^6 U87 cells (5×10^6 for HEK293T and 4×10^6 for CHO-K1) were plated in 10-cm culture dishes and 48 hours (24 hours for HEK293T and CHO-K1 cells) later co-transfected with pNBe vectors encoding GPCRs C-terminally tagged with SmBiT and human β-arrestin-1 (arrestin-2) or β-arrestin-2 (arrestin-3) or mini G proteins (mG, engineered GTPase domains of G_α subunits,) N-terminally fused to LgBiT[44-46]. 48 hours post-transfection cells were harvested, incubated 25 minutes at 37 °C with Nano-Glo Live Cell substrate diluted 200-fold and distributed into white 96-well plates (5×10^4 cells per well). Ligand-induced, β-arrestin and mini G recruitment to GPCRs was evaluated with a Mithras LB940 luminometer (Berthold Technologies, running on MicroWin 2010 5.19 software (Mikrotek Laborsysteme) for 20 minutes. For single dose screening experiments on all chemokine receptors, the results are represented as percentage of signal monitored with 100 nM of one known agonist chemokine listed in the IUPHAR repository of chemokine receptor ligands which was added as positive control (supplementary Table 3). For concentration-response curves, the signal recorded with a saturating concentration of full agonist for each receptor was set as 100 %. To evaluate the antagonist properties of ligands, full agonists of each receptor (50 nM BAM22 for MOR, 50 nM dynorphin A for KOR, 70 nM met-enkephalin for DOR, 70 nM nociceptin for NOP and 4 nM CXCL12 for ACKR3) were added after the 20-minute incubation with the ligands. Signal from wells treated with full agonist only was defined as 0 % inhibition and signals from wells treated with no agonist were used to set 100 % inhibition.

For dynorphin A scavenging experiments with U87 cells, 1.5×10^5 U87 or U87.ACKR3 cells were distributed per well in a white 96-well plate. After 15-minute incubation at 37 °C with 400 nM LIH383 or LIH383 control peptide (200 nM CXCL12 or CXCL10), dynorphin A was added at concentrations ranging from 0.15 nM to 3 µM and incubated for 25 minutes at 37 °C. 1.5×10^4 U87 cells, cotransfected 48 hours before the experiment with SmBiT-tagged KOR and LgBiT-tagged β-arrestin-1 or mini G_i and pre-incubated for 25 minutes with Nano-Glo Live substrate were then added per well and signal was measured for 20 minutes. For dynorphin A scavenging experiments with smNPC, 2×10^6 smNPC were pretreated for 15 minutes with 1.5 µM LIH383 or LIH383 control peptide (300 nM CXCL12 or CXCL10) before 4-hour incubation with 3 µM dynorphin A. Cells were centrifuged and the activity of the remaining dynorphin A in serially diluted supernatants was determined on U87 cells expressing SmBiT-tagged KOR and LgBiT-tagged mini G_i protein.

Label-free dynamic mass redistribution (DMR) assay

Dynamic mass redistribution (DMR) experiments were conducted using the Corning Epic (Corning) biosensor system[47-51]. In brief, 6×10^5 U87 cells were seeded in 6-cm dishes. 24 hours later cells were

transfected with pcDNA3.1-based expression plasmids coding for the respective chemokine (ACKR3, CXCR4) or opioid (KOR, NOP) receptors. 24 hours after transfection 1×10^4 cells per well were transferred to a 384-well Epic biosensor plate and incubated overnight at 37 °C.

Cells were then washed twice with Hanks' balanced salt solution (HBSS) (Life Technologies) containing 20 mM HEPES (Life Technologies) and subsequently incubated in the DMR-reader for 1.5 hours to achieve temperature equilibration (37 °C). Five minutes after equilibration of the baseline DMR traces, compounds were added to the biosensor plate. Alterations of ligand-induced DMR were monitored for at least 3000 seconds. For quantification, negative and positive areas under the curve (AUC) between 0 and 3000 seconds were used.

HTRF-based ERK1/2 phosphorylation assays

Homogeneous Time-Resolved Fluorescence (HTRF)-based phospho-ERK1/2 (extracellular signal regulated kinases 1 and 2) and total-ERK1/2 assays were performed using phospho-ERK1/2 (Thr202/Tyr204) and total-ERK1/2 cellular kits (Cisbio International). In short, for quantification of phosphorylated and total ERK1/2 protein, U87 cells stably expressing (or not) ACKR3 or CXCR4 were seeded in 96-well poly-D-lysine (PDL)-coated microtiter plates (Sigma-Aldrich) at a density of 3.5×10^4 cells per well. After overnight incubation, cells were starved for 4 hours at 37 °C in serum-free medium. Cells were then stimulated for the indicated time intervals with chemokine or opioid ligands. The supernatants were replaced with the lysis buffer provided and incubated 1.5 hours. Lysates were transferred to white 384-well plates and incubated with pERK1/2-specific (2 hours) or total-ERK1/2-specific (24 hours) antibodies conjugated with Eu^{3+}-cryptate donor and d2 acceptor at recommended dilutions. HTRF was measured using the Mithras LB 940 multimode reader (Berthold Technologies) equipped with 320 nm excitation filter and 620 nm (donor) and 665 nm (acceptor) emission filters.

Transcriptional Nanoluciferase reporter assays

Activation of the MAPK/ERK signaling pathway was evaluated using a serum response element (SRE) Nanoluciferase reporter assay. Activation of calcium-dependent signaling pathways was evaluated using a Nuclear Factor of Activated T-cell response element (NFAT-RE) Nanoluciferase reporter assay. For both assays, 1.2×10^6 U87 cells (5×10^6 for HEK293T, 4×10^6 for CHO-K1, 5×10^6 for smNPC) were seeded in 10-cm dishes and 48 hours (24 hours for HEK293T and CHO-K1 cells) later co-transfected with the pNanoLuc/SRE or pNanoLuc/NFAT-RE vectors (Promega), containing the Nanoluciferase gene downstream of SRE or NFAT-RE, and pcDNA3.1 encoding the respective chemokine or opioid receptors. 24 hours later, 2.5×10^4 cells/well (1×10^5 for HEK293T cells, 7×10^4 for CHO-K1 cells and 2.5×10^5 for smNPCs) were seeded in a white 96-well plate. 24 hours later, the medium was replaced by serum-free and phenol red-free DMEM (serum free DMEM/F12 for smNPCs) and further incubated for two

hours. Opioid peptides (500 nM) and chemokines (200 nM) were then added to the cells and incubated for six hours. 30 nM phorbol 12-myristate 13-acetate (PMA), 10 % FBS or 30 nM PMA, 1uM ionomycin, 10 % FBS were used as positive controls for SRE and NFAT-RE assays, respectively. Nano-Glo Live Cell substrate (Promega) was then added and luminescence was read over 20 minutes on a Mithras LB940 plate reader (Berthold Technologies).

Visualization of fluorescently labeled opioid-peptide uptake

Cells were distributed into 96-well plates (2×10^5 cells/well in Opti-MEM for U87 and U87-ACKR3 and 3×10^5 cells/well in N2B27 medium for smNPCs). After 15-minute incubation at 37 °C with LIH383 (3 µM) or Opti-MEM only, FAM-labeled dynorphin A (1-13) (250 nM), BAM22-Cy5 (400 nM), big dynorphin-Cy5 (400 nM) or nociception-FAM (1 µM) was added, incubated for 40 minutes at 37 °C and washed twice with FACS buffer. For comparison of labeled opioid peptide-uptake by ACKR3 or classical opioid receptors, 1.2×10^6 U87 cells were seeded in 10-cm dishes and transfected 48 hours later with 4 µg pcDNA3.1 plasmid encoding ACKR3 or KOR, MOR or NOP. 48 hours post transfection, cells were harvested and treated as described above. Dead cells were excluded using Zombie NIR or Zombie Green viability dye (BioLegend, #423106, dilution 1:1000) for FAM-labeled peptides and Cy5-labeled peptides, respectively. Images of 1×10^4 in-focus living single cells were acquired with an ImageStream MKII imaging flow cytometer (Amnis, running on the INSPIRE Mark II software (EMD Millipore)) using 40x magnification (60x magnification for smNPCs). Samples were analyzed using Ideas6.2 software. The number of spots per cell was determined using a mask-based software wizard.

Receptors detection and localization analysis

Intracellular and surface ACKR3 levels were analyzed by flow cytometry using ACKR3-specific mAb (12.5 µg per ml (dilution 1:40), clone 11G8 (R&D Systems, catalog #MAB42273) or a matched isotype control (12.5 µl per ml, (dilution 1:40) clone MG1-45, BioLegend, #401402) and phycoerythrin–conjugated $F(ab')_2$ fragment anti-mouse IgG (dilution 1:300, Jackson ImmunoResearch, #115-116-146). Dead cells were excluded using the Zombie NIR fixable viability dye (BioLegend, dilution 1:2000, catalog #423106). For intracellular staining, cells were treated with the BD Cytofix/Cytoperm Fixation/Permeabilization solution kit (BD Biosciences, catalog # 554714) according to manufacturer recommendations. Fluorescence intensity was quantified on a Novocyte Quanteon flow cytometer (ACEA Biosciences) using NovoExpress 1.4.1 (ACEA Biosciences) and samples were analyzed/processed using FlowJo 10.6.1.

For fluorescence imaging, 3×10^5 U87 cells/well of a 6-well plate were seeded and 48 hours later transfected with 0.4 µg plasmid encoding ACKR3 or the opioid receptors C-terminally tagged with mNeonGreen. 24 hours later, 1×10^5 cells were reseeded on 8-well chamber slides (µ-Slide 8 well, ibidi)

and grown overnight. Cells were then incubated 90 minutes in the presence or absence of opioid peptides (1 μM) (dynorphin A for ACKR3, BAM22 for MOR, met-enkephalin for DOR, dynorphin A for KOR and nociceptin 1-13 for NOP) washed with PBS and fixed with 3.5 % (w/v) paraformaldehyde for 20 minutes at room temperature. After three washes with PBS, nuclear staining was performed with Hoechst 33342 (1:1000) for 15 minutes at room temperature. Cells were again washed three times and imaged on a Zeiss LSM880 confocal microscope using a 63x oil-immersion objective using Zen Black 2.3 SP1 software (Zeiss). Representative cells from 10 image acquisitions of two independent experiments are shown.

Ligand-induced receptor delivery to endosomes

Opioid peptide-induced receptor-arrestin complex delivery to endosomes was monitored by β-galactosidase complementation using a PathHunter eXpress ACKR3 activated GPCR internalization assay (DiscoverX). In brief, U2OS cells stably expressing ACKR3, β-arrestin-2 fused to the enzyme acceptor of β-galactosidase and an endosome marker fused to the β-galactosidase ProLink donor peptide were seeded 24 hours before the experiment in a 96-well plate at a density of 1×10^4 cells per well. Opioid peptides (3 μM and 300 nM) were then added and after 4-hour incubation at 37 °C, luminescent signal was generated through addition of 55 μl β-galactosidase substrate (PathHunter Detection reagent). After 1-hour incubation at room temperature, chemiluminescent signal was measured on a Mithras LB940 plate reader (Berthold Technologies).

Ligand-induced changes in receptor cell surface levels

Determination of receptor surface expression level by NanoLuc complementation assay was performed using the Nano-Glo HiBiT extracellular detection system (Promega) according to manufacturer's protocol. In brief, 1.2×10^6 U87 cells were seeded on a 10-cm dish and 48 hours later transfected with 100 ng plasmid encoding ACKR3 or the respective opioid receptors N-terminally tagged with HiBiT, a small part of the Nanoluciferase with high affinity towards LgBiT. 48 hours later, 5×10^4 cells per well were seeded in 96-well plates and stimulated for the indicated time with CXCL12 (300 nM) or opioid peptides (1 μM) at 37 °C. Cells were then incubated with HiBiT extracellular reagent, consisting of Nanoluciferase extracellular substrate and LgBiT protein in HiBiT buffer. Light emission from complementation of LgBiT protein with remaining surface receptor-fused HiBiT was determined on a Mithras LB940 plate reader (Berthold Technologies). Signal was normalized to the measurement recorded at t = 1 min. Noteworthy the effect of nociceptin and derivatives could not be determined in this assay due to significant LgBiT protein cross-complementation by nociceptin. Where indicated, cells were treated with bafilomycin A1 (1.5 μM in 0.15 % DMSO) (Santa Cruz Biotechnology) or 0.15 % DMSO prior ligand stimulation (45 minutes) and during ligand stimulation (180 minutes).

For determination of receptor surface expression levels by flow cytometry, U87-ACKR3 or U87-KOR cells were stimulated with opioid peptides (1 µM) or CXCL12 (300 nM) for 60 minutes at 37 °C. The remaining surface-bound ligands were then removed by a brief wash with 150 mM NaCl, 50 mM glycine, pH 3 and twice with FACS buffer. Where indicated, cells were incubated for additional 120 minutes to allow surface receptor recovery. Cell surface levels of ACKR3 or KOR were then measured by flow cytometry using a saturating concentration (12.5 µg per ml, dilution 1:40) of receptor-specific mAb (clones 11G8 and 387301, R&D Systems, catalog #MAB42273 or #MAB3895 , respectively) and a secondary phycoerythrin–conjugated $F(ab')_2$ fragment anti-mouse IgG (Jackson ImmunoResearch, cat. #115-116-146, dilution 1:300). Dead cells were excluded using the Zombie NIR fixable viability dye (BioLegend, catalog #423106, dilution 1:2000). Mean fluorescence intensity was quantified on a Novocyte Quanteon flow cytometer (ACEA Biosciences) using NovoExpress 1.4.1 (ACEA Biosciences).

Ex vivo rat neuron firing rate

Adult male Wistar rats (6-8-week old) were housed at room temperature in groups of three or four with a 12:12 hour light-dark cycle. All animals had access to ad libitum food and water. All procedures were carried out in accordance with guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EE) and were accepted by the Ethics Committee for Animal Use of the University of Liège (protocol 2061). All efforts were made to minimize animal suffering.

The methods used for brain slice preparation and recording procedures, were as previously described[52]. Rats were anaesthetized with chloral hydrate (400 mg per kg, i.p.) and placed under a cap with oxygenated air (95 % O_2, 5 % CO_2) two minutes prior to decapitation. After decapitation, the brain was rapidly removed and placed in ice cold (~2 °C) oxygenated artificial cerebrospinal fluid (aCSF) of the following composition: NaCl 130 mM, KCl 3.5 mM, NaH_2PO_4 1.25 mM, $NaHCO_3$ 24 mM, Glucose 10 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1.25 mM. A block of tissue containing the pons was placed in a vibrating blade microtome (Vibratome 1000 Plus, Sectioning System) and a slice containing the locus coeruleus (LC) immediately rostral to the fourth ventricle and the VIIth nerve, used as anatomical landmarks, was cut coronally (400 µm thick). The slice was placed on a nylon mesh in a recording chamber (volume: 0.5 ml) where it was superfused by oxygenated aCSF (34.0 ± 0.5 °C) at a rate of 2 to 3 ml per min. The LC was recognized as a translucent region during transillumination, lateral to the fourth ventricle. All experiments were performed in oxygenated aCSF with synaptic blockers consisting of 10 µM CNQX, 10 µM SR95531, 1 µM MK801, and 1 µM CGP55845, which block AMPA/Kainate, GABAA, NMDA, and GABAB receptors, respectively. This ensured that the spontaneous firing of the neurons was only due to its endogenous pace making.

Extracellular single cell recordings of LC neurons were performed with glass microelectrodes filled with aCSF (resistance 10-20 MΩ). Signals were passed through an impedance adapter and were amplified 1000x using a homemade amplifier. They were displayed on a Fluke Combiscope oscilloscope and fed to an analog–digital interface (CED 1401, Cambridge Electronic Design, Cambridge, UK) connected to a computer. Data were collected and analyzed with the Spike 2 software (Cambridge Electronic Design, Cambridge, UK). All recorded neurons had a firing rate of 0.5 to 3 Hz with a good regularity (coefficient of variation of the interspike interval was 0.13 ± 0.01, n=18) and a cessation of firing during application of the α2-adrenergic receptor agonist clonidine (10-20 nM). The duration of the extracellularly recorded action potentials was 2-3 ms. Drugs and peptides were applied for at least 10 minutes. Neuron firing was recovered to initial rates after the treatment was stopped.

The mean firing rate over 1 minute was calculated during each condition. Next, the inhibition of firing by the peptides and drugs used (LIH383 and dynorphin) was quantified as the % of total inhibition. For this purpose, we considered the mean firing rate during the last minute of each condition (control, LIH383 alone, LIH383 plus a given concentration of dynorphin). The EC_{50} of dynorphin was obtained using the Hill equation (E/E_{max} = [dynorphin]/ EC_{50} (dynorphin) + [dynorphin]). One aberrant value (in the LIH383 3 µM group: 559 nM, which was > 2SD away from the mean value for this group) was omitted.

RNA extraction and quantitative PCR

Post-mortem samples from six brain regions of five patients suffering lethal non-head trauma were collected within 2-10 hours of death as previously reported[53]. Brain autopsies were performed after informed consent of the closest relatives. Informed consent was also given for the use of anonymized brain tissues and clinical and pathological information for research purposes. The study was approved by the Ethical Committee of the Faculty of Medicine, Chiang Mai University, Thailand (protocol 2012-038). Total RNA was extracted from biopsies using the AllPrep DNA/RNA mini kit (Qiagen) or from smNPC using RNeasy mini kit (Quiagen) and stored at -80 °C until cDNA synthesis. First-strand synthesis was performed in a two-step process. Initially samples were incubated with RNaseOUT (Invitrogen) at 65 °C for 5 minutes. The reverse transcription reaction was subsequently performed at 55 °C for 60 minutes using Superscript III RT (Invitrogen) and 2 µM dT20 primer (Eurogentec). Quantitative PCR was performed on a CFX96 thermal cycler (Bio-Rad) and analyzed with CFX Manager 3.1 (Bio-Rad). Thermal cycling was performed as follows: denaturation at 95 °C for 15 minutes, 40 cycles at 95 °C for 15 seconds, annealing for 30 seconds, elongation at 72 °C for 30 seconds and a final elongation for 10 minutes at 72 °C. For each primer (supplementary Table 8) the specificity of amplification was verified by melting curve analysis and visualization of PCR products on an agarose gel with SYBR Safe (Invitrogen). Relative PCR quantification was performed using the comparative threshold cycle

method[54] using the arithmetic mean of PPIA and GAPDH as stable housekeeping genes. Samples with Ct values greater than three standard deviations from the mean were excluded from further analysis.

Brainbank database analysis of gene expression

CNS gene expression data were extracted from Allen Institute, BrainSpan: Atlas of the Developing Human Brain[55] (http://www.brainspan.org/static/download.html, file: RNA-Seq Gencode v10 summarized to genes). The dataset contains RNA-Seq RPKM (reads per kilobase per million) values averaged to genes[56]. For detailed descriptions of sample preparation, tissue selection criteria and data normalization, see the technical white paper, Developmental Transcriptome (http://help.brain-map.org/display/devhumanbrain/Documentation). Depending on the brain region, gene expression data were extracted form 16-22 donors aged from 4 months to 40 years old. Prenatal samples of the database were excluded (pcw 8-37). Brain samples from male and female donors were equally represented.

Phylogenetic tree and sequence alignments

Phylogenetic tree was created using sequences and the tree generator from the G protein-coupled receptors database GPCRdb (https://www.gpcrdb.org/). For each GPCR, its full sequence was used to generate the tree using neighbor-joining distance calculation method. Design and formatting of the tree was done with CLC main workbench 7.9.1 using radial representation.

Data and statistical analysis

Concentration-response curves were fitted to the four-parameter Hill equation using an iterative, least-squares method (GraphPad Prism version 8.0.1). All curves were fitted to data points generated from the mean of at least three independent experiments.

All statistical tests, *i.e.* t-test, ordinary one way- or two-way ANOVA, Kruskal-Wallis test and post hoc analysis were performed with GraphPad Prism 8.0.1. Sample size was chosen to allow sufficient statistical power. P-values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, **** p<0.0001.

3. Results

3.1. ACKR3 is activated by a broad range of opioid peptides

In a recent study, we suggested that the proenkephalin-derived peptide BAM22 shares structural and functional features important for ACKR3 binding and activation with the N terminus of chemokine ligands[37]. Given that all endogenous opioid peptides show remarkable sequence homologies including the F/YGGFL/M motif at their N termini, as well as several positively charged residues throughout the

sequence (Table 1), we wondered whether BAM22 and the related peptides are the only opioid peptides able to activate ACKR3. Therefore, we screened a library of 58 opioid peptides (5 µM, supplementary Table 1) for their ability to induce β-arrestin-2 recruitment to ACKR3 (indicative of ACKR3 activation), and, additionally to CXCR4 and CXCR3, two classical chemokine receptors sharing ligands with ACKR3, which served as negative controls. Besides BAM22, BAM18 and Peptide E previously reported as ACKR3 ligands[1], our screening revealed that numerous other opioid peptides are capable of inducing β-arrestin-2 recruitment to ACKR3. These included adrenorphin, another proenkephalin-derived peptide, but also peptides from the nociceptin and dynorphin families (Figure 1a). Endorphins and endomorphins, however, did not activate ACKR3. None of these peptides acted as ACKR3 antagonist (supplementary Figure 1a) or induced β-arrestin-2 recruitment to CXCR4 or CXCR3 (Figure 1a).

We then sought to further characterize the interactions of the opioid peptides with ACKR3 to establish whether their activity towards this new receptor may be of physiological relevance. To this end, we performed pharmacological analysis, investigating the potency and efficacy of the different hits towards ACKR3 as well as the classical opioid receptors in β-arrestin-1 and β-arrestin-2 recruitment (Figure 1b-f, Table 1, supplementary Figure 1b). ACKR3 was activated by several endogenous opioid peptides such as dynorphin A, dynorphin A 1-13, big dynorphin, BAM22 or adrenorphin at low concentrations comparable to their activity on the classical opioid receptors. Higher concentrations of dynorphin B, nociceptin or nociceptin 1-13-amide were necessary for ACKR3 activation. Surprisingly, ACKR3 was also fully activated by the weak partial NOP agonist $Phe^1\psi(CH_2\text{-}NH)\text{-}Gly^2$-nociceptin-1-13 amide (FψG nociceptin 1-13) as well as by endogenous truncated dynorphin variants, dynorphin 2-13 and dynorphin 2-17 (Table 1), which do not activate the classical opioid receptors but were shown to have a physiological effect[57, 58]. However, ACKR3 seems to show a certain degree of selectivity as several peptides, like endorphins, short endomorphins and leu- or met-enkephalin did not trigger β-arrestin recruitment. This was further confirmed in different cellular backgrounds, such as HEK293T and CHO-K1 (supplementary Figure 1c and 1d) and in binding competition studies, showing that all of the identified ligands were able to compete with, and displace Alexa Fluor 647-labeled CXCL12 from ACKR3 (Figure 1g, supplementary Table 2).

These data reveal that ACKR3 is selectively activated by various endogenous opioid peptides from different families (Figure 1h) in a concentration range similar to that observed for activation and signaling via the long-established opioid receptors, strongly pointing to a physiological relevance of these newly identified ligand-receptor interactions.

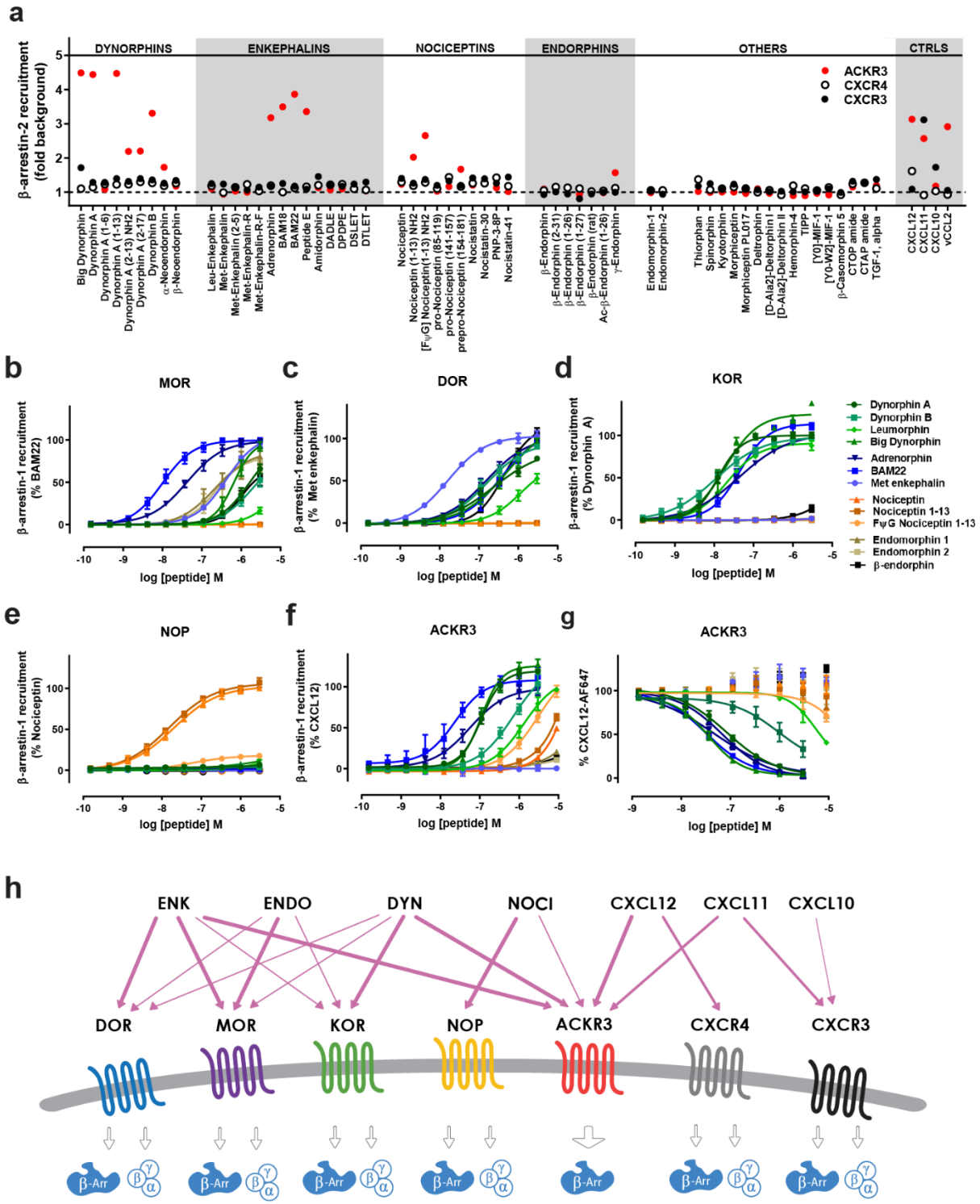


Figure 1. Opioid peptide library screening on ACKR3 and hit activity comparison (**a**) The ability of 58 compounds, including natural opioid peptides from the four opioid families, variants thereof and small molecule opioid receptor modulators, to induce β-arrestin-2 recruitment to ACKR3, CXCR4 and CXCR3 in U87 cells at a concentration of 5 µM. For full peptide names and sequences, see supplementary Table 1. Chemokines used as positive controls were added at a concentration of 300 nM. Results are expressed as fold change over vehicle-treated cells and presented as mean of two technical replicates for ACKR3 and CXCR4. A single measurement was performed for CXCR3. (**b** - **f**) Comparison of potency and efficacy of ACKR3-activating and other representative opioid peptides in inducing β-arrestin-1 recruitment to the opioid receptors MOR (b), DOR (c),

KOR (d), NOP (e) and ACKR3 (f) in U87 cells. Results are expressed as percentage of indicated agonist response. The corresponding EC_{50} and Emax values are summarized in Table 1. (**g**) Binding competition of ACKR3-activating and other representative opioid peptides with Alexa Fluor 647-labeled CXCL12 (5 nM) on U87-ACKR3 cells determined by flow cytometry. Results from b-g are presented as mean ± S.E.M of three or four independent experiments (n = 3 or 4). Peptides from the enkephalin, dynorphin, nociceptin and endorphin families are depicted in blue, green, orange and grey scale, respectively. (**h**) Schematic representation of the interactions of the four opioid receptors and chemokine receptors with their respective ligands. The newly identified opioid peptide pairings with ACKR3 are shown, highlighting the cross-family selectivity of ACKR3.

Table 1. Sequences of opioid peptides and their activity in β-arrestin-1 recruitment

Name	Sequence	ACKR3 EC_{50} nM - Max %	MOR EC_{50} nM - Max %	DOR EC_{50} nM - Max %	KOR EC_{50} nM - Max %	NOP EC_{50} nM - Max %
Dynorphin A	**YGGF**LRRIRPKLKWDNQ	110 (94.7 - 129) - 119	~1500 - 66	240 (115 - 2143) - 76	12.7 (10.5 - 15.2) - 100	NA - 6
Dynorphin A 2-17	-**GGF**LRRIRPKLKWDNQ	~2,000 - 87*	NA - 0	NA - 0	NA - 0	NA - 0
Dynorphin A 2-13	-**GGF**LRRIRPKLK-NH2	~6,000 - 83*	NA - 0	NA - 0	NA - 0	NA - 0
Dynorphin A 1-13	**YGGF**LRRIRPKLK	61.9 (50.4 - 76.1) - 94	~2000 - 67	319 (188 - 1038) - 81	2.5 (1.5 - 3.7) - 93	NA - 6
Dynorphin B	**YGGF**LRRQFKVVT	727 (385 - 8608) - 103	~2000 - 53	158 (101 - 331) - 90	10.9 (6.3 - 17.2) - 99	NA - 3
Leumorphin	**YGGF**LRRQFKVVTRSQEDPNAYSGELFDA	1320 (841 - 3050) - 96*	ND - 17	~3,000 - 53	21.4 (17.4 - 26.4) - 88	NA - 8
Big Dynorphin	**YGGF**LRRIRPKLKWDNQKR**YGGF**LRRQFKVVT	108 (89.3 - 133.9) - 127	652 (502 - 966) - 92	529 (251 - 8197) - 97	19.1 (12.9 - 30.6) - 138	ND - 11
Adrenorphin	**YGGF**MRRV-NH2	56.5 (36.6 - 95.0) - 96	41.6 (30.7 - 57.3) - 96	157 (104 - 310) - 96	43.5 (33.7 - 57.9) - 98	NA - 3
BAM22	**YGGF**MRRVGRPEWWMDYQKRYG	23.5 (11.0 - 53.1) - 112	10.0 (7.1 - 13.8) - 100	367 (193 - 2930) - 95	44.2 (33.6 - 58.2) - 111	NA - 2
Met enkephalin	**YGGF**M	NA - 0*	419 (350 - 528) - 95	16.3 (12.0 - 21.6) - 100	NA - 2	NA - 0
Nociceptin	F**GGF**TGARKSARKLANQ	> 10,000 - 49*	NA - 0	NA - 0	NA - 1	19.6 (13.4 - 28.5) - 100
Nociceptin 1-13	F**GGF**TGARKSARK-NH2	~10,000 - 63*	NA - 0	NA - 0	NA - 2	15.7 (10.7 - 22.1) - 107
FΨG nociceptin 1-13	[Fψ(CH2-NH)G]GFTGARKSARK-NH2	2966 (1660 - 22000) - 95*	NA - 0	NA - 0	NA - 2	56.5 (34.9 - 106.7) - 18
Endomorphin-1	YPWF-NH2	ND - 20*	216 (111 - 8640) - 95*	NA - 0	NA - 0	NA - 0
Endomorphin-2	YPFF-NH2	ND - 11*	231 (124 - 876) - 80*	NA - 0	NA - 0	NA - 0
β-endorphin	**YGGF**MTSEKSQTPLVTLFKNAIIKNAYKKGE	ND -13*	~1000 - 85*	441 (339 - 679) - 102	ND - 14	NA - 0
LIH383	F**GGF**MRRK-NH2	0.61 (0.19 - 1.17) - 99	NA - 1	NA - 1	NA - 9	NA - 1

EC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)
Max = maximum signal measured at 3 µM expressed as % of the positive control/reference peptide
*measured at 9 µM
ND: Not determinable since saturation was not reached
NA: No activity or activity below 10 % of positive control in the concentration range tested
YGGF motif conserved in most of the opioid peptides is bold. Positively charged residues are underlined
Full name of FΨG nociceptin 1-13: [Phe^1Ψ(CH_2-NH)-Gly^2]nociceptin-(1-13)-NH_2

3.2. ACKR3 is the only chemokine receptor activated by opioid peptides

Just like classical opioid receptors, many chemokine receptors have multiple ligands, which they often share with other receptors[21]. Thus, we wondered whether ACKR3 is the only member of the chemokine receptor family activated by opioid peptides. To this end, we tested all chemokine receptors for arrestin recruitment in response to the different ACKR3-recognizing opioid peptide ligands at a saturating concentration using the same Nanoluciferase complementation assay. None of the peptides induced similar β-arrestin-1 or β-arrestin-2 recruitment to any of the 21 other chemokine receptors (Figure 2 and supplementary Figure 2a). A weak induction of β-arrestin recruitment was detectable towards several receptors such as CCR3, CXCR3 and CX3CR1 treated with big dynorphin. However

contrary to ACKR3 or opioid receptors, their responses to big dynorphin were severely reduced compared to those achieved with their cognate chemokines (supplementary Figure 2b and supplementary Table 3). These data provide strong support for the notion that the capacity to recruit arrestins in response to endogenous opioid peptides is unique and distinguishing for ACKR3 among all chemokine receptor family members.

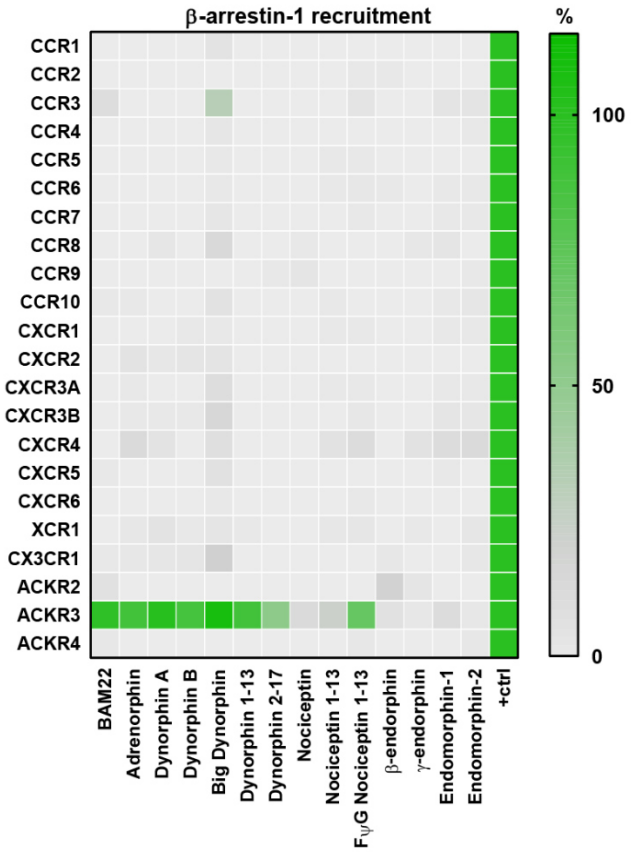


Figure 2. Specific activation of ACKR3 by opioid peptides. Agonist activity of opioid peptides (3 µM) representative of the four opioid families towards 18 classical and 3 atypical chemokine receptors evaluated in β-arrestin-1 recruitment assay in U87 cells. Results are expressed as percentage of signal monitored with saturating concentration of active chemokines (100 nM) used as positive controls (supplementary Table 3) and presented as mean of three independent experiments (n = 3).

3.3. Ligand SAR study reveals mixed ACKR3 opioid-binding pocket

The activity and selectivity of opioid peptides are generally proposed to be supported by distinct regions of the peptide. While the amino-terminal YGGF core, the message, is responsible for receptor activation through interactions with the binding pocket, the address, composed of the carboxy-terminal residues beyond the YGGF core, provides the structural basis for receptor selectivity. While the YGGF-L/M sequence is necessary and sufficient for high-affinity binding and modulation of DOR and MOR, this does not hold true for KOR[59] or ACKR3 as observed in our initial screening, where neither met- nor leu-enkephalin (YGGF-L/M) activated ACKR3.

To gain further insights into the binding and activation modes of ACKR3 compared to classical opioid receptors, we performed a structure-activity relationship (SAR) study based on the octapeptide adrenorphin (YGGFMRRV-NH_2, formerly metorphamide) (Figure 3a). Adrenorphin triggered arrestin recruitment to ACKR3, MOR, DOR and KOR with roughly the same potency (Figure 3b), providing a suitable base for investigating the activation mode of the four receptors. We performed an alanine scan of adrenorphin and introduced substitutions by closely related amino acids or other modifications such as N- and C-terminal extension, D-amino acid replacement or dimerization. We evaluated the ability of these modified peptides to activate ACKR3 and the opioid receptors in a β-arrestin-1 recruitment assay (Figure 3a). Interestingly, the message and address sequences were somewhat different for ACKR3 compared to classical opioid receptors, despite a similar trend in potency changes. ACKR3 appears to be more tolerant to modifications of the N-terminal tyrosine residue, critical for the activation of classical opioid receptors. Indeed, variants displaying a leucine or a phenylalanine retained the parental activity, with Y1F mutation, mimicking the nociceptin peptide N terminus, resulting in a tenfold improvement in potency (Figure 3a and 3c). However, similarly to classical receptors, the phenylalanine at position 4 of the YGGF-L/M core was found to be crucial for ACKR3 binding, as any mutation, with the exception of F4W, was detrimental for receptor activation. Methionine-to-leucine substitution at position 5, mimicking peptides of the dynorphin family, improved binding to KOR but significantly reduced the binding to MOR and ACKR3 (Figure 3d), whereas mutations at positions 6 in the di-arginine motif abolished the activity towards KOR and ACKR3, but largely improved the activity towards MOR (Figure 3e).

Based on the above SAR analysis we concluded that the interaction mode of ACKR3 with opioid peptides is in some aspects distinct from that of the classical opioid receptors, while at the same time ACKR3 shares important interaction determinants with all of the other four opioid receptors. This feature likely provides a molecular explanation why ACKR3 binds and responds to opioid peptides from different families.

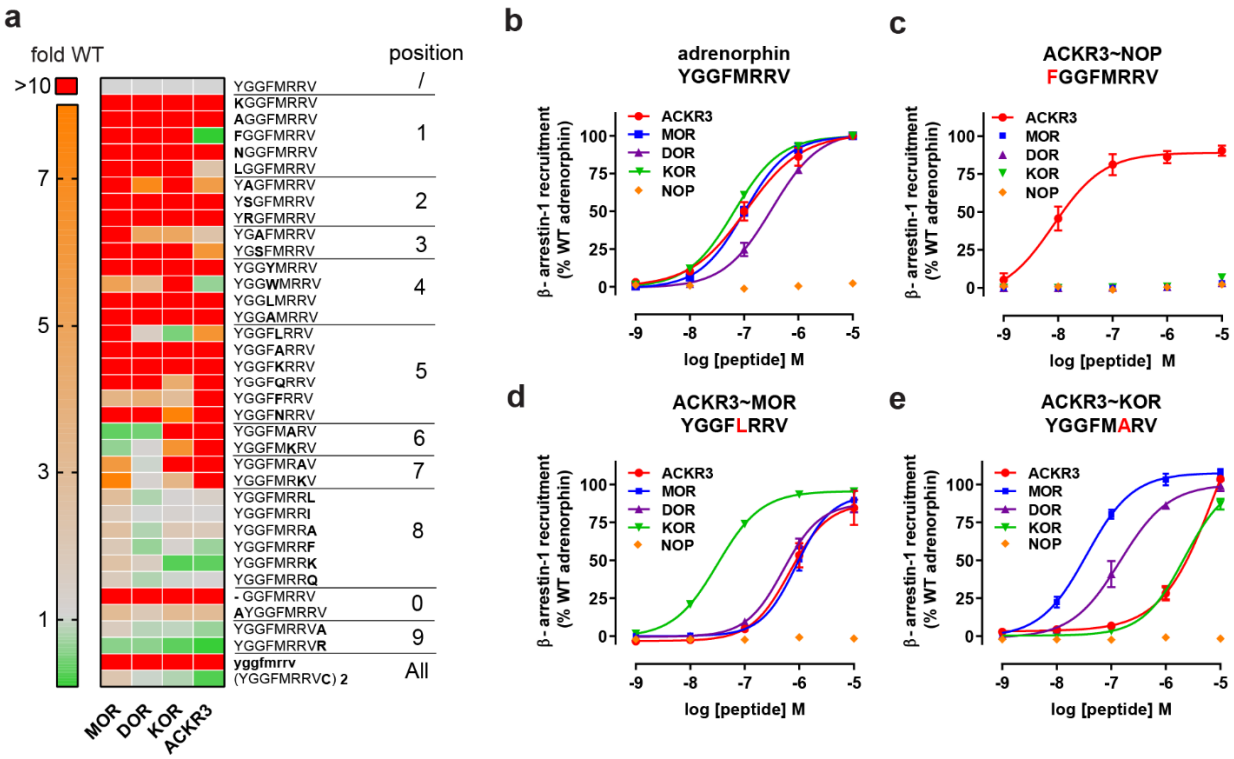


Figure 3. SAR analysis of adrenorphin variants on ACKR3 and classical opioid receptors. (**a**) Comparison of the impact of substitutions, truncations, extensions, D-amino acid replacement or dimerization on the agonist activity of adrenorphin towards ACKR3, MOR, DOR, and KOR. The agonist activity of each variant was evaluated in β-arrestin-1 recruitment assay in U87 cells and expressed as fold change over the activity of wild-type adrenorphin. None of the peptides showed agonist activity towards NOP. (**b - e**) Comparison of potency and efficacy of adrenorphin (b) and its variants bearing mutations Y1F (c), M5L (d) and R6A (e) to induce β-arrestin-1 recruitment to ACKR3 and the opioid receptors KOR, MOR, DOR and NOP in U87 cells. "~ " indicates a similar impact of the modification on the potency of the peptide towards ACKR3 and the indicated opioid receptor. Results represent the mean (a) or mean ± S.E.M (b - e) of three independent experiments (n = 3). Corresponding EC_{50} values are available in supplementary Table4.

3.4. ACKR3 is unresponsive to alkaloid opioids and opioid drugs

Based on its ability to respond to different families of endogenous opioid peptides and the binding mode similarities with classical opioid receptors, we wondered whether ACKR3 could also respond to non-endogenous opioid ligands commonly used to activate or inhibit the classical opioid receptors. Besides prototypical opioid tool compounds like D-Ala^2,D-Leu^5-Enkephalin (DADLE) or [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin (DAMGO), we tested approved pain medications such as morphine, fentanyl or buprenorphine in β-arrestin recruitment assays. All molecules showed their expected agonist or antagonist activities on their respective opioid receptors (Figure 4a). For morphine, only a weak β-arrestin recruitment to MOR was observed in line with previous reports[60]. At high concentrations, many of these molecules, although designed to target specifically one receptor, showed some activity towards other opioid receptors, similar to the endogenous ligands. However,

ACKR3 was not responsive to any of the molecules tested, even at high concentrations. Weak activation of ACKR3 was observed with the KOR agonist U50488 and antagonist nor-binaltrophimine, but with a much weaker potency compared to KOR.

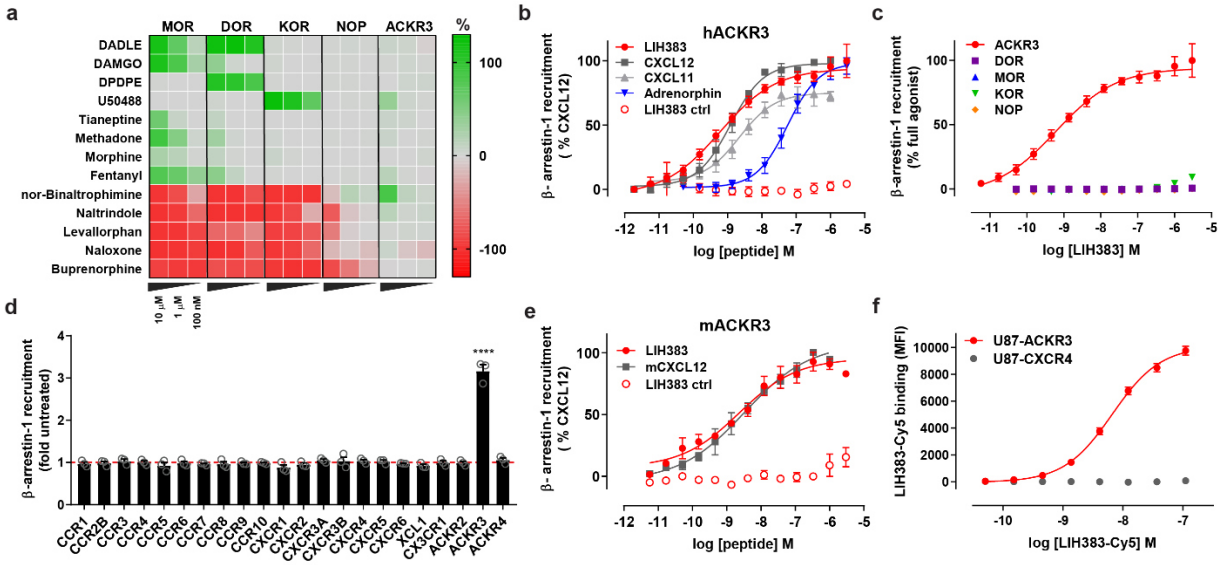


Figure 4. Activity of opioid modulators and development of LIH383 as ACKR3-selective agonist. (a) Agonist (green color scale) and antagonist (red color scale) activity towards ACKR3 of opioid modulators (10 µM, 1 µM and 100 nM) commonly used for research purposes or in clinic and comparison with the opioid receptors MOR, DOR, KOR and NOP monitored in a β-arrestin-1 recruitment assay. Antagonist activity was measured following addition of BAM22 (50 nM), met-enkephalin (70 nM), dynorphin A (50 nM), nociceptin (70 nM) and CXCL12 (4 nM) on MOR, DOR, KOR, NOP and ACKR3, respectively. (**b** and **e**) Agonist activity of LIH383 (FGGFMRRK) towards human (b) and mouse (e) ACKR3 and comparison with other endogenous ACKR3 chemokine ligands, and a control peptide (MRRKFGGF), consisting of the 8 amino acids building LIH383 in a different arrangement. **(c** and **d)** LIH383 selectivity evaluated by comparison of β-arrestin-1 recruitment to ACKR3 and the opioid receptors MOR, DOR, KOR and NOP (c) or all the other chemokine receptors known to recruit β-arrestin (3 µM) (d). (**f**) Selective binding of fluorescently labeled LIH383 (LIH383-Cy5) to ACKR3-expressing cells. All assays were performed in U87 cells. Results represent the mean (a) or mean ± S.E.M (b - f) of three to five independent experiments (n = 3 to 5) except for LIH383 on hACKR3 (n= 9). All fitted values are available in supplementary Table 6. For statistical analysis, one-way ANOVA with Bonferroni multiple comparison test was used. **** p< 0.0001.

These results show that although ACKR3 shares several endogenous opioid peptide ligands with classical opioid receptors, it is not modulated by opiate analgesics or synthetic opioid drugs targeting classical opioid receptors. This is in accordance with the absence of the binding pocket determinants in ACKR3 that are required for productive interaction with such ligands (supplementary Figure 3).

3.5. LIH383 is a highly selective subnanomolar agonist of ACKR3

Despite its attractiveness as drug target, there is still a clear lack of small, pharmacologically well-characterized and easily available molecules for specific ACKR3 modulation *in vitro* or *in vivo*. To overcome this limitation, we took advantage of the adrenorphin SAR data (Figure 3a) with the aim of

developing a highly potent and selective ACKR3 modulator. We designed a second generation of peptides, using the adrenorphin Y1F variant as scaffold as it showed a 10-fold increase in potency towards ACKR3 and over 100-fold reduction of potency towards the classical opioid receptors MOR, DOR and KOR as compared to WT adrenorphin (Figure 3a and 3c). Other mutations increasing the potency towards ACKR3 including F4W, V8F, V8K or 9R were further combined and the resulting peptides were tested in a β-arrestin recruitment assay (supplementary Tables 4 and 5). Of all the combinations tested, the octapeptide FGGFMRRK-NH_2 (designated LIH383) was the most potent ACKR3 agonist. It competed directly with CXCL12-AF647 for ACKR3 binding at low nanomolar concentrations (supplementary Figure 4a) and was more potent in inducing β-arrestin recruitment to ACKR3 (EC_{50} = 0.61 nM) than the full-length chemokine ligands CXCL12 or CXCL11 (EC_{50} = 1.2 nM and 2.2 nM, respectively) (Figure 4b). Importantly, no activation or inhibition of any other opioid receptor, nor of any other chemokine receptor could be detected upon LIH383 treatment, even at concentrations as high as 3 µM (Figure 4c and 4d, supplementary Figure 4b and 4c). Remarkably, LIH383 had equivalent activity on human and mouse ACKR3 (mACKR3) (Figure 4b and 4e). Moreover, fluorescent labelling at the C-terminal lysine of LIH383 did not alter its binding properties as shown by Cy-5-labeled LIH383 binding to ACKR3-expressing U87 cells, but not to native or CXCR4-expressing U87 cells (Figure 4f). Therefore, LIH383 is a highly attractive and versatile tool for specific ACKR3 modulation or detection of ACKR3-expressing cells in human and rodent models and is particularly suitable for investigating the biological consequences of opioid peptide interactions with ACKR3[34, 61].

3.6. ACKR3 does not signal in response to opioid peptides

To decipher the function and impact of ACKR3 on the opioid system, we tested the ability of opioid peptides to trigger downstream signaling through ACKR3 in U87 cells that have no endogenous expression of CXCR4[3]. We first applied a whole-cell optical biosensing approach based on dynamic mass redistribution (DMR), which enables to detect multiple downstream signaling events including all four major G protein pathways[47, 62 63]. In agreement with other studies[64-69], we did not detect any ACKR3-dependent signaling upon chemokine stimulation (Figure 5a). Likewise, no difference in DMR signal was observed between ACKR3-transfected and non-transfected cells in response to opioid peptides (Figure 5b-d and supplementary Figure 5a and 5b). In contrast, U87 cells expressing the G protein signaling-competent CXCR4, KOR or NOP, did show a strong DMR in response to CXCL12, dynorphin A/adrenorphin or nociceptin 1-13, respectively, in accordance with a robust activation of downstream signaling pathways by these receptors (Figure 5a-d). In line with these observations, we did not detect any interaction of ACKR3 with mini G (mG) proteins (mGi, mGs, mGq or mG12/13) upon chemokine or opioid peptide treatment, in contrast to CXCR4 or classical opioid receptors, which all

efficiently recruited mini G_i (Figure 5e and supplementary Figure 5d). Moreover, ERK phosphorylation levels monitored by Homogeneous Time Resolved Fluorescence (HTRF) remained unchanged upon ligand stimulation of cells stably expressing ACKR3, whereas a strong increase in ERK phosphorylation was observed between 2 and 120 minutes after CXCL12 stimulation of cells stably expressing CXCR4 (Figure 5f) with total ERK levels remaining unchanged (supplementary Figure 5c). These results were further corroborated by the absence of activation of the MAPK/ERK-dependent Serum Response Element (SRE) upon opioid peptide or chemokine stimulation in ACKR3-positive U87 cells (Figure 5g, left panel) or HEK293T and CHO-K1 cells (supplementary Figure 5e), in contrast to the robust signal increase in CXCR4- or classical opioid receptor-expressing cells upon stimulation with the respective ligands. Similar absence of signaling through ACKR3 in response to opioid or chemokine ligands was shown for calcium-dependent Nuclear Factor of Activated T-cell Response Element (NFAT-RE) activation (Figure 5g, right panel).

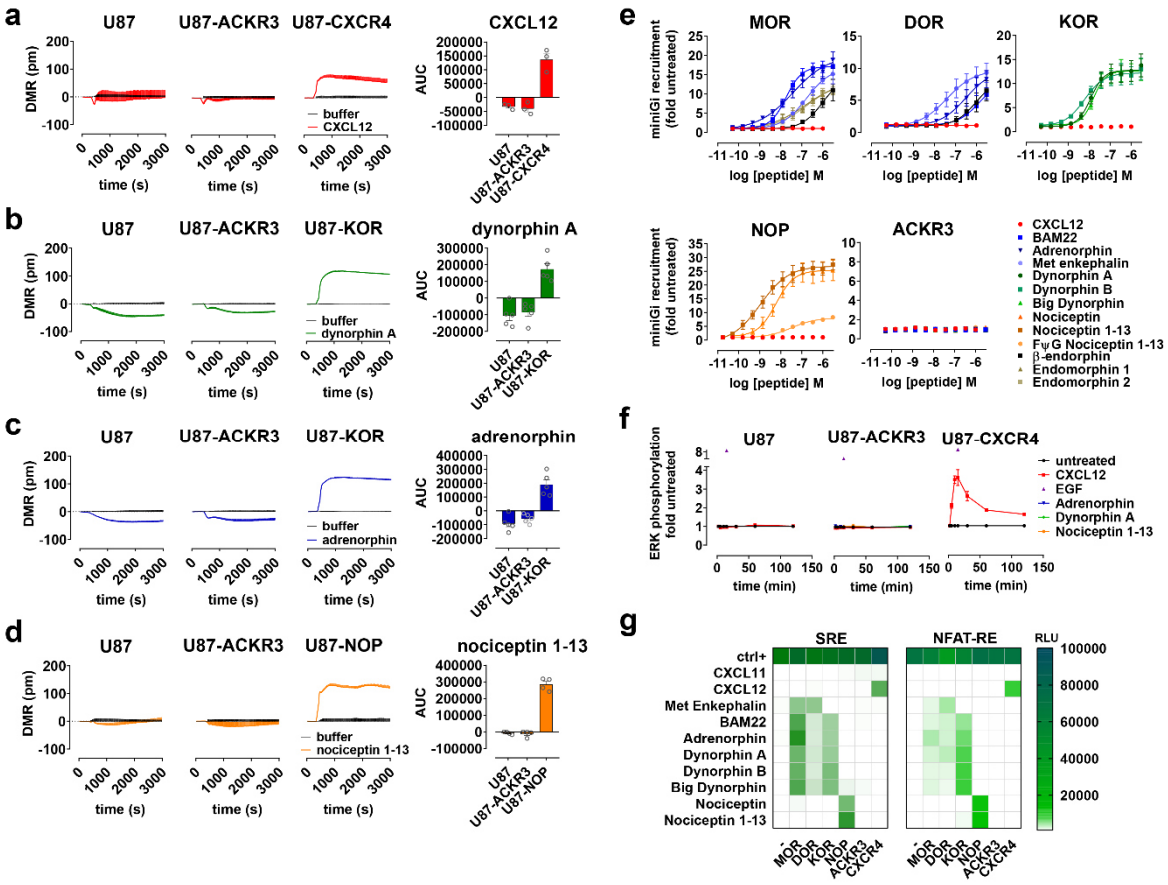


Figure 5. Absence of ACKR3 signaling in response to opioid and chemokine ligands. (a -d) DMR profiles of U87 cells expressing (or not) ACKR3, CXCR4 or classical opioid receptors (KOR and NOP) stimulated by the chemokine CXCL12 (200 nM) (a) or opioid peptides (500 nM) dynorphin A (b), adrenorphin (c) and nociceptin 1-13 (d). Left panels: representative DMR profiles determined over 3000 seconds of three to five independent experiments. Right panel: area under the curve (AUC) ± S.E.M. of three to five independent experiments (n=3 to 5). **(e)** Comparison of mini G_i recruitment to ACKR3 and classical opioid receptors (MOR, DOR, KOR or NOP) in response to CXCL12 and opioid peptides monitored in U87 cells. The corresponding EC_{50} and Emax values are summarized

in supplementary Table 7. **(f)** Kinetic analysis of ERK1/2 phosphorylation in U87 cells stably expressing ACKR3 or CXCR4 stimulated by CXCL12 and opioid peptides. EGF was used as positive control. **(g)** Comparison of activation of SRE (ERK1/2) and NFAT-RE (Ca^{2+}) signaling cascades (green color scale, relative light units, RLU) in U87 cells (-) or U87 expressing ACKR3, CXCR4 or classical opioid receptors (MOR, DOR, KOR or NOP) in response to chemokines CXCL12 and CXCL11 (200 nM), opioid peptides (500 nM) or positive controls (30 nM PMA, 10 % FBS for SRE and 30 nM PMA, 1 µM ionomycin, 10 % FBS for NFAT-RE). Results represent the mean ± S.E.M. (e - f) or the mean (g) of three to five independent experiments (n = 3 to 5)

These data demonstrate that opioid peptides induce β-arrestin recruitment to ACKR3 without triggering signaling typical to G protein-coupled receptors suggesting that ACKR3 may act as a scavenger for opioid peptides in a manner akin to that observed for its chemokine ligands.

3.7. ACKR3 mediates efficient uptake of various opioid peptides

To investigate the ability of ACKR3 to scavenge opioid peptides, we first measured the uptake of fluorescently labeled opioid peptides of different families by cells expressing ACKR3 or the corresponding classical opioid receptors using imaging flow cytometry.

For dynorphin A (1-13), a clear intracellular accumulation of the fluorescently labeled peptide after 40-minute stimulation could be observed in U87-ACKR3 cells, with a notably higher number of distinguishable vesicle-like structures and mean fluorescent intensity compared to U87 cells or U87-ACKR3 cells pre-incubated with LIH383 at a saturating concentration (Figure 6a) demonstrating that ACKR3 can mediate the uptake of opioid peptides. Moreover, the uptake of dynorphin A (1-13) by ACKR3 was more efficient compared to that of KOR, the main classical opioid receptor for this peptide, despite the lower potency of dynorphin A (1-13) towards ACKR3 (Figure 6b and Table 1). Similar observations were made for labeled big dynorphin, the precursor of dynorphin A and B, and for BAM22, a peptide from the enkephalin family. Indeed, despite its similar potency towards the two receptors, BAM22 was markedly more internalized by ACKR3-positive than by MOR-positive cells. The low-affinity ligand nociceptin was also internalized by ACKR3 to a degree equivalent to the corresponding classical opioid receptor NOP (Figure 6b). Importantly, this ACKR3-driven intracellular accumulation of opioid peptides was also associated with a reduction of their availability in the extracellular space. For instance, we found that the apparent potency of dynorphin A in inducing KOR activation was reduced in the presence of ACKR3-expressing cells. This effect was reversed when ACKR3-expressing cells were pre-treated with LIH383 or CXCL12 but not LIH383ctrl peptide or irrelevant chemokine CXCL10, illustrating the plausible scavenging function of ACKR3 (Figure 6c and supplementary Figure 6).

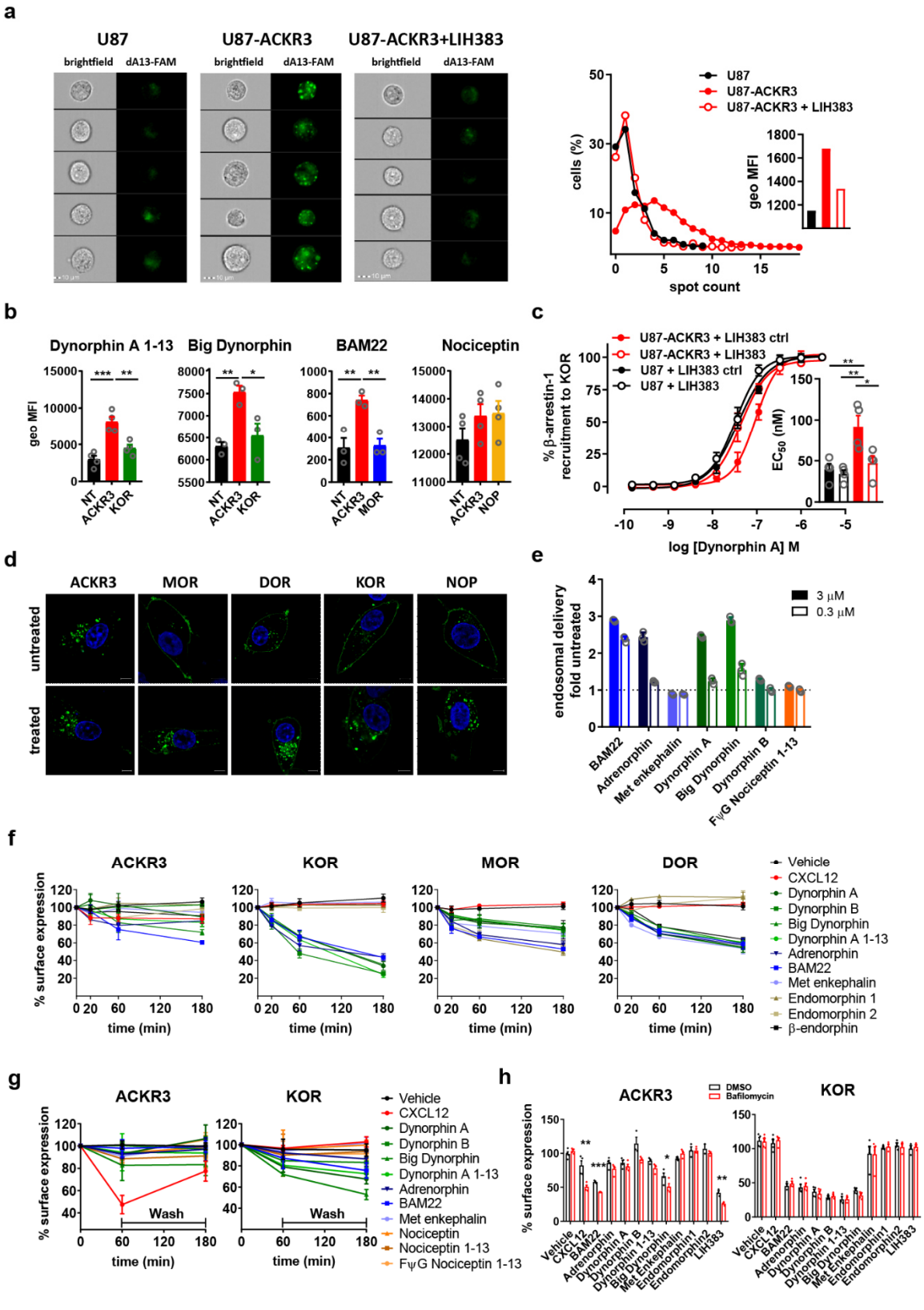


Figure 6. Uptake of opioid peptides and atypical localization and trafficking of ACKR3 (a) Uptake of dynorphin A (1-13) by ACKR3-expressing cells visualized by imaging flow cytometry. Left panels: U87, U87-ACKR3 or U87-ACKR3 cells pre-treated with LIH383 (3 µM) stimulated with (FAM)-labeled dynorphin A (1-13) (250 nM, dA13-FAM, green channel). Five representative cells per condition are shown. Scale bar: 10µm. Right panel: Percentage of cells with a given number of distinguishable vesicle-like structures (spots) and the geometrical mean

fluorescence intensity (MFI, green channel) (inset). Data are representative of three independent experiments. **(b)** Uptake of opioid peptides (dynorphin A (1-13)-FAM (250 nM), big dynorphin-Cy5 (400 nM), BAM22-Cy5 (400 nM) or nociceptin-FAM (1 µM)) by U87 cells (NT) or U87 cells transfected with ACKR3 or classical opioid receptors analyzed by imaging flow cytometry as described in (a). **(c)** ACKR3-mediated depletion of extracellular dynorphin A. U87 or U87-ACKR3 cells pre-treated with LIH383 (400 nM) or LIH383ctrl were incubated with dynorphin A. Cell supernatant was added on U87 cells expressing KOR-SmBiT and LgBiT-beta-arrestin-1. (inset): EC_{50} values. **(d)** Cellular localization of ACKR3 and classical opioid receptors fused to Neongreen fluorescent protein (green) stimulated or not by opioid peptides (1 µM) monitored by fluorescent confocal microscopy. Nuclear DNA was Hoechst-stained (blue). Pictures are representative of 10 acquired images from two independent experiments. Scale bar: 5µm. **(e)** Ligand-induced receptor-arrestin delivery to endosomes monitored by β-galactosidase complementation assay in U2OS cells stably expressing ACKR3. Results are expressed as mean ± SD of three technical replicates. **(f - h)** Kinetics of ligand-induced internalization of ACKR3 and classical opioid receptors (f and h, HiBiT technology) and (g, flow cytometry). **(f)** U87 cells expressing N-terminally HiBiT-tagged receptors were stimulated with opioid peptides (1 µM) or CXCL12 (300 nM) for indicated times. Remaining membrane receptors were quantified with soluble LgBiT protein. **(g)** ACKR3 and KOR internalization and recycling in U87 cells after ligand stimulation (1 µM for opioid peptides, 300 nM for CXCL12) followed by acid wash, monitored by flow cytometry. **(h)** Effect of bafilomycin A1 (1.5 µM) on endosomal trafficking/cycling of ACKR3 and KOR following ligand stimulation (1 µM) monitored by HiBiT technology. If not otherwise indicated, results are presented as mean ± S.E.M of three to five independent experiments (n=3 to 5). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by one-way ANOVA with Bonferroni correction (b), by two-way ANOVA: interaction between cell line and LIH383 treatment with Tukey's post hoc test (c), and by two-tailed unpaired t-test (h).

In line with this scavenging function, ACKR3 showed an atypical cellular localization, internalization and trafficking pattern compared to classical opioid receptors. In agreement with previous reports[37, 69], we observed that a much higher proportion of ACKR3 was present intracellularly compared to the cell surface. In contrast, classical opioid receptors MOR, DOR, KOR and NOP were mainly localized at the plasma membrane (Figure 6d). Moreover, despite an efficient uptake of various opioid peptides and their delivery to the early endosomes (Figure 6e), the overall reduction of ACKR3 at the cell surface was much less pronounced than for classical opioid receptors, likely reflecting the rapid cycling of ACKR3 between the plasma membrane and intracellular compartments (Figure 6f and 6d). Similar to what was reported for chemokines[69, 70], we found that agonist removal after stimulation led to progressive increase of ACKR3 at the plasma membrane, while for classical receptors like KOR such recovery was not observed (Figure 6g). This was further corroborated by results obtained with bafilomycin A1, an inhibitor of vacuolar-type H^+-ATPases. Previous studies showed that low endosomal pH is needed for chemokine dissociation from ACKR3 and efficient receptor recycling and resensitization[69, 70]. We observed that treatment with bafilomycin A1 resulted in decreased receptor recovery at the plasma membrane following stimulation with diverse opioid peptides, whereas it had no effect on surface levels of KOR (Figure 6h).

Altogether, these results demonstrate that ACKR3 can support a rapid and efficient uptake of opioid peptides of different families through continuous receptor cycling between the intracellular

compartments and the plasma membrane, leading to a progressive depletion of extracellular opioid peptides, thereby limiting their availability for classical receptors.

3.8. ACKR3 regulates the availability of opioid peptides in CNS

To support a physiological relevance of the observed opioid peptide scavenging capacity of ACKR3, we then analyzed, using the Brainspan database (www.brainspan.org), its gene expression profile in comparison with classical opioid receptors in different brain regions corresponding to important centers for opioid activity. In agreement with previous studies[27, 71], we found that not only was *ACKR3* expressed in many of these regions such as amygdala, hippocampus or medial prefrontal cortex, but more interestingly we noticed that its expression was often higher (up to 100 fold) than that of MOR (*OPRM1*), KOR (*OPRK1*), DOR (*OPRD1)* and NOP (*OPRL1*) in the same region (Figure 7a and supplementary Figure 7). These data were further confirmed by qPCR on human brain samples, where additional opioid centers such as dentate gyrus or locus coeruleus showed a similar high *ACKR3* expression (Figure 7b).

Considering the expression of ACKR3 in the same regions of the CNS as the classical opioid receptors and its ability to efficiently internalize opioid peptides without inducing downstream G protein mediated signaling, we wondered whether ACKR3 might influence classical opioid receptor signaling by regulating the availability of their ligands. To validate this hypothesis and the inability of ACKR3 to trigger signaling in a more physiological context, we used small molecule neural precursor cells (smNPCs)[41], that endogenously express ACKR3 but no classical opioid receptors (Figure 7c). We confirmed that just like U87-ACKR3 cells, smNPCs express higher proportion of ACKR3 intracellularly compared to the cell surface (Figure 7d) and that they are able to accumulate labeled dynorphin A (1-13) (Figure 7e) without activating the ERK signaling pathway (Figure 7f). In line with U87 cell results, this uptake was also associated with a decrease of the extracellular dynorphin A concentration and consequently its ability to signal through its corresponding classical opioid receptor (Figure 7g).

To ultimately confirm the scavenging function of ACKR3 for opioid peptides, we monitored *ex vivo* the inhibition of spontaneous neuronal firing in slices of rat locus coeruleus, one of the brain regions where ACKR3 is found together with KOR and MOR (Figure 7b)[72]. Treatment with dynorphin A led to a concentration-dependent inhibition of firing with total inhibition obtained with 1 µM (Figure 7h). However, the same concentration of dynorphin A did not lead to a change in neuronal firing rate when pretreated with naloxone, indicating that the dynorphin A-induced inhibition of the firing can be attributed to a classical opioid receptor.

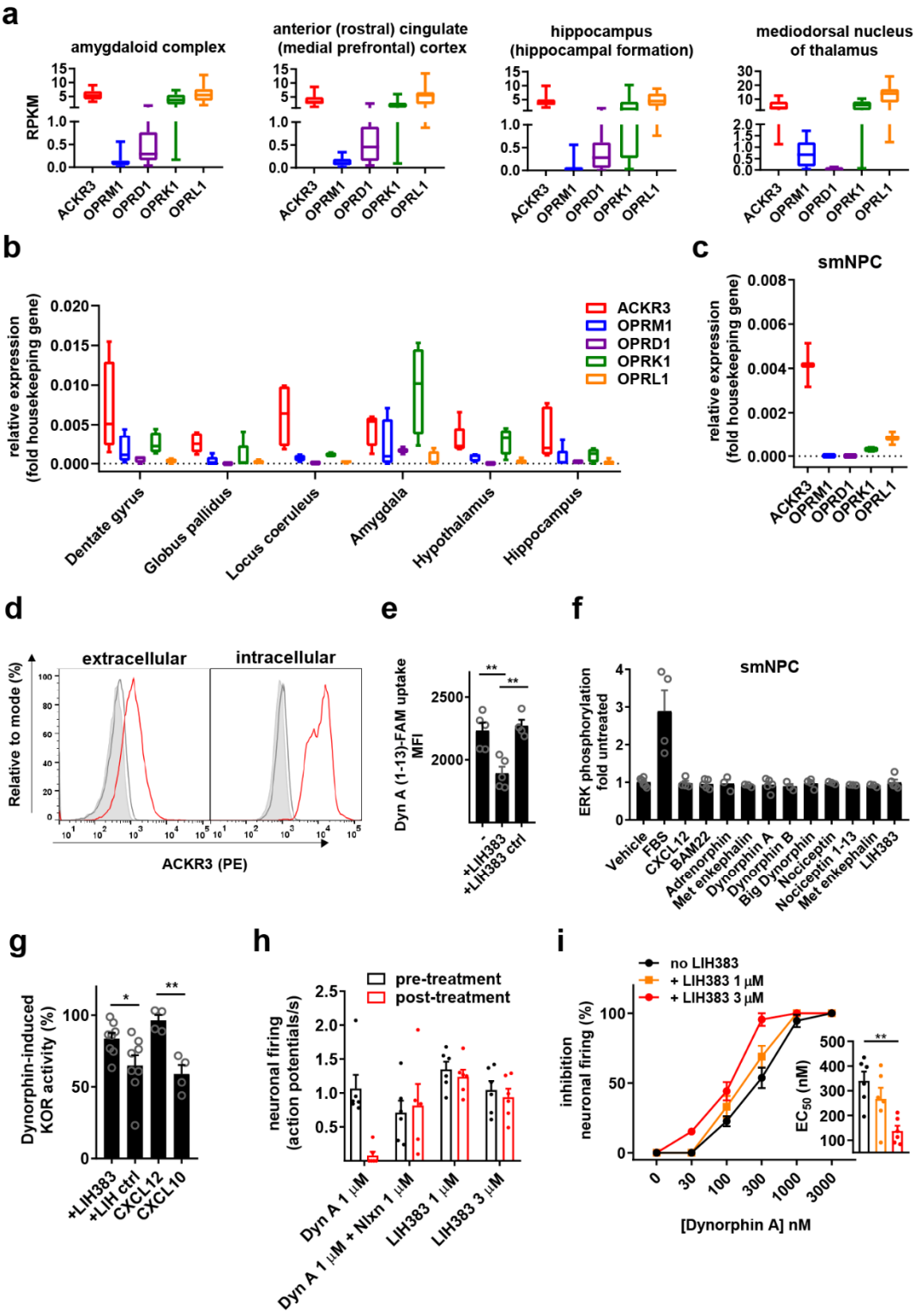


Figure 7. ACKR3-mediated regulation of opioid peptide availability for classical receptors (a – c) Relative gene expression of ACKR3 and classical opioid receptors in smNPCs and different brain regions corresponding to centers important for opioid peptide activity. Box plots encompass 25th to 75th percentile with median as central mark and whiskers extending to most extreme data points. (a) RNA-Seq RPKM (reads per kilobase per million) values from the open-source brainspan.org database (n=16 for amygdaloid complex and mediodorsal nucleus of thalamus, n=15 for hippocampus and n=17 for anterior cingulate cortex). (b and c) mRNA expression determined by qPCR on five human adult brains (b) or two preparations of smNPCs (c) normalized to the arithmetic mean of PPIA and GAPDH as housekeeping genes. (**d**) Extracellular and intracellular expression of ACKR3 monitored by

flow cytometry using ACKR3-specific mAb antibody (11G8, red) or a matched isotype control (MG1-45, grey) and a PE-conjugated secondary antibody in comparison to unstained cells (filled light grey), representative of two experiments with 10,000 gated cells. (**e**) Uptake of 250 nM (FAM)-labeled dynorphin A (1-13) by smNPCs pre-treated with LIH383 or LIH383ctrl (3 µM) analyzed by imaging flow cytometry. (**f**) SRE (ERK1/2) signaling cascade activation in smNPC in response to various ligands (500 nM) or 10 % FBS as positive control. (**g**) ACKR3-mediated depletion of extracellular dynorphin A. SmNPCs, pre-treated with LIH383, LIHctrl (1.5 µM), CXCL12 or CXCL10 (300 nM), were incubated with dynorphin A (3 µM) and the activity of dynorphin A remaining in the cell supernatants was probed on U87 cells expressing SmBiT-tagged KOR and LgBiT-tagged mini G_i. Representative 30x supernatant dilution is shown. **(h** and **i)** *Ex vivo* rat locus coeruleus inhibition of neuronal firing induced by LIH383 alone, dynorphin A in the absence or presence of naloxone (h) or increasing concentrations of dynorphin A in the presence or absence of LIH383 (i) (inset): EC_{50} values. For (e - i), data are presented as mean ± S.E.M of independent experiments (n =3 to 5 except in (g) for LIH and LIHctrl (n = 8) and all animal experiments (h and i) (n = 6)). * p< 0.05, ** p< 0.01 by one-way ANOVA with Bonferroni correction (e and g) and Kruskal-Wallis with two-sided Dunn's test (i).

Treatment with LIH383 at concentration as high as 3 µM however did not lead to significant inhibition of neuronal firing, further confirming the inability of ACKR3 to trigger classical G protein signaling in this region of the CNS (Figure 7h). Interestingly, pretreatment of locus coeruleus neurons with LIH383 (1 or 3 µM) to selectively block the scavenging capacity of ACKR3 resulted in an improved potency of dynorphin A towards its classical receptors (Figure 7i). This observation is in line with our *in vitro* data and suggests that, in a physiological environment and at endogenous receptor abundance, ACKR3 exerts a scavenging function to regulate opioid peptide availability and thereby fine-tune the signaling through their classical opioid receptors.

4. Discussion

More than 40 years after the identification of the classical opioid receptors MOR, DOR and KOR and 20 years after the deorphanization of NOP, this study provides strong evidence that ACKR3 is an additional and broad spectrum opioid receptor. Besides sharing many endogenous ligands, ACKR3 and the classical opioid receptors are all expressed in different important centers of opioid activity in the CNS. Our study shows however, that ACKR3 is an atypical opioid receptor and does not induce classical G protein signaling upon opioid peptide binding but rather internalizes these peptides in order to regulate their availability for classical opioid receptors.

ACKR3 was deorphanized as a chemokine receptor in 2006 based on its ability to bind CXCL12 and CXCL11. Our study demonstrates that in contrast to this rather narrow range of chemokine ligands, ACKR3 is a highly promiscuous opioid receptor, binding and scavenging peptides belonging to different subfamilies, primarily enkephalins and dynorphins, with potencies similar to those of classical opioid receptors. The function and signaling capacity of ACKR3 have since long been a matter of debate[29, 31, 66]. Our study, by using a range of different assay platforms - monitoring various early and late signaling steps typical of GPCRs - provides strong evidence that ACKR3, in contrast to classical opioid receptors

and CXCR4, is not able to trigger detectable ERK activation or any other canonical G protein signaling in response to opioid peptides or chemokines, corroborating its atypical silent behavior. Moreover, our study shows that ACKR3 is not only capable of binding active opioid peptides but also their precursors, such as big dynorphin, as well as inactive N-terminally truncated peptides, such as dynorphin A 2-17, suggesting that it acts as an important multilevel rheostat of the opioid system. Although it is difficult to judge on the physiological relevance of these interactions that only occur at relatively high concentrations, it should be noted that certain opioid peptides, including dynorphin have been shown to reach micromolar local concentrations in certain areas of the CNS[73, 74]. Indeed, in addition to the core-dependent inactivation of opioid peptides by broad-range proteolytic enzymes cleaving bonds within the N-terminal YGGF sequence[75 76 77], scavenging by ACKR3 provides an alternative way to fine tune the opioid system by specific address-dependent regulation of opioid peptide availability.

The identification of ACKR3 as a major regulator of the opioid system opens additional therapeutic opportunities. Indeed, drugs targeting the opioid system remain among the most widely prescribed analgesics for severe pain but their use frequently leads to tolerance, dependence or depression. There is thus an urgent need to find means to modulate the opioid system by drugs with alternative mechanisms of action and improved safety profiles. Our results showed that none of the small drugs targeting the classical opioid receptors such as morphine, fentanyl or naloxone activated or inhibited ACKR3. Interestingly, blocking ACKR3 with LIH383 positively impacted on the availability and signaling of opioid peptides through classical receptors in a rat *ex-vivo* model, providing an original and indirect alternative to modulate the system. This concept is endorsed by previous *in vivo* results showing that mice treated with CCX771, a small ACKR3 modulator, exhibit anxiolytic-like behavior[1]. However, our results clearly point towards an arrestin-dependent opioid peptide scavenging function of ACKR3 in the CNS, rather than signal transduction, which is supported by the recent reconsideration of arrestins as having merely a regulatory role in GPCR signaling [63, 78, 79].

Besides ACKR3, other receptors with pharmacological properties incompatible with the classical receptors MOR, DOR and KOR were proposed to bind opioid peptides. For instance, the N-methyl-D-aspartate (NMDA) receptor and the bradykinin receptors were reported to bind to big dynorphin and dynorphin A, respectively[80-82]. Other opioid activities were attributed either to the non GPCR Zeta (ς) opioid receptor that binds met-enkephalin with high affinity, to the undefined opioid receptors Epsilon (ε) and Lamba (λ) or to other unknown receptors[83 84 85]. In the light of the present study, it is tempting to speculate that some of these activities may be attributed to the scavenging function of ACKR3[86, 87]. Recently, the orphan receptor MRGPRX2 (Mas-related GPCR X2 or MRGX2) was also proposed as an atypical opioid receptor[73, 88, 89]. It is expressed on mast cells and small diameter neurons in dorsal root

and trigeminal ganglia and induces classical G protein signaling in response to micromolar concentrations of pro-dynorphin-derived peptides and well-known synthetic opioid agonists. Its designation as an atypical opioid receptor stems from its restrained selectivity for dynorphin peptides, its unique preference for dextromorphinans and dextrobenzomorphans and its insensitivity to classical opioid receptor antagonists[73]. ACKR3, by analogy to its classification in the ACKRs subfamily, owes its designation as atypical to the characteristics that distinguish it from any other opioid peptide receptor. These include its inability to induce G protein signaling in response to ligand stimulation, its continuous recycling after opioid peptide binding, and efficient ligand depletion from extracellular space but also its broad-spectrum selectivity for opioid peptides, in the nanomolar range, and its unresponsiveness to alkaloid opioids and synthetic opioid drugs.

Similarly to the classical opioid receptors, ACKR3 is highly conserved among species and through the evolution indicating important functions. A detailed comparative sequence analysis revealed that ACKR3 does not harbor the residues delimiting the orthosteric morphinate binding pocket[90] of classical opioid receptors but does retain residues that are highly conserved in chemokine receptors[91] (supplementary Figure 3a and 3b). However, although structurally and genetically linked to the chemokine receptor family[92,91, 93, 94], phylogenetic analysis does not associate ACKR3 directly with the classical or atypical chemokine receptors, but rather places it on a separate branch in between the chemokine and the opioid receptors (supplementary Figure 3c). Noteworthy, sequence analysis revealed the conservation of a four-residue loop within the N terminus of KOR (C_{16}APSAC_{21}) and ACKR3 (C_{21}NSSDC_{26})[37, 95]. Considering that few other GPCRs bear a similar loop, one might speculate that this loop derives from a common ancestor serving a similar purpose for both receptors.

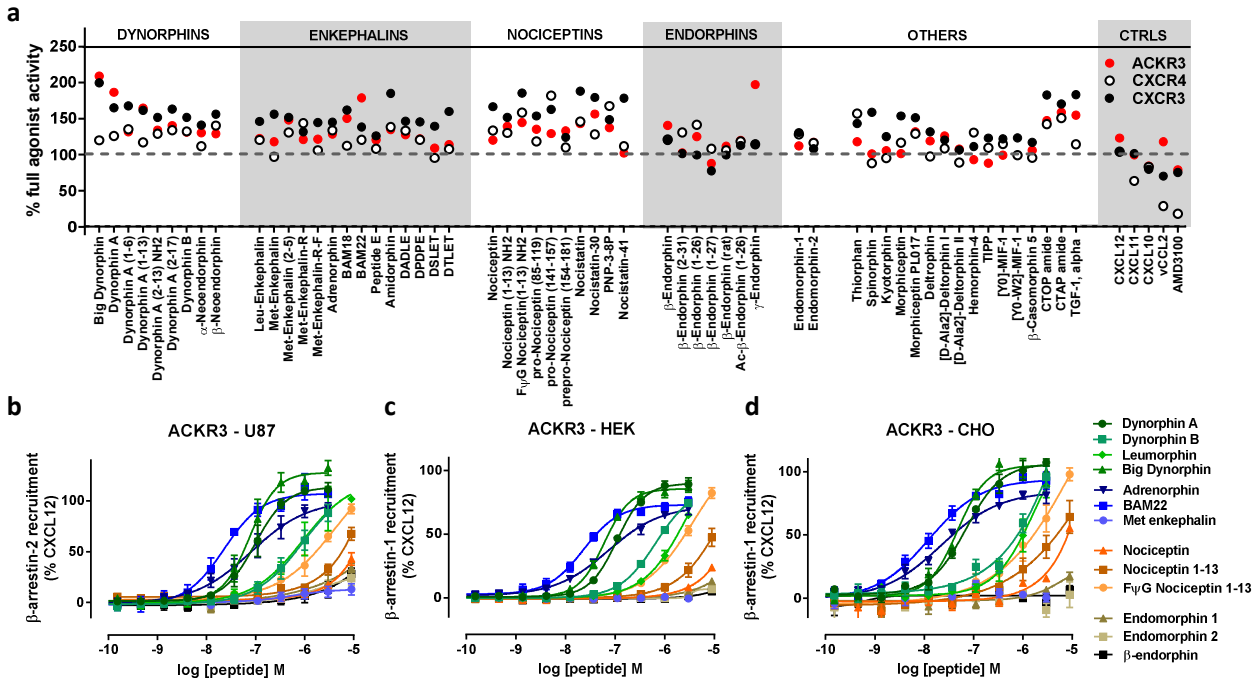
Besides the brain, ACKR3 is abundantly expressed in numerous other tissues, including the adrenal glands where a link between ACKR3 and BAM22 has previously been suggested[1]. The interplay between ACKR3 and opioids may therefore apply to other physiological systems and in particular the immune system, as subsets of lymphocytes, monocytes or macrophages, proposed to express ACKR3, have also been shown to secrete and respond to opioid peptides[96-100]. The results of this study further strengthen the proposed crosstalk between the chemokine and the opioid systems but, by identifying a common receptor directly used by ligands of the two systems, extend it far beyond the concept of simple allosteric modulation through heterodimer formation[101 16, 102]. How this dual ligand scavenging function impacts on each other remains to be elucidated. Moreover, although the results of the present study add another piece to the growing body of evidence that ACKR3 is unable to trigger G protein signaling, it cannot be excluded that, in certain cell types or cellular contexts, ACKR3 could induce G protein-dependent or -independent signaling[32]. Finally, in analogy to its regulatory function

within the chemokine system (when co-expressed with CXCR4) the ability of ACKR3 to heterodimerize with classical opioid receptors and to modulate their signaling properties remain to be investigated[103].

The unique dual chemokine-opioid peptide scavenging activity in the nanomolar range of ACKR3 represents a rare example of functional promiscuity among GPCRs (Figure 1h). So far, few other receptor ligand dualities have been described such as those between dopamine and noradrenergic receptors[104] and many such reports remain to be independently confirmed. This cross reactivity may stem from a common ancestor, co-evolution or the high degree of similarity between the opioid core and the N-terminal sequences of the ACKR3-binding chemokines.

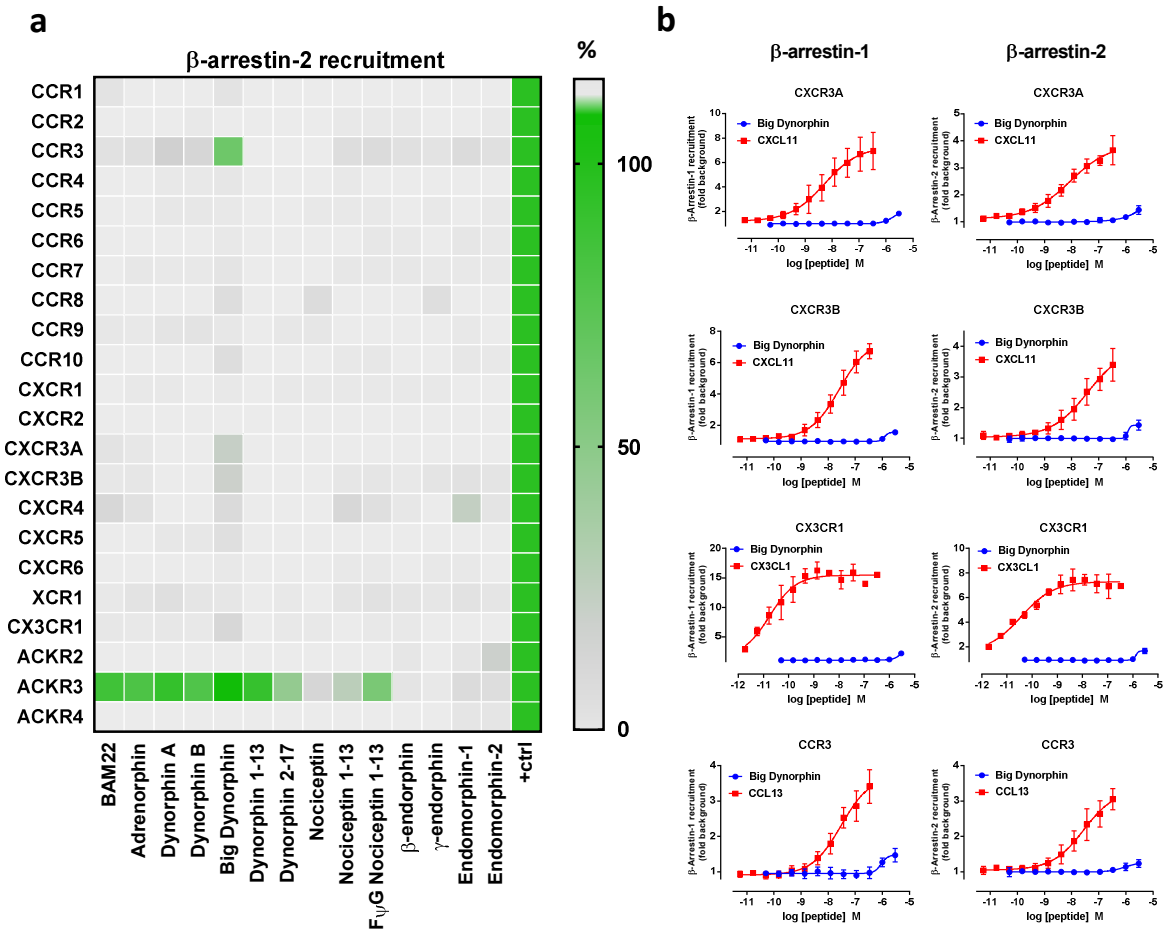
In conclusion, the identification of ACKR3 as negative regulator of opioid peptide function adds another level of complexity and fine-tuning to the opioid system but also opens additional therapeutic opportunities. Considering its ligand binding properties, lack of G protein signaling, and scavenging functions, we propose ACKR3 as a promiscuous, atypical opioid receptor (AOR) and as the first opioid scavenger receptor.

5. Supplementary Data

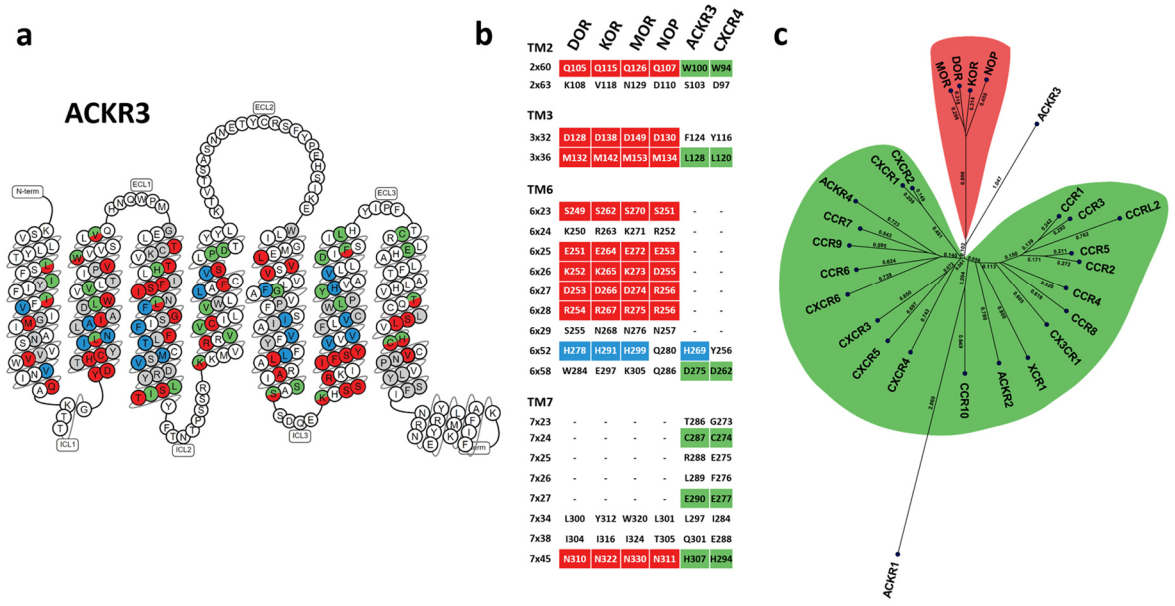


Supplementary Figure 1. Opioid peptide library screening in antagonist mode and confirmation of ACKR3 activation in different cell lines. (**a**) Antagonistic activity of 58 compounds including natural opioid peptides from the four opioid families, variants thereof and small molecule opioid receptor modulators (5 µM) towards ACKR3, CXCR4 and CXCR3 evaluated using β-arrestin-2 recruitment assay in U87 cells. For full peptide names and sequences, see supplementary Table 1. Antagonist activity was measured in the presence of CXCL12 (4 nM for ACKR3, 35 nM for CXCR4) or CXCL11 (50 nM for CXCR3) and results are presented as mean of two technical replicates for ACKR3 and CXCR4. A single measurement was performed for CXCR3. (**b-d**) Comparison of potency

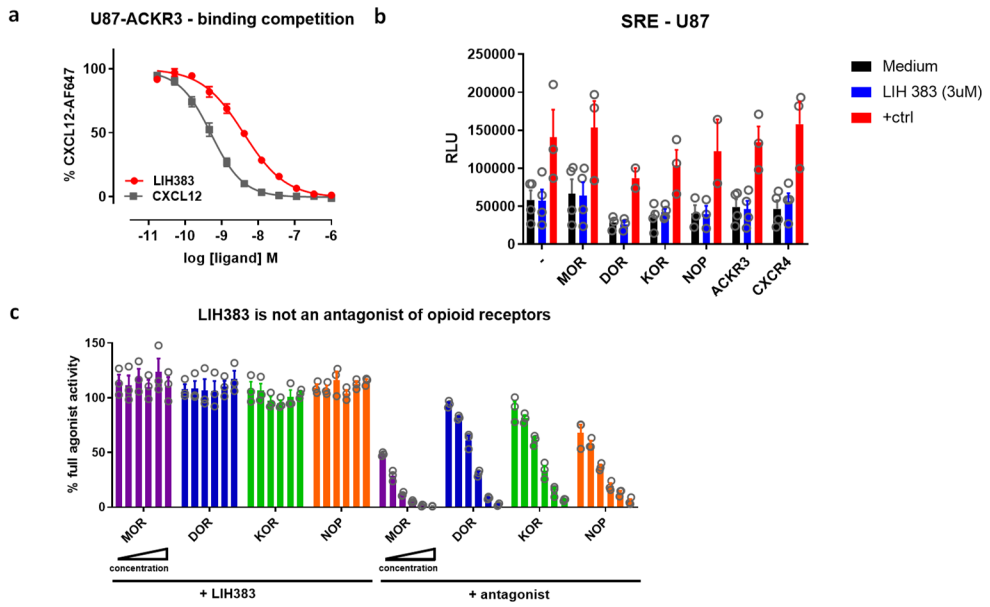
and efficacy of different opioid peptides in inducing β-arrestin-2 recruitment to ACKR3 in U87 cells (**b)** or β-arrestin-1 recruitment to ACKR3 in HEK cells (**c)** or in CHO cells (**d**). Results are presented as mean ± S.E.M of independent experiments (n = 3-5). The corresponding EC_{50} and Emax values are summarized in supplementary Table 9.



Supplementary Figure 2. Specific activation of ACKR3 by opioid peptides. (a) Agonist activity of opioid peptides (3 µM) representative of the four opioid families towards 18 classical and 3 atypical chemokine receptors evaluated in β-arrestin-2 recruitment assay. Results are expressed as the percentage of signal recorded with agonist chemokines used as positive controls (supplementary Table 3) at a concentration of 100 nM and presented as mean of independent experiments (n=3) (**b**) Comparison of potency and efficacy of big dynorphin with a full agonist chemokine inducing the recruitment of β-arrestin-1 (left) or β-arrestin-2 (right) to the chemokine receptors CXCR3A, CXCR3B, CX3CR1 or CCR3 in U87 cells. Results are expressed as fold change over untreated and presented as mean ± S.E.M of independent experiments (n =3). The corresponding EC_{50} and Emax values are summarized in supplementary Table 10.

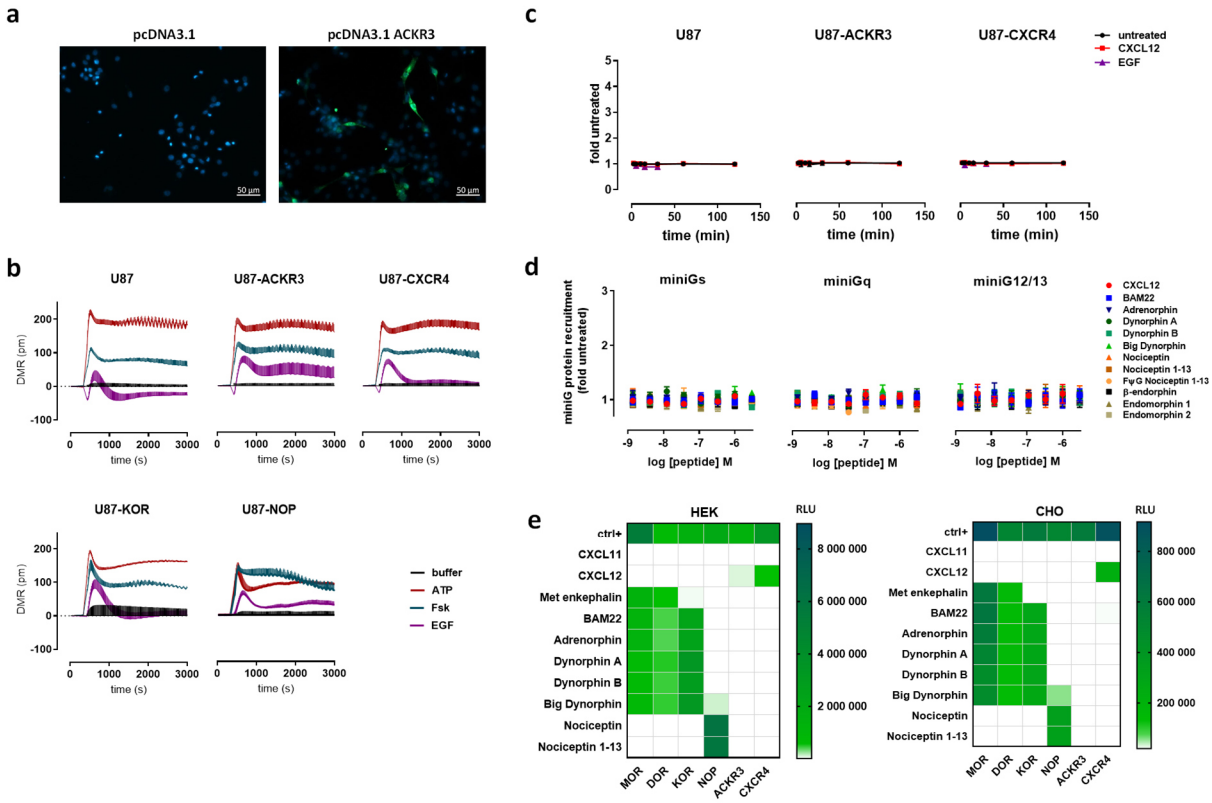


Supplementary Figure 3. Phylogenetic analysis and comparison of residues conserved in ACKR3, opioid and chemokine receptors. (**a**) Snake diagram depicting ACKR3 topology and identifying the positions occupied by residues conserved only in ACKR3 and CXCR4 (green), conserved in opioid receptors but not in ACKR3 (red), conserved separately in chemokine receptors and opioid receptors (green /red), conserved in at least three of the four opioid receptors and ACKR3 (blue) or in opioid receptors as well as ACKR3 and CXCR4 (grey) (GPCRdb). (**b**) Comparison of particular positions such as positions delineating the orthosteric binding pocket of opioid receptors (2x60, 3x32, 3x36, 6x52, 7x34, 7x38 and 7x45) or CXCR4 (2x63, 6x58 and 7x38), the bottom of TM6 (6x23 to 6x29), the top of TM7 (7x23 to 7x27) or key conserved position in chemokine receptors (7x38). (**c**) Phylogenetic tree of the human chemokine and opioid receptors showing that ACKR3 stands on a separate branch in between the chemokine and the opioid receptors (GPCRdb).

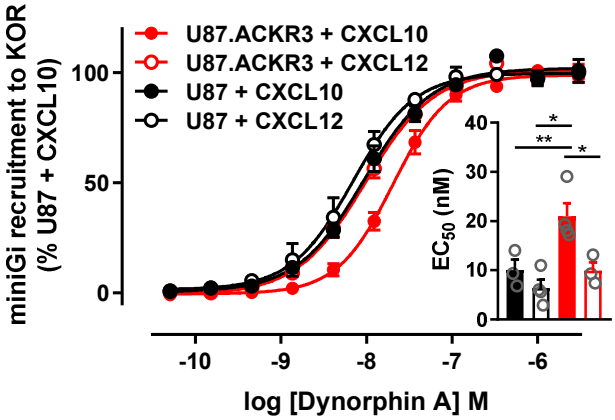


Supplementary Figure 4. Absence of activity and signaling by LIH383 towards classical opioid receptors and ACKR3-binding potency of LIH383. (**a**) Binding competition of increasing concentrations of ACKR3-activating CXCL12 or LIH383 with Alexa Fluor 647-labeled CXCL12 (5 nM) on U87-ACKR3 cells determined by flow cytometry.

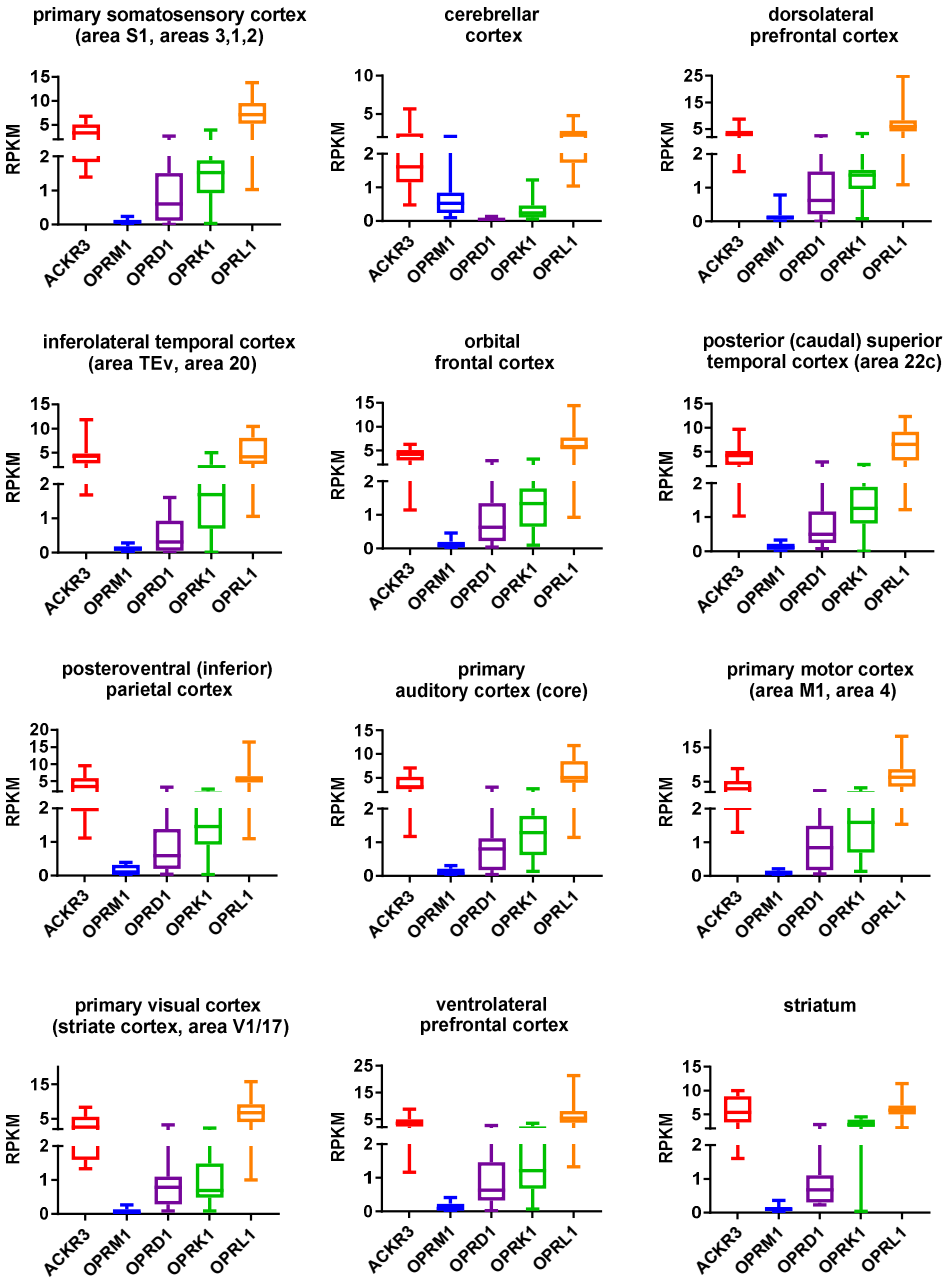
(**b**) Activation of SRE (ERK1/2) signaling cascade in U87 cells (-) or U87 expressing ACKR3, CXCR4 or classical opioid receptors (MOR, DOR, KOR or NOP) in response to 3 µM LIH383 or positive controls (+ctrl, 30 nM PMA, 10 % FBS). Medium only was used as reference. (**c**) Dose dependent antagonist activity of LIH383 (12.3-3000 nM) towards classical opioid receptors MOR, DOR, KOR and NOP determined by β-arrestin-1 recruitment. Naloxone (4.1-1000 nM) for MOR, DOR and KOR or buprenorphine (111-27000 nM) for NOP were used as positive control antagonists. Antagonist activity was measured following addition of BAM22 (50 nM), met-enkephalin (70 nM), dynorphin A (50 nM), nociceptin (70 nM) on MOR, DOR, KOR and NOP, respectively. Results are presented as mean ± S.E.M of independent experiments (n = 3-5).



Supplementary Figure 5. Absence of biased/alternative ACKR3 signaling in response to opioid peptides in different cell lines. (**a**) Verification of effective ACKR3 transfection by fluorescence microscopy for DMR experiments. Adherent U87 cells transfected with pcDNA3.1-ACKR3 or an empty pcDNA3.1 vector were fixed and stained with an ACKR3-specific primary mouse antibody (clone 11G8) and a secondary Cy2-conjugated anti-mouse antibody (green). Nuclei were stained with DAPI (blue). Pictures are representative of three independent transfections used for DMR experiments. Scale bar: 50µm. (**b**) DMR profiles of U87 cells expressing (or not) ACKR3, CXCR4 or classical opioid receptors (KOR and NOP) stimulated with 100 µM of adenosine triphosphate (ATP), forskolin (Fsk) or epidermal growth factor (EGF) determined over 3000 seconds. Representative profiles of three to five independent experiments are shown. (**c**) Kinetic analysis of total ERK in U87 cells stably expressing ACKR3 or CXCR4 stimulated by CXCL12 or EGF. Results represent the mean of independent experiments (n=2). **(d)** mini G_s, mini G_q and mini $G_{12/13}$ recruitment to ACKR3 in response to CXCL12 and opioid peptides monitored in U87 cells. Results represent the mean ± S.E.M of independent experiments (n = 3-5). (**e**) Activation of SRE (ERK1/2) signaling cascade in HEK (left panel) or CHO (right panel) cells expressing or not ACKR3, CXCR4 or classical opioid receptors MOR DOR KOR or NOP in response to chemokines CXCL12 and CXCL11, opioid peptides or positive controls (30 nM PMA, 10 % FBS). Results represent the mean of independent experiments (n = 3).



Supplementary Figure 6. Regulation by ACKR3 of opioid peptide availability for classical receptors. Dynorphin A-induced concentration-dependent recruitment of mini G_i to KOR in the presence of tenfold excess of U87 or U87-ACKR3 cells (1.5×10^5) pre-treated for 15 minutes with CXCL12 (200nM) to block ACKR3, or the irrelevant chemokine CXCL10 (200nM). The determined EC_{50} values are represented as bars in the inset. Results are presented as mean ± S.E.M of independent experiments (n=3 for U87 + CXCL10 and U87 ACKR3 + CXCL12 and n=4 for both other conditions). * $p< 0.05$, ** $p< 0.01$ by Two-way ANOVA: interaction between cell line and chemokine treatment with Tukey's post hoc test.



Supplementary Figure 7. Relative gene expression of ACKR3 and classical opioid receptors in different brain regions. RNA-Seq RPKM (reads per kilobase per million) values from the open-source brainspan.org database containing data of different brain regions from 14–22 donors (n=14 for striatum, n=15 for dorsolateral prefrontal cortex, n=16 for primary somatosensory cortex and for primary motor cortex, n=17 for orbital frontal cortex and for primary auditory cortex, n=18 for primary visual cortex, n=19 for ventrolateral prefrontal cortex and for posteroventral parietal cortex, n=21 for cerebellar cortex and for inferolateral temporal cortex, n=22 for posterior superior temporal cortex). Box plots encompass 25th to 75th percentile with median as central mark and whiskers extending to most extreme data points.

Supplementary Table 1. Sequences, full names and family of endogenous opioid peptides and variants thereof used in experiments reported in Figure 1a and supplementary Figure 1a.

Name/Abbreviation used in Fig 1A			
Dynorphins	Big Dynorphin	Dynorphin, big (Human, Rat, Mouse, Porcine)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
	Dynorphin A	Dynorphin A (Human, Rat, Mouse, Porcine)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
	Dynorphin A (1-6)	Dynorphin A (1-6) / Endorphin (1-6), alpha-Neo / Leu-Enkephalin-Arg (Human, Rat, Mouse, Porcine)	Tyr-Gly-Gly-Phe-Leu-Arg
	Dynorphin A (1-13)	Dynorphin A (1-13) (Human, Rat, Mouse, Porcine)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys
	Dynorphin A (2-13) NH2	Dynorphin A (2-13) amide (Human, Rat, Mouse, Porcine)	Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-NH2
	Dynorphin A (2-17)	Dynorphin A (2-17) (Human, Rat, Mouse, Porcine)	Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
	Dynorphin B	Dynorphin B / Rimorphin (Human, Rat, Mouse s, Porcine)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
	α-Neoendorphin	Endorphin, alpha-Neo (Porcine)	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys
	β-Neoendorphin	Endorphin, beta-neo	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro
Enkephalins	Leu-Enkephalin	Leu-Enkephalin	Tyr-Gly-Gly-Phe-Leu
	Met-Enkephalin	Met-Enkephalin	Tyr-Gly-Gly-Phe-Met
	Met-Enkephalin (2-5)	[Des-Tyr1]-Met-Enkephalin	Gly-Gly-Phe-Met
	Met-Enkephalin-R	Met-Enkephalin-Arg	Tyr-Gly-Gly-Phe-Met-Arg
	Met-Enkephalin-R-F	Met-Enkephalin-Arg-Phe	Tyr-Gly-Gly-Phe-Met-Arg-Phe
	Adrenorphin	Metorphinamide / Adrenorphin	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-NH2
	BAM18	BAM-18P (Bovine)	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln
	BAM22	BAM-22P (Bovine)	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly
	Peptide E	Peptide E / 3200-Dalton Adrenal Enkephalin-Containing Peptide (Bovine)	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu
	Amidorphin	pro-Enkephalin A (104-129) amide / Amidorphin (Bovine)	Tyr-Gly-Gly-Phe-Met-Lys-Lys-Met-Asp-Glu-Leu-Tyr-Pro-Leu-Glu-Val-Glu-Glu-Ala-Asn-Gly-Gly-Glu-Val-Leu-NH2
	DADLE	[D-Ala2, D-Leu5]-Enkephalin	Tyr-D-Ala-Gly-Phe-D-Leu
	DPDPE	[D-Pen2, D-Pen5]-Enkephalin	Tyr-D-Pen-Gly-Phe-D-Pen Disulfide bridge: Pen2-Pen5
	DSLET	[D-Ser2]-Leu-Enkephalin-Thr / Delta-Receptor Peptide / DSLET	Tyr-D-Ser-Gly-Phe-Leu-Thr
	DTLET	[D-Thr2]-Leu-Enkephalin-Thr / DTLET	Tyr-D-Thr-Gly-Phe-Leu-Thr
Nociceptins	Nociceptin	Orphanin FQ / Nociceptin (Human, Rat, Mouse, Ox)	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln
	Nociceptin (1-13) NH2	Orphanin FQ (1-13) amide / Nociceptin (1-13) amide (Human, Rat, Mouse, Ox)	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH2
	[F-ψ-G]Nociceptin(1-13) NH2	[Phe psi-Gly]-Orphanin FQ (1-13) amide / [Phe psi-Gly]-Nociceptin (1-13) amide (Human, Rat, Mouse, Ox)	[Phe1ψ(CH2-NH)-Gly2]-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH2
	pro-Nociceptin (85-119)	pro-Orphanin FQ (85-119) / pro-Nociceptin (85-119) / Nocistatin-35 (Rat)	Met-Pro-Arg-Val-Arg-Ser-Val-Val-Gln-Ala-Arg-Asp-Ala-Glu-Pro-Val-Ala-Asp-Glu-Ala-Asp-Val-Glu-Gln-Lys-Gln-Leu-Gln
	pro-Nociceptin (141-157)	pro-Orphanin FQ (141-157) / pro-Nociceptin (141-157)	Phe-Ser-Glu-Phe-Met-Arg-Gln-Tyr-Leu-Val-Leu-Ser-Met-Gln-Ser-Ser-Gln
	pregro-Nociceptin (154-181)	pregro-Orphanin FQ (154-181), free acid (Rat) / pregro-Orphanin FQ (160-187) free acid (Mouse)	Phe-Ser-Glu-Phe-Met-Arg-Gln-Tyr-Leu-Val-Leu-Ser-Met-Gln-Ser-Ser-Gln-Arg-Arg-Arg-Thr-Leu-His-Gln-Asn-Gly-Asn-Val
	Nocistatin	pregro-Orphanin FQ (111-127) / Nocistatin / PNP-3 (Bovine)	Thr-Glu-Pro-Gly-Leu-Glu-Glu-Val-Gly-Glu-Ile-Glu-Glu-Gln-Lys-Gln-Leu-Gln
	Nocistatin-30	pregro-Orphanin FQ (98-127) / Nocistatin-30 (Human)	Met-Pro-Arg-Val-Arg-Ser-Leu-Phe-Glu-Glu-Glu-Glu-Glu-Pro-Glu-Pro-Gly-Met-Glu-Glu-Ala-Gly-Glu-Met-Glu-Glu-Lys-Gln-Leu-Glu-Gln-
	PNP-3-8P	PNP-3-8P (Bovine)	Glu-Ile-Glu-Glu-Lys-Gln-Leu-Gln
	Nocistatin-41	PNP-2/3 / Nocistatin-41 (Mouse)	Met-Pro-Arg-Val-Arg-Ser-Leu-Val-Glu-Val-Arg-Asp-Ala-Glu-Pro-Gly-Ala-Asp-Ala-Glu-Pro-Gly-Ala-Asp-Asp-Ala-Glu-Glu-Val-Glu-Glu-Lys-Gln-Leu-Gln
Endorphins	β-Endorphin	Endorphin, beta (Human)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
	β-Endorphin (2-31)	Endorphin, beta (2-31) (Human)	Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-
	β-Endorphin (1-26)	Endorphin, beta (1-26) (Human)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala
	β-Endorphin (1-27)	Endorphin, beta (1-27) (Human)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr
	β-Endorphin (rat)	Endorphin, beta (Rat)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Val-His-Lys-Lys-Gly-Gln
	Ac - β-Endorphin (1-26)	acetyl-Endorphin, beta (1-26) (Human)	Ac-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala
	γ-Endorphin	Endorphin, gamma / Lipotropin, beta (61-77) (Human, Bovine, Ovine, Camel)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu
Endomorphins	Endomorphin 1	Endomorphin 1	Tyr-Pro-Trp-Phe-NH2
	Endomorphin 2	Endomorphin 2	Tyr-Pro-Phe-Phe-NH2
Others	Thiorphan	Thiorphan / D,L-3-mercapto-2-benzylpropanoyl-Glycine	D,L-3-Mercapto-2-Benzylpropanoyl-Glycine
	Spinorphin	Spinorphin (Bovine)	Leu-Val-Val-Tyr-Pro-Trp-Thr
	Kyotorphin	Kyotorphin	Tyr-Arg
	Morphiceptin	Casomorphin (1-4), amide beta / Morphiceptin	Tyr-Pro-Phe-Pro-NH2
	Morphiceptin PL017	[N-MePhe, D-Pro4]-Morphiceptin / PL017	Tyr-Pro-MePhe-D-Pro-NH2
	Deltorphin	Dermenkephalin / Deltorphin (Phyllomedusa s sauvagei)	Tyr-D-Met-Phe-His-Leu-Met-Asp-NH2
	[D-Ala2]-Deltorphin I	[D-Ala2]-Deltorphin I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH2
	[D-Ala2]-Deltorphin II	[D-Ala2]-Deltorphin II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH2
	Hemorphin-4	Hemorphin-4 (H-4) (Human, Bovine)	Tyr-Pro-Trp-Thr
	TIPP	TIPP / delta-Opioid Antagonist	Tyr-Tic-Phe-Phe
	[Y0-W2]-MIF-1	[Tyr0, Trp2]-Melanocyte Inhibiting Factor 1 (MIF-1)	Tyr-Pro-Trp-Gly-NH2
	[Y0]-MIF-1	[Tyr0]-Melanocyte Inhibiting Factor 1 (MIF-1)	Tyr-Pro-Leu-Gly-NH2
	β-Casomorphin 5	Casomorphin (1-5), beta	Tyr-Pro-Phe-Pro-Gly
	CTOP amide	CTOP amide (Cys2, Tyr3, Orn5, Pen7) / Somatostatin Analog	D-Phe-Cys-Tyr-D-Trp-Om-Thr-Pen-Thr-NH2 Disulfide bridge: Cys2-Pen7
	CTAP amide	CTAP amide (Cys2, Tyr3, Arg5, Pen7)	D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 Disulfide bridge: Cys2-Pen7
	TGF-1, alpha	Transforming Growth Factor 1 (TGF-1), alpha (Rat)	Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol

Supplementary Table 2. Sequences of selected opioid peptides and their binding competition with CXCL12 AF647 on ACKR3 reported in Figure 1g and supplementary Figure 4a

Name	Sequence	Binding ACKR3	
		IC_{50} (CI) nM	Min %
Dynorphin A	**YGGF**LRRIRPKLKWDNQ	85.3 (64.2-118)	6
Dynorphin A 2-17	-**GGF**LRRIRPKLKWDNQ	~10,000	55*
Dynorphin A 2-13-NH2	-**GGF**LRRIRPKLK-NH2	> 10,000	75*
Dynorphin A 1-13	**YGGF**LRRIRPKLK	122 (96.3 - 162)	9
Dynorphin B	**YGGF**LRRQFKVVT	~1000	33
Leumorphin	**YGGF**LRRQFKVVTRSQEDPNAYSGELFDA	~6000	41*
Big Dynorphin	**YGGF**LRRIRPKLKWDNQKR**YGGF**LRRQFKVVT	32.8 (25.4 - 41.9)	4
Adrenorphin	**YGGF**MRRV-NH2	41.2 (28.2 - 60.1)	7
BAM22	**YGGF**MRRVGRPEWWMDYQKRYG	31.3 (27.6 - 35.5)	3
Met enkephalin	**YGGF**M	NB	110*
Nociceptin	F**GGF**TGARKSARKLANQ	NB	106*
Nociceptin 1-13-NH2	F**GGF**TGARKSARK-NH2	> 10,000	78*
[Phe1Ψ(CH2-NH)-gly2]nociceptin-(1-13)-NH2	[Fψ(CH2-NH)G]GFTGARKSARK-NH2	> 10,000	71*
Endomorphin-1	YPWF-NH2	> 10,000	81*
Endomorphin-2	YPFF-NH2	NB	102*
β-endorphin	**YGGF**MTSEKSQTPLVTLFKNAIIKNAYKKGE	NB	125*
LIH383	F**GGF**MRRK-NH2	4.0 (3.1 - 5.1)	1

IC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)

NB: No binding competition with 5 nM CXCL12-AF647

Min = minimal remaining AF647 signal measured at 3 µM non-labeled competitor and expressed as % of signal measured for 5 nM CXCL12-AF647 without competitor

*measured at 9 µM

YGGF motif conserved in most of the opioid peptides is shown in bold. Positively charged residues are underlined

Supplementary Table 3. Activity of positive controls used in Figure 2 and Supplementary Figure 2a

Receptor	Positive ctrl	β-arrestin-1	β-arrestin-2
		Fold untreated ± SEM	Fold untreated ± SEM
CCR1	CCL3	2.01 ± 0.35	1.64 ± 0.14
CCR2B	CCL2	9.49 ± 1.69	7.15 ± 1.05
CCR3	CCL13	2.13 ± 0.19	1.88 ± 0.15
CCR4	CCL22	8.20 ± 0.64	5.49 ± 0.14
CCR5	CCL5	17.00 ± 0.77	9.07 ± 0.24
CCR6	CCL20	13.26 ± 4.37	6.86 ± 0.52
CCR7	CCL19	7.45 ± 1.27	5.94 ± 0.77
CCR8	CCL1	2.91 ± 0.26	1.56 ± 0.28
CCR9	CCL25	5.71 ± 1.38	5.69 ± 1.63
CCR10	CCL27	4.97 ± 1.61	3.05 ± 0.52
CXCR1	CXCL8	30.77 ± 5.68	14.54 ± 1.08
CXCR2	CXCL8	2.46 ± 0.44	2.20 ± 0.24
CXCR3A	CXCL11	8.16 ± 0.62	3.30 ± 0.17
CXCR3B	CXCL11	6.05 ± 0.93	3.49 ± 0.36
CXCR4	CXCL12	1.69 ± 0.04	1.37 ± 0.09
CXCR5	CXCL13	4.85 ± 0.28	3.78 ± 0.10
CXCR6	CXCL16	29.50 ± 5.45	16.52 ± 1.85
XCR1	XCL2	4.75 ± 1.52	3.26 ± 1.07
CX3CR1	CX3CL1	10.24 ± 1.72	8.66 ± 0.86
ACKR2	CCL5	1.42 ± 0.09	1.33 ± 0.11
ACKR3	CXCL12	3.03 ± 0.23	2.79 ± 0.36
ACKR4	CCL19	6.02 ± 0.77	4.39 ± 0.54

Values correspond to fold increase over untreated and represent the mean of three independent experiments ± standard error of the mean.

Supplementary Table 4. Sequences of adrenophin-derived peptides and activity in β-arrestin-1 recruitment towards human opioid receptors reported in Figure 3.

Sequence	Position	Receptor			
		MOR	DOR	KOR	ACKR3
		EC_{50} (CI) nM	EC_{50} (CI) nM	EC_{50} (CI) nM	EC_{50} (CI) nM
YGGFMRRV	Ref.	101 (83.0 - 125)	323 (258 - 409)	65.6 (56.5 – 75.9)	109 (70.7 – 182)
KGGFMRRV	1	NA	NA	NA	NA
AGGFMRRV	1	NA	NA	NA	>10,000
FGGFMRRV	1	NA	NA	NA	9.33 (5.12 – 16.0)
NGGFMRRV	1	NA	NA	NA	NA
LGGFMRRV	1	NA	NA	NA	261 (nc – 573)
Y**A**GFMRRV	2	1094 (776 – 4207)	2317 (nc – 75020)	NA	611 (397 – 1052)
Y**S**GFMRRV	2	NA	NA	NA	~10,000
Y**R**GFMRRV	2	NA	NA	NA	~5,000
YG**A**FMRRV	3	>10,000	1581 (nc – 4986)	315 (253 – 392)	263 (163 – 425)
YG**S**FMRRV	3	>10,000	>10,000	>10,000	668 (465 – 1049)
YGG**Y**MRRV	4	NA	NA	NA	~5,000
YGG**W**MRRV	4	522 (361 – 725)	1018 (844 – 1436)	>10,000	70.3 (38.3 – 136)
YGG**L**MRRV	4	NA	NA	NA	~10,000
YGG**A**MRRV	4	NA	NA	NA	~10,000
YGGFLRRV	5	880 (624 – 1572)	513 (403 – 639)	31.9 (28.4 – 25.8)	684 (368 – 1682)
YGGF**A**RRV	5	>10,000	>10,000	656 (555 -775)	~5,000
YGGF**K**RRV	5	NA	NA	NA	~10,000
YGGF**Q**RRV	5	~5000	>10,000	283 (221 – 366)	~5,000
YGGF**F**RRV	5	363 (225 – nc)	1313 (nc – 5420)	194 (81.3 – 900)	1535 (nc – 4196)
YGGF**N**RRV	5	NA	NA	508 (441 – 586)	~10,000
YGGFM**A**RV	6	35.4 (27.7 – 45.2)	147 (98.5 – 238)	2238 (nc – 12290)	~5,000
YGGFM**K**RV	6	63.8 (50.6 – 79.3)	360 (248 – 533)	416 (355 – 485)	~5,000
YGGFMR**A**V	7	682 (227 – nc)	308 (214 – 461)	1250 (1001 – 2131)	NA
YGGFMR**K**V	7	786 (567 – 1250)	378 (260 – 565)	230 (212 – 251)	~10,000
YGGFMRR**L**	8	294 (235 – 366)	254 (184 – 354)	71.2 (59.6 – 84.5)	153 (104 – 242)
YGGFMRR**I**	8	188 (nc – 260)	320 (215 – 470)	76.9 (55.9 – 105)	115 (81.1 – 177)
YGGFMRR**A**	8	265 (nc – 448)	260 (nc – 427)	132 (59.8 – 343)	201 (109 – 478)
YGGFMRR**F**	8	198 (177 – 220)	212 (171 – 261)	80.9 (66.9 – 96.5)	73.5 (45.2 – 119)
YGGFMRR**K**	8	252 (187 – 342)	527 (383 – 713)	19.2 (14.9 – 24.8)	37.0 (19.1 – 75.0)
YGGFMRR**Q**	8	183 (143 – 240)	259 (180 – 381)	63.1 (53.0 – 74.6)	110 (43.6 – 499)
- GGFMRRV	0	NA	NA	NA	>10,000
AYGGFMRRV	0	308 (238 – 396)	637 (240 – 19890)	205 (157 – 270)	330 (nc – 1394)
YGGFMRRV**A**	9	174 (nc – 270)	272 (186 – 403)	57.5 (49.6 – 66.2	72.3 (50.7 – 101)
YGGFMRRV**R**	9	58.7 (46.1 – 73.5)	193 (136 – 283)	21.9 (20.3 – 23.7)	12.6 (7.47 – 21.2)
yggfmrrv	Daa	NA	NA	NA	>10.000
(YGGFMRRVC)2	dimer	271 (188 – 389)	253 (154 – 337)	79.4 (44.7 – 138)	52.9 (17.9 – 173)

EC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)
NA: No activity or activity below 10 % of positive control in the concentration range tested
nc; not calculable
Note that all peptides showed no activity towards NOP
Peptides presented in Figure 3 b-e are marked in red

Supplementary Table 5. Potencies of peptide variants combining mutation Y1F with other modifications in β-arrestin-1 recruitment to ACKR3.

2nd generation peptides	ACKR3	
Sequence	**EC_{50} (nM)**	**pEC_{50} ± SEM**
FGGFMRR**K** (LIH383)	0.61	9.21 ± 0.17
FGGFMRR**KR**	1.12	8.96 ± 0.20
FGGFMRRV**R**	3.53	8.45 ± 0.10
FGG**W**MRR**K**	4.39	8.36 ± 0.12
FGG**W**MRRV**R**	6.16	8.21 ± 0.14
FGG**W**MRR**KR**	7.34	8.14 ± 0.13
FGG**W**MRRV	10.52	7.98 ± 0.62
FGGFMRR**F**	10.96	7.96 ± 0.29
FGGFMRR**FR**	16.34	7.79 ± 0.24
FGG**W**MRR**FR**	19.07	7.72 ± 0.22
FGG**W**MRR**F**	19.10	7.72 ± 0.20
FGGFMRRV (Y1F adrenorphin)	9.33	8.03 ± 0.11
YGGFMRRV (adrenorphin)	108.7	6.96 ± 0.09
MRRKFGGF (LIH383 ctrl)	inactive	

Modified amino acids with respect to peptide Y1F-adrenorphin (FGGFMRRV) are underlined and in bold

Supplementary Table 6. Activity of LIH383, adrenorphin and chemokines in β-arrestin-1 recruitment to human and mouse ACKR3 and classical human opioid receptors reported in Figure 4 b-f.

Name	β-arrestin-1											
	hACKR3		MOR		DOR		KOR		NOP		mACKR3	
	EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %
LIH383	0.61 (0.19 – 1.17)	99	NA	1	NA	1	NA	9	NA	1	2.31 (0.008 – 7.56)	95
LIH383 Ctrl	NA	4	-	-	-	-	-	-	-	-	ND	17
CXCL12	1.21 (0.83 – 1.74)	100	-	-	-	-	-	-	-	-	-	-
CXCL11	2.20 (0.78 – 4.81)	75	-	-	-	-	-	-	-	-	-	-
Adrenorphin	56.5 (36.6 – 95.0)	96	41.6 (30.7 – 57.7)	96	157 (104 – 310)	96	43.5 (33.7 – 57.9)	98	NA	3	-	-
mCXCL12	-	-	-	-	-	-	-	-	-	-	3.32 (1.17 – 33.6)	100
LIH383-Cy5	6.65 (5.52 – 8.29)	100	-	-	-	-	-	-	-	-	-	-

EC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)
ND: Not determinable since saturation was not reached
NA: No activity or activity below 10 % of positive control in the concentration range tested
Max = maximum signal measured at 3 µM expressed as % of the reference peptide
- = not analyzed

Supplementary Table 7. Activity of selected opioid peptides in mini G_i recruitment to ACKR3, MOR, KOR, DOR and NOP reported in Figure 5e.

Name	Sequence	mini Gi									
		ACKR3		MOR		DOR		KOR		NOP	
		EC50 (CI) nM	Max %	EC50 (CI) nM	Max %	EC50 (CI) nM	Max %	EC50 (CI) nM	Max %	EC50 (CI) nM	Max %
CXCL12		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
BAM22	**YGGF**MRRVGRPEWWMDYQKRYG	NA	NA	17.8 (12.2 – 26.5)	100	1324 (547 - nc)	63	-	-	-	-
Adrenorphin	**YGGF**MRRV-NH2	NA	NA	38.0 (14.23 – 43840)	110	315 (92.6 - nc)	89	-	-	-	-
Met enkephalin	**YGGF**M	NA	NA	170 (97.7 – 453)	89	39.4 (11.7 - nc)	100	-	-	-	-
Dynorphin A	**YGGF**LRRIRPKLKWDNQ	NA	NA	-	-	-	-	11.8 (6.49 – 22.0)	100	-	-
Dynorphin B	**YGGF**LRRQFKVVT	NA	NA	-	-	-	-	6.06 (1.99 – 14.8)	95	-	-
Big Dynorphin	**YGGF**LRRIRPKLKWDNQ**KRYGGF**LRRQFKVVT	NA	NA	-	-	-	-	16.1 (8.98 – 28.7)	94	-	-
Nociceptin	F**GGF**TGARKSARKLANQ	NA	NA	-	-	-	-	-	-	5.97 (2.45 – 12.3)	100
Nociceptin 1-13-NH2	F**GGF**TGARKSARK-NH2	NA	NA	-	-	-	-	-	-	1.06 (0.008 – 2.97)	108
[Phe1Ψ(CH2-NH)-gly2]nociceptin-(1-13)-NH2	[Fψ(CH2-NH)G]GFTGARKSARK-NH2	NA	NA	-	-	-	-	-	-	32.2 (21.6 – 53.8)	33
β-endorphin	**YGGF**MTSEKSQTPLVTLFKNAIIKNAYKKGE	NA	NA	1021 (342 - nc)	64	931 (208 - nc)	70	-	-	-	-
Endomorphin-1	YPWF-NH2	NA	NA	162 (37.9 - nc)	66	-	-	-	-	-	-
Endomorphin-2	YPFF-NH2	NA	NA	175 (53.4 - nc)	63	-	-	-	-	-	-

EC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)
NA: No activity or activity below 10 % of positive control in the concentration range tested
Max = maximum signal measured at 3 µM expressed as % of the reference peptide
nc: not calculable
- = not analyzed
YGGF motif conserved in most of the opioid peptides is shown in bold. Positively charged residues are underlined

Supplementary Table 8. Sequences and annealing temperatures of qPCR primers used for receptor mRNA quantification in human brain samples and smNPC represented in Figure 7b and 7c.

Gene	T_a	Primer Sequence	Reference
ACKR3	57°C	F: 5'-TCGGCAGCATTTTCTTCCTC-3' R: 5'-GCAGTAGGTCTCATTGTTGGAC-3'	*Ikeda et al, 2013*[1]
OPRD	60°C	F: 5'-TCGGGCTCCAAGGAGAAG-3' R: 5'-TCGTCGAGGAAAGCGTAGA-3'	*Ikeda et al, 2013*[1]
OPRK	61°C	F: 5'-CTGCATCTGGCTGCTGTCGT-3' R: 5'-GACGCAGGATCATCAGGGTGTAG-3'	*Ikeda et al, 2013*[1]
OPRM	58°C	F: 5'-CCGTGTGCTATGGACTGA-3' R: 5'-GAAATGCATAAAGGACTGGGTT-3'	*Ikeda et al, 2013*[1]
OPRL	62°C	F: 5'-CTCGGCTGGTGCTGGTGGTA-3' R: 5'-CGTGCAGAAGCGCAGAATGG-3'	*Li et al, 2013*[105]
PPIA	60°C	F: 5'-TCCTGGCATCTTGTCCATG-3' R: 5'-CCATCCAACCACTCAGTCTTG-3'	*Schote et al, 2007*[106]
GAPDH	60°C	F: 5'-GAAGGTGAAGGTCGGAGTC-3' R: 5'-GAAGATGGTGATGGGATTTC-3'	*Schote et al, 2007*[106]

All primer pairs have been validated and used in previous studies (see references [1, 105, 106])

Supplementary Table 9. Sequences of selected opioid peptides and their activity in β-arrestin-1 or β-arrestin-2 recruitment to ACKR3 in different cell lines reported in supplementary Figure 1 b-d.

Name	Sequence	β-arrestin-2		β-arrestin-1			
		ACKR3-U87		ACKR3-HEK		ACKR3-CHO	
		EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %
Dynorphin A	**YGGF**LRRIRPKLKWDNQ	97.6 (70.5 – 138)	111	110 (97.2 – 125)	89	70.5 (54.5 – 93.9)	107
Dynorphin B	**YGGF**LRRQFKVVT	908 (373 - nc)	88	673 (572 – 831)	74	~1000	96
Leumorphin	**YGGF**LRRQFKVVTRSQEDPNAYSGELFDA	902 (342 - nc)	92*	2708 (1302 – 92760)	65*	1723 (700 - nc)	88*
Big Dynorphin	**YGGF**LRRIRPKLKWDNQKR**YGGF**LRRQFKVVT	76.6 (54.9 – 109.2)	132	60.7 (52.9 – 69.8)	87	47.5 (36.1 – 61.5)	100
Adrenorphin	**YGGF**MRRV-NH2	81.5 (40.1 – 342)	93	72.6 (51.1 – 114)	69	22.5 (15.0 – 34.8)	81
BAM22	**YGGF**MRRVGRPEWWMDYQKRYG	22.9 (15.4 – 35.6)	106	23.2 (17.8 – 30.6)	75	12.5 (9.10 – 19.6)	93
Met enkephalin	**YGGF**M	ND	13*	NA	0*	NA	1*
Nociceptin	F**GGF**TGARKSARKLANQ	>10,000	43*	ND	24*	~10,000	55*
Nociceptin 1-13-NH2	F**GGF**TGARKSARK-NH2	~10,000	68*	~10,000	47*	~10,000	65*
[Phe1Ψ(CH2-NH)-gly2]nociceptin-(1-13)-NH2	[Fψ(CH2-NH)G]GFTGARKSARK-NH2	4344 (1429 - nc)	92*	3761 (2339 – 9706)	82*	~5000	97*
Endomorphin-1	YPWF-NH2	ND	34*	ND	13*	ND	17*
Endomorphin-2	YPFF-NH2	ND	24*	ND	7*	ND	3*
β-endorphin	**YGGF**MTSEKSQTPLVTLFKNAIIKNAYKKGE	ND	28*	ND	5*	ND	7*

EC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)
ND: Not determinable since saturation was not reached
NA: No activity or activity below 10 % of positive control in the concentration range tested
nc: not calculable
Max = maximum signal measured at 3 µM expressed as % of signal measured for CXCL12
*measured at 9 µM
YGGF motif conserved in most of the opioid peptides is shown in bold. Positively charged residues are underlined

Supplementary Table 10. Off-target activity of selected opioid peptides in β-arrestin-1 and β-arrestin-2 recruitment towards human chemokine receptors reported in supplementary Figure 2b

Receptor	β-arrestin-1				β-arrestin-2			
	Positive Ctrl		Big Dynorphin		Positive Ctrl		Big Dynorphin	
	chemokine – EC_{50} (CI) nM	Max	EC_{50} (CI) nM	Max	chemokine – EC_{50} (CI) nM	Max	EC_{50} (CI) nM	Max
CXCR3A	CXCL11 – 5.14 (2.03 – 72.7)	6.93	ND	1.83	CXCL11 – 8.65 (4.26 – 57.1)	3.65	ND	1.43
CXCR3B	CXCL11 – 25.6 (16.1 – 61.8)	6.71	ND	1.57	CXCL11 – 40.2 (14.1 – nc)	3.40	ND	1.42
CX3CR1	CX3CL1 – 0.02 (0.000005 – 0.06)	14.00	ND	2.20	CX3CL1 – 0.04 (0.0002 – 0.12)	7.09	ND	1.68
CCR3	CCL13 – 30.1 (15.5 – 195)	3.41	ND	1.47	CCL13 – 28.7 (11.2 – 13220)	3.05	ND	1.23

EC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)
ND: Not determinable since saturation was not reached
Max = signal measured at 300 nM for chemokines or 3 µM for big dynorphin expressed as fold untreated
nc: not calculable

6. Highlights

- ACKR3 is activated by various endogenous opioid peptides from at least three different families: Efficient β-arrestin recruitment to ACKR3 is especially induced by enkephalins and dynorphins and to a lower extend by nocicepins.
- ACKR3 is the only chemokine receptor with dual chemokine- and opioid activity, which is also underlined in phylogenetic analysis, where ACKR3 is intermediate between chemokine and opioid receptors.
- A structure-activity relationship (SAR) assay using adrenorphin as reference peptide showed that ACKR3 has a unique opioid-binding pocket, sharing important interaction determinants with all of the other opioid receptors.
- ACKR3 is not modulated by prototypical opioid drugs like morphine, fentanyl, naloxone, methadone, DADLE or DAMGO.
- In order to specifically modulate ACKR3, the afore gathered SAR-based information on critical residues for efficient and specific ACKR3 binding and activation of opioid peptides was used to develop a novel small peptide ACKR3 agonist, baptized LIH383 (patent application filed on "Novel Selective ACKR3 Modulators And Uses Thereof"; Publication number: WO/2020/225070, Inventors: Andy Chevigné, Max Meyrath, Martyna Szpakowska).
- No form of ACKR3-mediated signaling could be detected in any of the assays performed (dynamic mass redistribution of stimulated cells, G protein interaction, phosphorylation of ERK and intracellular calcium increase) and in none of three different cell lines tested.
- Uptake of opioid peptides by ACKR3 is significantly more efficient than by classical opioid receptors. This can be explained by the atypical cellular localization, internalization and trafficking properties of ACKR3, with large intracellular ACKR3 pools constantly recycling to the cell surface, mediating efficient ligand scavenging. Contrarily to this, classical opioid receptors are mainly localized on the cell membrane, and are internalized upon ligand binding.
- In human, ACKR3 is co-expressed with classical opioid receptors in brain regions with important opioid activities, like amygdala, locus coeruleus or hippocampus, often even at much higher (up to 100 fold) levels than classical receptors.
- In slices of rat locus coeruleus, dynorphin A dependent inhibition of neuronal firing was significantly more potent when the slices were pretreated with the ACKR3 specific agonist LIH383, while treatment with LIH383 alone had no effect. This points towards a specific regulatory scavenging function of ACKR3 towards endogenous opioid peptides, which can be modulated using specific ACKR3-targeting molecules.

Altogether, these findings greatly enlarge the array of ACKR3 ligands and functions, from a narrow spectrum of chemokines CXCL12 and CXCL11, to a large variety of endogenous opioid peptides. The proof of concept, that ACKR3 can be modulated in the brain to increase the availability of endogenous opioid peptides for classical opioid receptors, opens new therapeutic avenues as alternative to modulating the classical opioid receptors using drugs with severe side effects like tolerance and dependence.

This study also led to several follow-up projects and publications: Firstly, together with the Center for Drug Discovery, RTI international, we could show that conolidine, a natural analgesic alkaloid used in traditional Chinese medicine, targets ACKR3, thereby providing additional proof of a correlation between ACKR3 and pain modulation and the concepts worked out in our main study described in this chapter. This follow up project could show that among over 240 screened receptors, only ACKR3 became activated by the natural analgesic conolidine with micromolar potency (determined in a β-arrestin recruitment assay). Conolidine was able to compete with CXCL12 binding and to restrain BAM22 uptake by ACKR3. Therefore, in analogy to LIH383, conolidine may inhibit the scavenging functions of ACKR3, consequently increasing the availability of pain relief-inducing endogenous opioid peptides for the classical opioid receptors. Although not discussed in further detail here, the interested reader can find a copy of this recently accepted manuscript entitled: *The natural analgesic conolidine targets the newly identified opioid scavenger ACKR3/CXCR7* (published in *Signal transduction and Targeted Therapy*, by Szpakowska, M., Decker, AM., **Meyrath. M**., Palmer, CB., Blough, BE., Namjoshi, OA., Chevigné, A.*)* at the end of this thesis.

Next, various tools that we developed for this study, especially different mini G-proteins coupled to smBIT of the Nanoluciferase in order to analyze receptor interaction with G-proteins could be used in a collaboration with the group of Prof. Christophe Stove from the University of Ghent, leading to a publication in *ACS Pharmacology & Translational Sciences* (Wouters, E., Walraed, J., Robertson, M.J., **Meyrath, M**., Szpakowska, M., Chevigne, A., Skiniotis, G., and Stove, C. *Assessment of Biased Agonism among Distinct Synthetic Cannabinoid Receptor Agonist Scaffolds*. ACS Pharmacol Transl Sci, 3, 285-295 (2020). A research article, that won't be discussed in detail here, since it was not a focus of this PhD project.

Finally, the idea of therapeutically targeting ACKR3 as alternative to modulating classical opioid receptors and possibly reducing side effects in the treatment of pain or other opioid-related disorders prompted us to undergo an extensive literature research on other receptors which were described to have some opioid-related activity besides their first-described function. These are mainly the bradykinin receptors B1 and B2, the ion channel NMDAR, as well as the two Mas-related G protein-

coupled receptors MrgX1 and MrgX2. All of these receptors, although not belonging to the family of opioid receptors, are also activated by endogenous opioid peptides and mediate a direct effect on opioid-related processes. Therapeutic targeting of these receptors might be, as for ACKR3, an interesting and promising alternative to the classical, but flawed approaches that are in use since decades. A comparative overview of the different opioid-related or "non-classical" opioid receptors and the possibility of modulating them in order to treat opioid-related disorders are discussed in a review, which will be submitted to *Pharmacology and Therapeutics* entitled *opioid-related-receptors: unconventional biology and therapeutic opportunities,* by Palmer, CP., **Meyrath, M**., Chevigné, A., Szpakowska, M..

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Chapter 4: Beyond a chemokine receptor: Characterization of novel unconventional ACKR3 ligands

Part2: ACKR3 as a scavenger for proadrenomedullin derived peptides

Adapted from: **Meyrath, M.,** Palmer, CB., Reynders, N., Vanderplasschen, A., Ollert, M., Bouvier, M., Szpakowska, M.*, Chevigné, A.* (2021) "*Proadrenomedullin N-terminal 20 peptides (PAMPs) are agonists of the atypical chemokine receptor ACKR3/CXCR7*" **ACS Pharmacol. Transl. Sci.** 4, 813-823
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After the discovery that ACKR3 is a broad-spectrum scavenger for a family of non-chemokine ligands, we wondered whether peptides from other families might share a similar fate with opioid peptides. One major candidate was the proangiogenic peptide adrenomedullin (ADM), which we already started characterizing back in 2016, but temporarily put on hold to focus on the characterization of opioid peptide-ACKR3 interactions. Already back in 1995, a first study came out claiming that ACKR3 (at that time still named RDC-1) was interacting with ADM and the related calcitonin-gene-related peptide (CGRP), leading to an increase of cAMP[1]. This observation seems nowadays quite odd, considering that none of the chemokine receptors are coupled to G_s and that ACKR3 is generally considered not to be G protein-coupled at all. On the other hand, the main CGRP and ADM receptor, calcitonin receptor-like receptor (CLR), as heterodimer with a receptor activity modifying protein (RAMP), is indeed G_s coupled. Furthermore, later studies could not confirm that ACKR3 (at that time called RDC1) is a CGRP receptor[2]. However, twenty years later, in 2014, another study described ACKR3 as a receptor for ADM[3]. In this study, the authors claimed that ACKR3 is not inducing any ADM-related signaling, but that the receptor acts as an ADM rheostat, reducing its availability for the signaling receptor CLR/RAMP. The authors demonstrated that *Ackr3* knockout in mice recapitulates an *Adm* overexpression phenotype, and that genetic knockout of *Ackr3*, which is normally lethal in mice due to lymphatic and cardiac hyperproliferation, can be counter-balanced by genetic reduction of *Adm*[3], showing that ACKR3 plays a regulatory role of ADM abundance. However, other groups, ours included, had trouble to confirm ADM as a potent ACKR3 ligand[4] (and personal communication). The project gained of interest again, once we realized that a second peptide released during ADM maturation,

called Proadrenomedullin N-terminal 20 peptide (PAMP) was a weak agonist of ACKR3. The characterization of the binding and activation mode of PAMP, and especially further processed PAMP-12 revealed many similarities to ACKR3's activation mode towards opioid peptides, which indeed share some similarities in size and sequence.

Abstract

Adrenomedullin (ADM) and proadrenomedullin N-terminal 20 peptide (PAMP) are two peptides with vasodilative, bronchodilative and angiogenic properties, originating from a common precursor, proADM. Previous studies proposed that the atypical chemokine receptor ACKR3 might act as a low-affinity scavenger for ADM, regulating its availability for its cognate receptor calcitonin receptor-like receptor (CLR) in complex with a receptor activity modifying protein (RAMP). In this study, we compared the activation of ACKR3 by ADM and PAMP, as well as other related members of the calcitonin gene-related peptide (CGRP) family. Irrespective of the presence of RAMPs, ADM was the only member of the CGRP family to show moderate activity towards ACKR3. Remarkably, PAMP, and especially further processed PAMP-12, had a stronger potency towards ACKR3 than ADM. Importantly, PAMP-12 induced β-arrestin recruitment and was efficiently internalized by ACKR3 without inducing G protein or ERK signaling *in vitro*. Our results further extend the panel of endogenous ACKR3 ligands and broaden ACKR3 functions to a regulator of PAMP-12 availability for its primary receptor Mas-related G protein-coupled receptor member X2 (MrgX2).

1. Introduction

Atypical chemokine receptors (ACKRs) are vital regulators of the spatiotemporal distribution of chemokines. ACKRs mediate chemokine internalization, degradation, sequestration or transcytosis without inducing classical G protein-mediated signaling[5]. ACKR3, formerly named CXCR7, is expressed ubiquitously but is most abundantly present in different brain regions, adrenal glands, lymphatic and blood vasculature, heart and on various subsets of immune cells[6, 7]. ACKR3 is a selective scavenger for two endogenous chemokines, CXCL12 and CXCL11, which are also the ligands of CXCR4 and CXCR3, respectively, and for the human herpesvirus 8 (HHV-8)-encoded chemokine vCCL2, as well as the pseudo-chemokine MIF[8-10]. Recently, it has also been shown that ACKR3 is a high-affinity scavenger for a broad spectrum of opioid peptides and modulates their availability for classical opioid receptors[11, 12].

ACKR3 regulates embryogenesis, haematopoiesis, neuronal migration, angiogenesis and cardiac development[8, 11, 13]. Genetic knockout of *Ackr3* in mice is associated with cardiomyocyte hyperplasia and disrupted lymphangiogenesis, usually leading to perinatal death due to cardiac valve and ventricular septal defects[14, 15]. However, these defects do not correlate with the CXCL12-CXCR4 signaling axis, suggesting that ACKR3 interaction with ligands other than CXCL12 may be responsible for this phenotype. Interestingly, recent studies proposed that besides its chemokine and opioid ligands, ACKR3 acts as a molecular rheostat for the proangiogenic peptide adrenomedullin (ADM)[3, 16]. Indeed, *Ackr3* knockout recapitulates the *Adm* overexpression phenotype, and genetic reduction of

Adm expression counterbalances lymphatic and cardiac abnormalities observed in *Ackr3* knockout mice[3].

Adrenomedullin is a 52-amino acid peptide, acting as a vital paracrine factor to promote cardiac development, vasodilation, and formation of blood and lymph vessels[17, 18]. Due to its proangiogenic properties, ADM is also a key player in tumor growth[19]. ADM belongs to calcitonin/calcitonin gene-related peptide (CGRP) family that also includes α-CGRP and β-CGRP, intermedin/adrenomedullin 2 (IMD/ADM2), amylin (AMY) and calcitonin (CT)[20]. ADM binds and activates the G protein-coupled receptor (GPCR) calcitonin receptor-like receptor (CLR), which can only be exported to the cell surface upon heterodimerization with one of the three accessory membrane proteins called receptor activity modifying proteins (RAMPs)[2, 21, 22]. RAMP interactions also define the pharmacological profile of CLR. While a complex with either RAMP2 or RAMP3 generates a selective ADM receptor, dimerization with RAMP1 creates a receptor for calcitonin gene-related peptide (CGRP) with only low affinity for ADM[20].

Adrenomedullin is generated through the proteolysis of a precursor molecule called proadrenomedullin (proADM), which also gives rise to the proadrenomedullin N-terminal 20 peptide (PAMP) (Figure 1A)[23]. PAMP is a 20-amino acid peptide involved in similar processes as ADM but differs in size and sequence and has no activity towards the ADM receptor complex CLR/RAMPs. Instead, the Mas-related G protein-coupled receptor member X2 (MrgX2 or MRGPRX2) was proposed as the receptor for PAMP as well as for its endogenously processed form, PAMP-12, consisting of amino acids 9–20[24, 25]. It is still unknown whether the observed physiological effects of PAMP rely exclusively on MrgX2 or on additional receptors. Although the vast majority of studies focus on ADM rather than on PAMP functions, both peptides are often found in the same regions and exert similar effects[26, 27], suggesting that they may act in parallel. However, the roles and the receptors of PAMP are largely under-investigated.

Although important biological and genetic links have been established between ADM expression and ACKR3[3, 14], the exact regulatory role of ACKR3 in ADM signaling, the pharmacological properties of ADM towards ACKR3 as well as the possible impact of ACKR3 on other proADM-derived peptides and ligands of the CGRP family have not been comprehensively assessed[4, 16].

In this study, we demonstrate that ADM is the only member of the CGRP family that activates ACKR3, with moderate micromolar-range activity. Remarkably, we found that PAMP, the second active peptide released during proADM maturation, has an activity towards ACKR3 that is comparable to ADM. Especially its truncated endogenous analog PAMP-12 shows a greater potency towards ACKR3 than ADM, which is comparable to the high-nanomolar range activity towards its previously identified receptor MrgX2. ACKR3 induces β-arrestin recruitment and drives PAMP-12 internalization, but in

contrast to MrgX2, does not induce classical G-protein signaling or ERK phosphorylation. Our data suggest that the *ADM*-encoded PAMP-12 peptide is an additional endogenous ligand of ACKR3 and cast light on the potential role of PAMP-12, along with ADM, on the phenotypes observed in *Adm* knockout animals or overexpression experiments.

2. Materials and Methods

Chemokines and Peptides

All chemokines were purchased from PeproTech. Alexa Fluor 647-labeled CXCL12 (CXCL12-AF647) was obtained from Almac. ADM and all other peptides from the CGRP family, as well as PAMP and PAMP(12-20) were acquired from Bachem. PAMP-12 was purchased from Phoenix Pharmaceuticals. PAMP-12 variants and (5-FAM)-labeled PAMP were synthesized by JPT. These peptides contain a free amine at the N terminus and an amide group at the C terminus.

Cell culture

HEK293T cells were purchased from ATCC and grown in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 Units per ml and 100 µg per ml). HEK293T cells stably expressing ACKR3 (HEK-ACKR3) were generated by transfection with a pIRES vector encoding the human ACKR3 and maintained under puromycin (5 µg per ml) selective pressure.

Binding competition assay

The assay was performed as previously described[4, 28]. In brief, HEK-ACKR3 cells were distributed into 96-well plates (2×10^5 cells per well) and incubated with a mixture of CXCL12-AF647 (5 nM) and unlabeled peptides at indicated concentrations in FACS buffer (PBS, 1% BSA, 0.1% NaN_3) for 90 minutes on ice. After two washing steps, cells were incubated for 30 minutes at 4°C with Zombie Green viability dye (BioLegend). After two washing steps, cells were resuspended in FACS buffer and mean fluorescence intensity (MFI) was measured from 10,000 gated cells using a BD LSR Fortessa flow cytometer. Signal obtained for CXCL12-AF647 in the absence of unlabeled ligands was defined as 100% binding and signal for CXCL12-AF647 in the presence of 1 µM unlabeled CXCL12 was set to 0%.

Nanoluciferase complementation-based assay (NanoBiT)

Ligand-induced recruitment of β-arrestin, mini G_i or mini G_q proteins (engineered GTPase domain of G_α subunit[29, 30]) to the receptors was monitored using NanoBiT technology (Promega), as previously described[4, 31]. 5×10^6 HEK293T cells were seeded in 10-cm dishes and 24 hours later co-transfected with pNBe plasmids encoding the receptor C-terminally fused to SmBiT and β-arrestin, mini G_i or mini G_q N-terminally fused to LgBiT. After 24 hours, cells were detached and incubated for 25 minutes at 37

°C with Nano-Glo Live Cell substrate diluted 200-fold, distributed into white 96-well plates (1×10^5 cells per well) and treated with indicated concentrations of peptides. Luminescence was recorded during 20 minutes with a Mithras LB940 luminometer (Berthold Technologies). For concentration-response curves, the signal recorded with a saturating concentration of full agonist for each receptor was set as 100 %. For receptor screening experiments, results were expressed as fold vehicle and an agonist chemokine (100 nM) listed in the IUPHAR repository of chemokine receptor ligands was included as positive control for each receptor.

Nanoluciferase Bioluminescence Resonance Energy Transfer (NanoBRET)

Ligand-induced receptor-arrestin delivery to endosomes was monitored by NanoBRET. In brief, 5×10^6 HEK293T cells were seeded in 10-cm dishes and 24 hours later co-transfected with plasmids encoding ACKR3, β-arrestin-2 N-terminally tagged with Nanoluciferase and FYVE domain of endofin, interacting with phosphatidylinositol 3-phosphate (PI3P) in early endosomes [32, 33], N-terminally tagged with mNeonGreen. After 24 hours, cells were detached and distributed into black 96-well plates (1×10^5 cells per well) and treated with saturating concentrations of ligands (3 µM for peptides or 300 nM for chemokines). After 30-minute incubation at 37°C, coelenterazine H (10 µM) was added and donor emission (460 nm) and acceptor emission (535 nm) were immediately measured on a Mithras LB940 plate reader (Berthold Technologies).

For receptor dimerization experiments, HEK293T cells were seeded in a 12-well plate (5×10^5 cells per well). After 24 hours, cells were transfected with 5 ng donor-encoding pNLF vector (RAMP, CD8, ACKR3 or CXCR4 C-terminally tagged with Nanoluciferase) and increasing concentrations of acceptor-encoding pNeonGreen vector (ACKR3, CXCR4 or CLR C-terminally tagged with mNeonGreen). An empty pcDNA3.1 vector was added to the different transfection mixes in order to maintain a constant total amount of DNA. 24 hours post transfection, cells were detached and seeded in black 96-well plates (1×10^5 cells per well). The signal of mNeonGreen was first quantified (ex. 485 nm, em. 535 nm) and used to determine the acceptor/donor ratio. After coelenterazine H (10 µM) addition, donor emission (460 nm) and acceptor emission (535 nm) were immediately measured on a Mithras LB940 plate reader (Berthold Technologies). BRET ratios were plotted against the determined acceptor/donor ratio and the data fitted using a nonlinear regression equation for one site-specific binding.

Inducible Nanoluciferase reporter gene transcription assays

Activation of the MAPK/ERK signaling pathway was evaluated using a Serum Response Element (SRE) Nanoluciferase reporter assay. Activation of calcium-dependent signaling pathways was evaluated using a Nuclear Factor of Activated T-cell Response Element (NFAT-RE) Nanoluciferase reporter assay. In brief, 6×10^6 HEK293T cells were seeded in 10-cm dishes and 24 hours later co-transfected with a

pcDNA3.1 encoding either ACKR3 or MrgX2 and pNanoLuc/SRE or pNanoLuc/NFAT-RE vectors (Promega) containing the Nanoluciferase gene downstream of a SRE or NFAT-RE. 24 hours later, cells were detached and seeded in white 96-well plates (1×10^5 cells per well). 24 hours later, medium was replaced by phenol-free DMEM and after 2-hour incubation, chemokines, peptides or positive control (30 nM phorbol 12-myristate 13-acetate (PMA) + 10% FBS and additionally 1µM ionomycin for NFAT-RE) were added. After 6 hours (SRE) or 8 hours (NFAT-RE), Nano-Glo Live Cell substrate (Promega) was added and luminescence was read during 20 minutes on a Mithras LB940 plate reader (Berthold Technologies).

HTRF-based determination of ERK1/2 phosphorylation

HTRF-based phospho-ERK1/2 (extracellular signal regulated kinases 1 and 2) assay was performed using the phospho-ERK1/2 (Thr202/Tyr204) cellular kit (Cisbio International). In brief, 6×10^6 HEK293T cells were seeded in 10-cm dishes and transfected 24 hours later with pcDNA3.1 plasmid encoding ACKR3 or MrgX2. 24 hours post transfection, cells were detached and seeded in 96-well plates (1×10^5 cells per well). 24 hours later, cell culture medium was replaced by phenol-free DMEM and after 90-minute incubation, cells were stimulated with PAMP-12 (3µM), vehicle or epidermal growth factor (EGF, 100nM) as positive control for the indicated time intervals. Supernatants were replaced with the provided lysis buffer and 45 minutes later the lysates were transferred to a white 384-well plate. After a 2-hour incubation with pERK1/2-specific antibodies conjugated to Eu^{3+}-cryptate donor and d2 acceptor at recommended dilutions, HTRF signal was measured on a Tecan GENios pro plate reader equipped with 340 nm excitation filter and 612 ± 10 nm (donor) and 670 ± 25 nm (acceptor) emission filters.

Visualization of PAMP-12–FAM uptake by imaging flow cytometry

HEK293T or HEK-ACKR3 cells were harvested in Opti-MEM and distributed into 96-well plates (3×10^5 cells per well). After a 15-minute incubation at 37 °C with CXCL10, CXCL12 or Opti-MEM only, FAM-labeled PAMP-12 was added to a final concentration of 3 µM and incubated for 45 minutes at 37 °C, then washed twice with FACS buffer. For comparison of labeled PAMP-12 uptake by ACKR3 or MrgX2, 6×10^6 HEK293T cells were seeded in 10-cm dishes and transfected 24 hours later with 4 µg of pcDNA3.1 plasmid encoding ACKR3 or MrgX2. 24 hours post transfection, cells were harvested and treated as described above. Dead cells were excluded using Zombie NIR viability dye (BioLegend). Images of 1×10^4 in-focus, living single cells were acquired with an ImageStream Mark II imaging flow cytometer (Amnis) equipped with an extended depth of field (EDF) module and using 60X magnification. Samples were analyzed using Ideas6.2 software. The number of spots per cell was determined using a mask-based software wizard.

Data and statistical analysis

Concentration-response curves were fitted to the four-parameter Hill equation using an iterative, least-squares method (GraphPad Prism version 8.0.1). All curves were fitted to data points generated from the mean of at least three independent experiments. Statistical tests, *i.e.* t-test and ordinary one way ANOVA and post hoc analysis were performed with GraphPad Prism 8.0.1.. P-values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p< 0.0001.

3. Results and Discussion

3.1 ADM is the only CGRP family member showing activity towards ACKR3

In order to characterize the activity and pharmacology of ADM towards ACKR3, we first measured its ability to induce β-arrestin-2 recruitment to ACKR3 using a Nanoluciferase complementation-based assay (NanoBiT). We additionally included other structurally and functionally related peptides of the CGRP family, namely α-CGRP, intermedin (IMD), amylin (AMY) and calcitonin (CT) to investigate the selectivity of ACKR3. Among these peptides, only ADM showed moderate activity towards ACKR3, triggering at the highest concentration tested (9 µM) about 50% of the maximum response observed with the full agonist CXCL12 (Figure 1B). No activity was detected with any of the other members of the CGRP family. However, although ACKR3 can heterodimerize with all three RAMP isoforms (supplementary Figure 1), co-expression of ACKR3 with RAMPs did not improve its responsiveness to ADM, as already suggested in a recent study[16], or to any other CGRP family ligands (Figure 1C and supplementary Table1). In contrast, CLR activation was only observed upon co-expression of one of the three RAMP isoforms, as previously described[2, 20] (Figure 1D and supplementary Table1).

These results confirm that ADM is a weak agonist of ACKR3 and that its activity and pharmacology are not influenced by the presence of RAMPs[16]. However, the apparent 300-fold lower ADM activity towards ACKR3 compared to CLR/RAMP2 or CLR/RAMP3 may question the physiological relevance of ACKR3 as an ADM receptor and suggest that the regulatory role of ACKR3 in the ADM signaling axis is either indirect, occurs in a particular microenvironment or requires additional so far unknown accessory proteins[34].

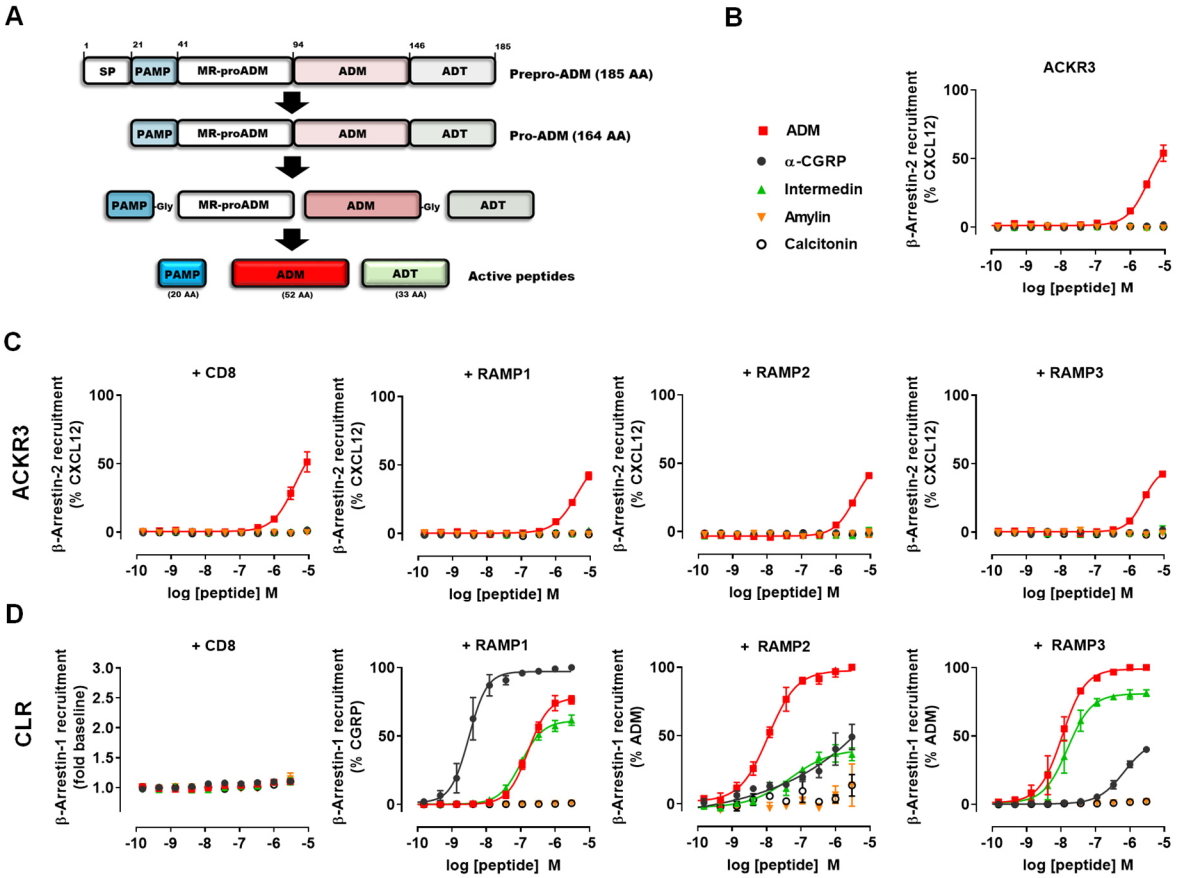


Figure 1. ADM is the only CGRP-family member with limited ACKR3 activity not influenced by RAMPs. (A) Schematic overview of preproADM processing into at least two active peptides (Proadrenomedullin N-terminal 20 peptide (PAMP), adrenomedullin (ADM)) and adrenotensin (ADT), whose bioactivity has to be confirmed. AA: Amino Acids, MR-proADM: Mid-regional proadrenomedullin, SP: Signal Peptide. **(B-D)** Efficacy and potency of different CGRP family members in inducing β-arrestin recruitment towards ACKR3 (B and C) or CLR (D) in HEK cells in the absence of regulatory proteins (B), or in the presence of one of the three RAMPs or CD8 used as negative control protein (C and D) using NanoBiT technology. Results are expressed as percentage of full agonist response and represent the mean ± S.E.M of three independent experiments (n = 3).

3.2 proADM-derived PAMP-12 has a stronger potency towards ACKR3 than mature ADM

Considering the strong biological link between *ACKR3* and *ADM*, we wondered whether ACKR3 might be activated by other peptides originating from the proADM precursor, namely proadrenomedullin N-terminal 20 peptide (PAMP) and adrenotensin (ADT), a sparsely characterized peptide suggested to exert angiogenic activity on its own[35] (Figure 1A). Surprisingly, while no activity of ADT could be detected, PAMP induced slightly lower β-arrestin-2 recruitment towards ACKR3 (EC_{50} > 10 µM) as the mature ADM (EC_{50} ≈ 5–10 µM) (Figure 2A and B). Examining further processed forms of PAMP, we found that PAMP-12, consisting of amino acids 9–20 (FRKKWNKWALSR-NH_2) (Figure 2A), showed a much greater potency (EC_{50} = 839 nM) towards ACKR3 compared to PAMP and ADM and acted as a full ACKR3 agonist for β-arrestin-2 recruitment (Figure 2B). PAMP-12 is often considered as the main active

form of PAMP, since it shows stronger *in vivo* effects and activates MrgX2 with higher potency (EC_{50} = 785 nM) than the full-length PAMP (EC_{50} = 6.2 µM) (Figure 2C and supplementary Table 1)[24, 25, 36]. PAMP(12-20), consisting of amino acids 12–20 (KWNKWALSR-NH_2) had a reduced potency (EC_{50} > 10 µM) towards ACKR3, comparable to full-length PAMP, indicating that the determinants for PAMP-12 activity lie within its N-terminal residues (FRK). These results were confirmed in a β-arrestin-1 recruitment assay (supplementary Figure 2) and in a binding competition assay, where all identified peptides displaced CXCL12-AF647 from ACKR3, with PAMP-12 being by far the most potent competitor (Figure 2D). Of note, co-expression with RAMPs did not modify the activity of PAMP-derived peptides towards ACKR3 and no activity of PAMP peptides was detected on CLR or CLR/RAMP complexes (Supplementary Figure 2).

Importantly, while PAMP and PAMP(12-20) had reduced potency towards ACKR3 compared to their classical receptor MrgX2, PAMP-12 had equivalent potencies towards both receptors (Figure 2B and C and Supplementary Table1). In agreement with the literature, PAMP-12 was also the most potent PAMP peptide towards MrgX2 in our β-arrestin-2 recruitment assay, although its apparent potency was lower than described previously, which may be due to the different receptor activation readouts used[24]. Noteworthy, ADM did not show any activity towards MrgX2 (Figure 2C) while substance P, another 11-amino acid (RPKPQQFFGLM-NH_2) ligand of MrgX2, had no activity towards ACKR3 (Figure 2B), demonstrating that not all ligands are interchangeable between ACKR3 and MrgX2.

3.3 ACKR3 is the only chemokine receptor activated by proADM-derived peptides

The promiscuity of chemokines for their receptors is remarkable as many chemokines bind to several receptors, while a single chemokine receptor can have multiple ligands. In order to evaluate the selectivity of the proADM-derived ligands for ACKR3, we screened ADM and PAMP-derived peptides (PAMP, PAMP-12 and PAMP(12-20)) in a β-arrestin-2 recruitment assay towards all known classical and atypical human chemokine receptors. Our results show that ADM and PAMP-derived peptides are selective for ACKR3 and do not activate any of the other 24 chemokine receptors tested (Figure 2E), while MrgX2 is only activated by PAMP and its variants but not by mature ADM. Of note, no activity towards GPR182, the GPCR phylogenetically closest to ACKR3, and a debated adrenomedullin receptor[37, 38] could be detected upon ligand treatment in this assay. These results indicate that ACKR3 is the only receptor with dual ADM-PAMP activation capacity.

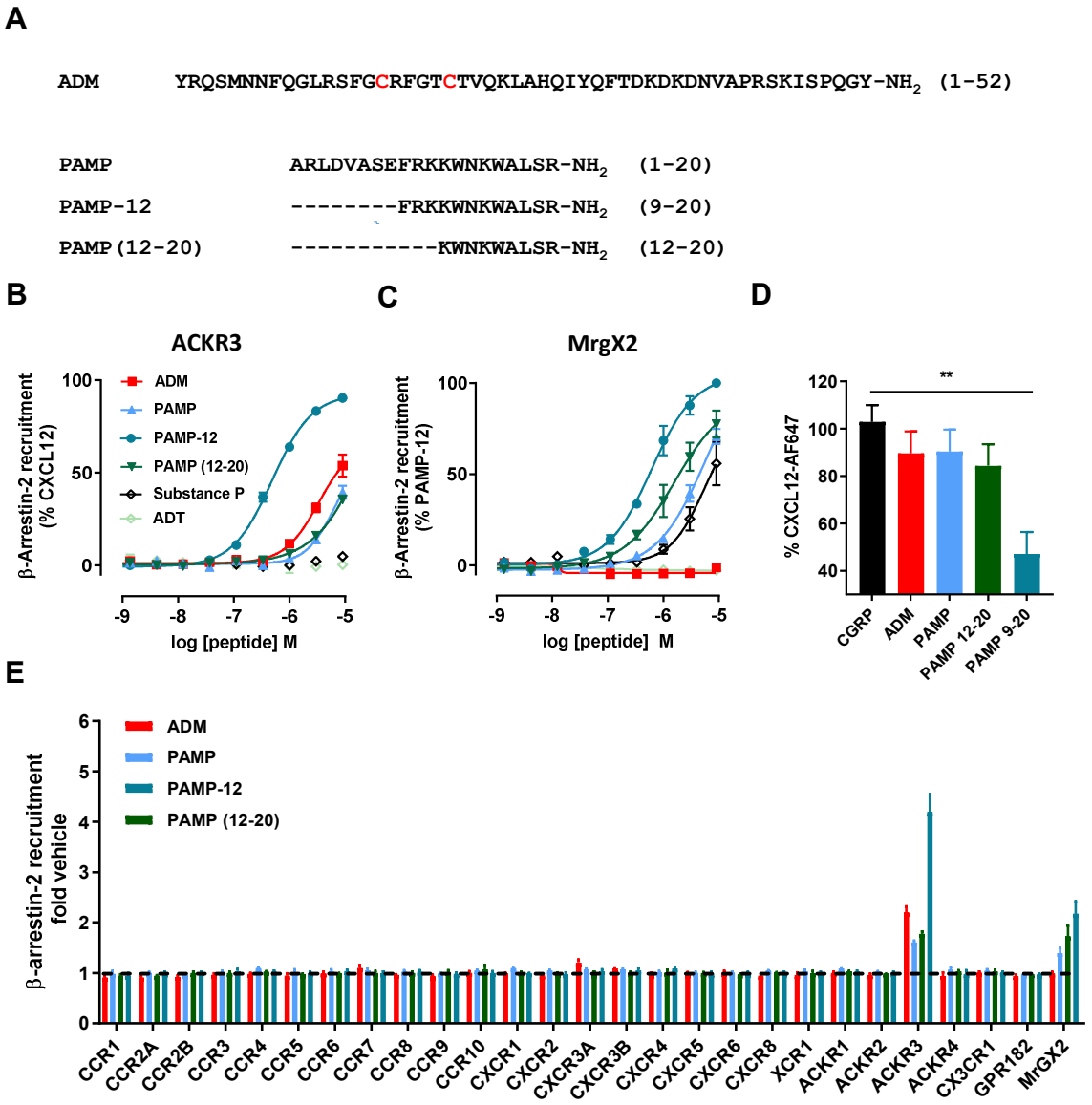


Figure 2. PAMP peptides have comparable activity towards ACKR3 and MrgX2 and no activity towards any other chemokine receptor. (A) Sequences of ADM and the three PAMP variants tested: full-length PAMP, comprising amino acids 1–20; PAMP-12, consisting of 12 amino acids from 9–20; and PAMP(12-20), comprising the last 9 amino acids of PAMP. For ADM, cysteine residues involved in a disulfide bridge forming a 4-residue intrapeptide arch are depicted in red. **(B-C)** Comparison of potency and efficacy of different active PAMP variants, ADM, ADT and substance P in inducing β-arrestin-2 towards ACKR3 (B) or MrgX2 (C) in HEK cells, normalized to % activity of their respective full agonists. **(D)** Binding competition of CGRP, ADM and PAMP variants (9 µM) with Alexa Fluor 647-labeled CXCL12 (5 nM) on HEK-ACKR3 cells determined by flow cytometry. **(E)** Agonist activity of ADM and different PAMP variants (3 µM) towards all chemokine receptors, as well as MrgX2 and GPR182 monitored in a β-arrestin-2 recruitment assay. Results are expressed as fold change over vehicle. For each receptor, an agonist chemokine (100 nM) listed in the IUPHAR repository of chemokine receptor ligands was used as positive control. Results from B-E are represented as mean ± S.E.M of three independent experiments. ** p<0.01 by one-way ANOVA with Bonferroni multiple comparison test.

3.4 PAMP-12 SAR analysis pinpoints different key residues for activation of ACKR3 compared to MrgX2

In order to gain a deeper insight into the activation mechanism of ACKR3 by this new class of ligands, we performed a comparative structure-activity relationship (SAR) analysis using as basis the most active peptide, PAMP-12, that shows comparable potencies on the two receptors (Figure 3A and B). In addition to a complete alanine scan, we compared the impact of different single amino acid substitutions and N-terminal extensions of PAMP-12, as well as several truncations of PAMP on the activation of ACKR3 and MrgX2, using β-arrestin-2 recruitment as readout (Figure 3A and supplementary Table2).

This analysis revealed that although a similar trend in potency shift towards the two receptors was observed for modifications at multiple positions, including W5, W8, L10 or R12, important differences could be highlighted. For instance, a phenylalanine at the first position of the peptide is required for a strong activity towards ACKR3 (Figure 3A and C). This is in stark contrast to MrgX2 (Figure 3D), but in full agreement with what we recently found in an adrenorphin SAR study, where the opposite Y1F mutation led to a 10-fold increase in potency of the peptide towards ACKR3[12]. Similarly, the SAR revealed that R2 is crucial for PAMP-12 activity towards ACKR3. Together, these observations are in line with the previously measured differences in potency between PAMP-12 and PAMP(12-20) and further confirm that the determinants for PAMP-12 activity towards ACKR3 mainly lie within its N-terminal residues. Of all modifications, only lysine substitutions K3R, K4A and K7R improved the potency towards ACKR3, while being neutral for MrgX2. This also aligns with the previously reported adrenorphin SAR study, where the opposite R7K mutation was detrimental for ACKR3 activation[12]. Of note, many mutations had only a minor impact on ACKR3 and MrgX2, pointing towards a high propensity for activation of both receptors towards PAMP-12. However, other truncated PAMP variants including PAMP(1-17), PAMP(16-20), PAMP(13-20) and PAMP(4-20) showed no activity towards ACKR3, highlighting some degree of selectivity of ACKR3 towards this class of ligands. Overall, this analysis shows that MrgX2 and ACKR3, while both showing ligand promiscuity, have somewhat different binding pockets for PAMP peptides.

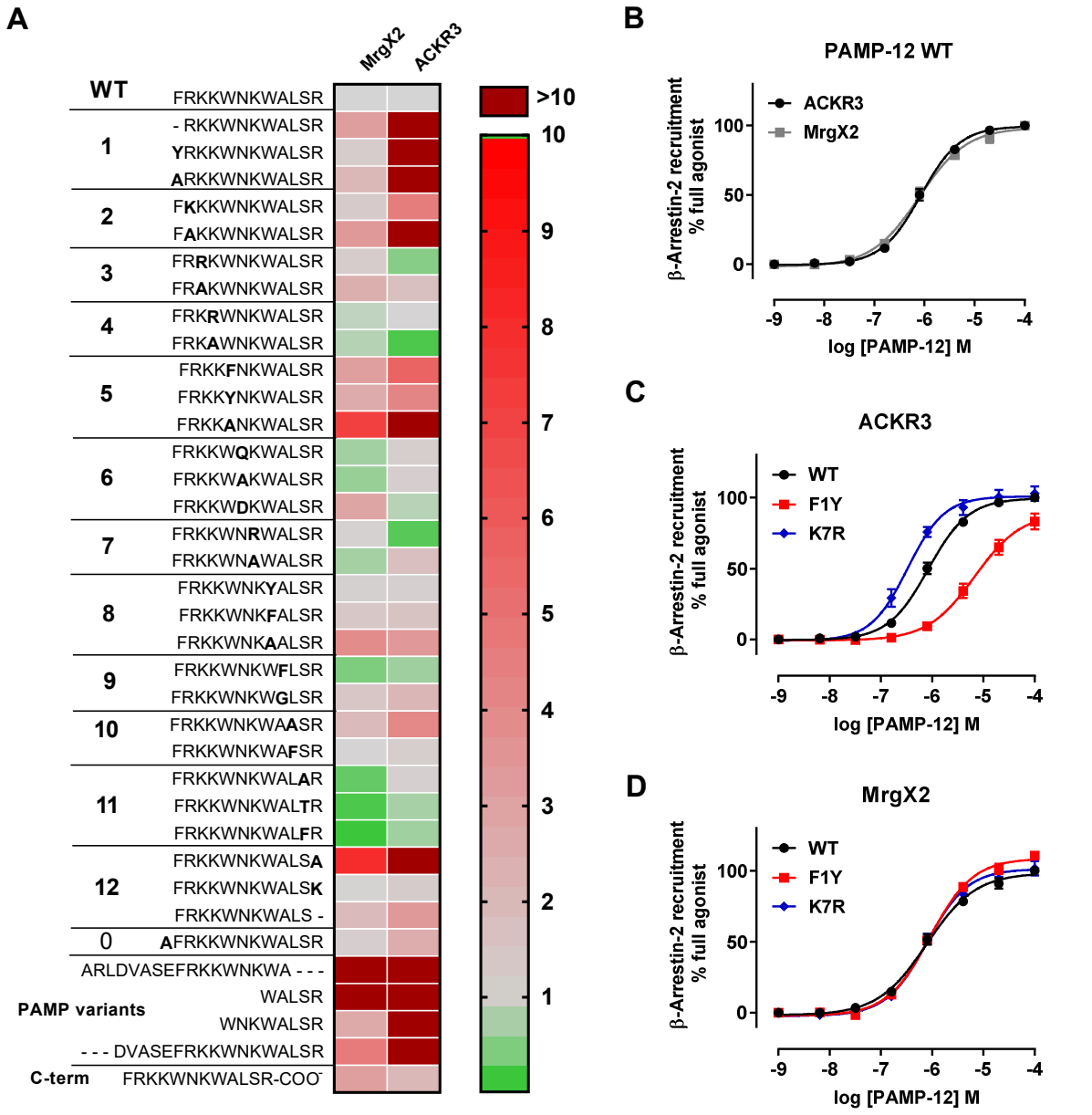


Figure 3. SAR analysis of PAMP-12 variants on ACKR3 and MrgX2. (A) Comparison of the impact of substitutions or truncations on the agonist activity of PAMP-12 towards ACKR3 and MrgX2. The agonist activity of each variant was evaluated in a β-arrestin-2 recruitment assay in HEK cells and expressed in a heat map as fold change in EC_{50} values with respect to wild-type PAMP-12. Four variants of PAMP, PAMP(1-17), PAMP(16-20), PAMP(13-20) and PAMP(4-20) were also included. (**B-D**) Comparison of potency and efficacy of PAMP-12 (**B**) and its variants bearing mutations F1Y or K7R in inducing β-arrestin-2 recruitment to ACKR3 (**C**) and MrgX2 (**D**)in HEK cells. Results represent the mean (A) or mean ± S.E.M (B-D) of three independent experiments (n = 3). Corresponding pEC_{50} values are available in supplementary Table 2.

3.5 ACKR3 mediates PAMP-12 uptake without inducing signaling events

The ability of ACKR3 to signal upon ligand binding is still highly debated and may be cell type-dependent. While some studies reported signaling capacity for ACKR3, especially β-arrestin-dependent ERK phosphorylation[39, 40], others suggested that ACKR3 acts as a non-signaling scavenger

receptor[12, 41]. In order to assess the ability of ACKR3 to signal in response to proADM-derived peptides, we first explored the possibility that ACKR3 could couple to G proteins in response to ADM or PAMP variants by monitoring the mini G_i and mini G_q recruitment to the receptor in a Nanoluciferase complementation-based assay. Contrarily to MrgX2, for which all PAMP variants increased mini G_i and mini G_q interactions with the receptor in a concentration-dependent manner, we did not detect ligand-induced interactions between ACKR3 and mini G_i or mini G_q (Figure 4A and B). Furthermore, we did not observe any increase in ERK phosphorylation upon PAMP-12 treatment, or any activation of the MAPK/ERK-dependent Serum Response Element (SRE) and of the calcium-dependent Nuclear Factor of Activated T-cell Response Element (NFAT-RE) upon ADM or PAMP stimulation in ACKR3-transfected cells, in contrast to MrgX2-transfected cells (Figure 4C and D).

Our data suggest that ADM, PAMP, PAMP(12-20) and especially PAMP-12 can trigger β-arrestin recruitment to ACKR3, without inducing classical downstream signaling. In line with these data, recent studies proposed ACKR3 as a scavenging receptor for ADM, reducing ADM levels to regulate its activity[3, 16]. We therefore wondered whether ACKR3 might play a similar role for PAMP peptides. To this end, using NanoBRET, we first investigated whether the ACKR3/β-arrestin complex is internalized and delivered to early endosomes upon receptor activation by PAMP peptides[33]. We observed a robust BRET signal upon treatment of ACKR3-expressing cells with CXCL12, ADM and PAMP peptides, but not with negative controls CXCL10 or Substance P, indicative of a specific delivery of the ligands to endosomes upon binding to ACKR3 (Figure 4E). Using imaging flow cytometry, we could also demonstrate that ACKR3 is able to internalize fluorescently labeled PAMP-12. We observed a clear intracellular accumulation of fluorescently labeled PAMP-12 after 45-minute stimulation of HEK-ACKR3 cells, with a notably higher number of distinguishable vesicle-like structures and a higher mean fluorescent intensity compared to naïve HEK cells (Figure 4F, G and H). Preincubation of HEK-ACKR3 cells with CXCL12, but not with the control chemokine CXCL10, reduced PAMP-12 accumulation to background levels, suggesting a specific ACKR3-driven uptake (Figure 4F, G and H). Moreover, despite a similar potency of PAMP-12 towards MrgX2 and ACKR3, MrgX2-positive cells showed significantly less peptide uptake than ACKR3-positive cells, underscoring the scavenging capacity of ACKR3 (Figure 4I).

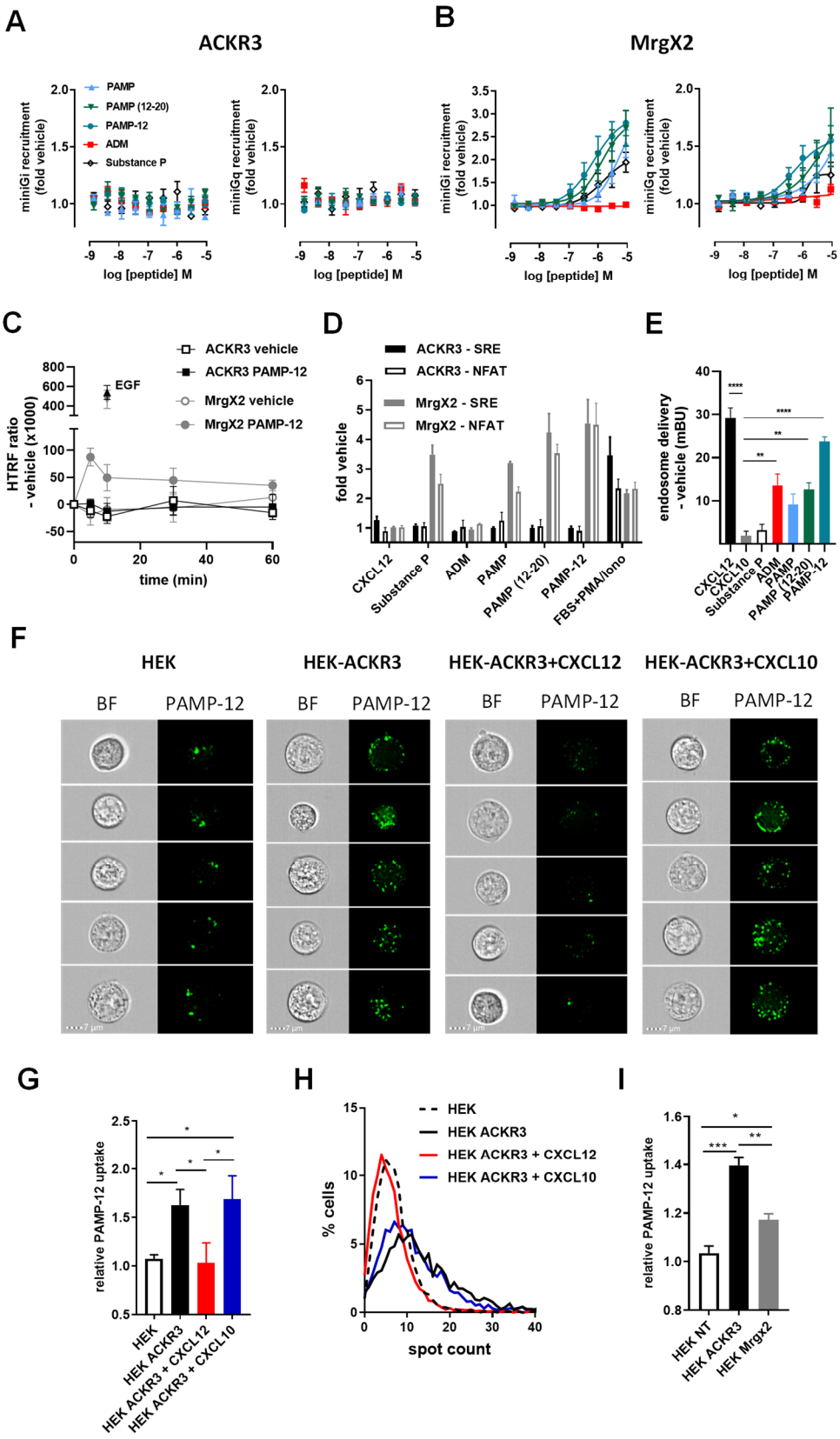


Figure 4. ACKR3 internalizes PAMP-12 without inducing G protein or ERK-signaling. (A-B) Comparison of mini G_i and mini G_q recruitment to ACKR3 (A) and MrgX2 (B) in response to ADM, substance P and PAMP variants monitored in HEK cells using NanoBiT technology. (**C**) Kinetic analysis of ERK1/2 phosphorylation in HEK cells transfected with ACKR3 or MrgX2 encoding plasmids treated with vehicle or PAMP-12 (3 µM). EGF (100 nM) was

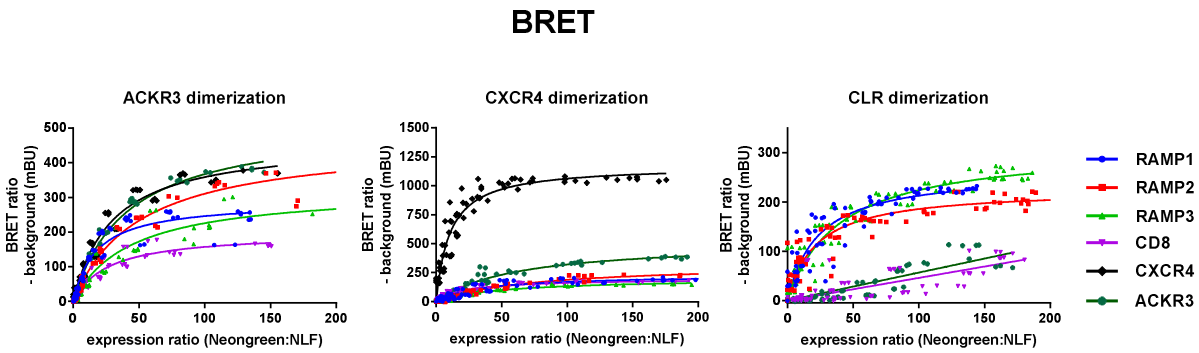
used as positive control. (**D**) Activation of ERK (SRE) and Ca^{2+} (NFAT) signaling cascades in HEK cells expressing ACKR3 or MrgX2 in response to CXCL12 (300 nM), ADM, substance P or PAMP variants (3 µM) or positive control (30 nM PMA, 10% FBS for SRE and 30 nM PMA, 1µM ionomycin, 10% FBS for NFAT). (**E**) ACKR3 delivery to endosomes induced by peptides (3 µM) and chemokines (300 nM) monitored in HEK cells by NanoBRET using Nanoluciferase-tagged β-arrestin-2 and mNeonGreen-tagged FYVE domain of endofin, which binds phosphatidylinositol 3-phosphate (PI3P) in early endosomes. Results are expressed in miliBRET units (mBU). (**F-I**) Uptake of fluorescently labeled PAMP-12 (PAMP-12-FAM, 3 µM). (**F**) Uptake of PAMP-12-FAM by ACKR3-positive or -negative HEK cells pretreated (or not) with CXCL12 or CXCL10 (200 nM) visualized by imaging flow cytometry. Five representative HEK or HEK-ACKR3 cells are shown. Scale bar: 7 µm. (**G**) PAMP-12-FAM uptake for conditions described in (F), quantified by mean fluorescence intensity (MFI) and normalized to signal obtained for non-transfected HEK cells. (**H**) Percentage of cells with a given number of distinguishable vesicle-like structures (spots) for conditions determined in (F). (**I**) PAMP-12-FAM uptake by HEK cells, transiently transfected with equal amounts of ACKR3 or MrgX2 encoding plasmids or an empty vector (NT) quantified by MFI. For all panels, results represent the mean ± S.E.M. of at least three independent experiments ($n \geq 3$) except for F and H, where one representative experiment of three independent repetitions is shown. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by one-way ANOVA with Bonferroni (E) or Tuckey's multiple comparison test (G and I).

In conclusion, our data suggest that, similarly to chemokines and endogenous opioid peptides, ACKR3 is able to reduce PAMP-12 availability by efficiently internalizing the peptide without inducing further signaling events.

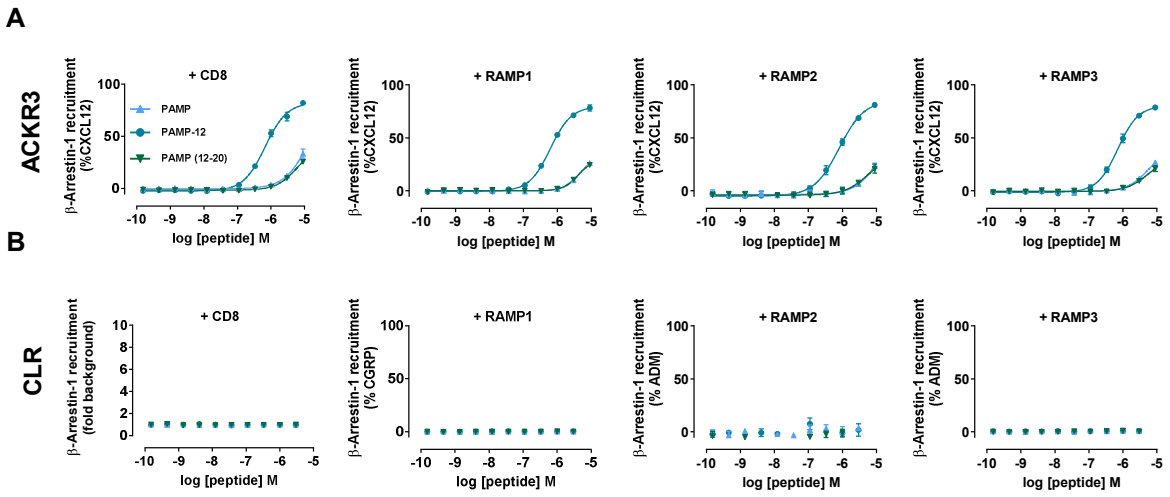
Although PAMP peptides are involved in a variety of physiological processes like vasodilation, angiogenesis, cell migration, apoptosis, or degranulation of mast cells, not much is known about their pharmacological activity or their physiological regulation. Here, we describe a mechanism of PAMP-12 regulation via scavenging by ACKR3, which may restrain peptide availability for its signaling receptor MrgX2. Interestingly, although MrgX2 and ACKR3 are not closely related phylogenetically or functionally, both receptors were recently described to be activated by a variety of small endogenous opioid peptides such as dynorphins, making PAMPs their second shared family of ligands[12, 42].

Regardless of the regulatory role of ACKR3 towards ADM[3], its scavenging capacity for PAMP-12 may be superior and partly explain why ADM and PAMP, despite their common precursor, show different and non-equimolar tissue distribution in regions where ACKR3 is highly expressed[6, 43, 44]. This spatiotemporal regulation of PAMP-12 may follow a similar mechanism as that described for CXCL12 and recently for opioid peptides and should be the focus of future *in vivo* investigations[45]. Of note, even though *Ackr3* knockout in mice shows a similar phenotype as *Adm* overexpression, a recent study revealed that $ADM^{\Delta PAMP/\Delta PAMP}$ mice, which carry the ADM but lack the PAMP-coding sequence, had no obvious anomalies, pointing towards a more complex (possibly dual ADM/PAMP-12) or non-homeostatic scavenging role of ACKR3 in the regulation of these peptides[46]. Altogether, our study expands the list of endogenous ACKR3 ligands and sheds light on the complex regulation of the availability of proADM-derived peptides.

4. Supplementary Data



Supplementary Figure 1. ACKR3 interacts specifically with all three RAMP isoforms. BRET values measured in HEK293T cells co-transfected with a constant amount of pNLF vector encoding Nanoluciferase-tagged donor (RAMPs, CD8, CXCR4 or ACKR3) and increasing amounts of pNeonGreen vector encoding mNeonGreen-tagged acceptor (ACKR3, CXCR4 or CLR). Results represent individual measurements performed in triplicates of three independent experiments and curves were fitted with the one-site specific binding equation using GraphPad Prism.



Supplementary Figure 2. RAMPs do not influence the responsiveness of ACKR3 and CLR to PAMP variants (A-B) Efficacy and potency of PAMP variants in inducing β-arrestin-1 recruitment to ACKR3 (A) and CLR (B) in HEK cells co-transfected with pcDNA3.1 encoding one of the three RAMPs or CD8 as negative control protein using NanoBiT technology. Results represent the mean ± S.E.M or three independent experiments (n = 3).

Supplementary Table 1. Sequences of proADM-derived peptides and substance P and their potency in β-arrestin recruitment towards ACKR3, MrgX2 and CLR/RAMP

Peptide	Sequence	Receptor				
		ACKR3	MrgX2	CLR/RAMP1	CLR/RAMP2	CLR/RAMP3
		pEC_{50} ±S.E.M	pEC_{50} ±S.E.M	pEC_{50} ±S.E.M	pEC_{50} ±S.E.M	pEC_{50} ±S.E.M
ADM	YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY-NH_2	approx. 5.0 - 5.3	NA	6.78 ± 0.04	7.95 ± 0.06	7.97 ± 0.06
PAMP(1-20)	ARLDVASEFRKKWNKWALSR-NH_2	< 5.0	5.21± 0.26	NA	NA	NA
PAMP(12-20)	KWNKWALSR-NH_2	< 5.0	5.80 ± 0.20	NA	NA	NA
PAMP-12	FRKKWNKWALSR-NH_2	6.08 ± 0.03	6.11± 0.05	NA	NA	NA
substance P	RPKPQQFFGLM-NH_2	NA	approx. 5.0 - 5.3	NA	NA	NA
AMY	KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY-NH_2	NA	NA	NA	NA	NA
IMD	TQAQLLRVGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH_2	NA	NA	6.99 ± 0.04	7.32 ± 0.21	7.81± 0.08
CT	CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP-NH_2	NA	NA	NA	NA	NA
α-CGRP	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-NH_2	NA	NA	8.51± 0.06	ND	6.17 ± 0.09

NA: no activity detected in the concentration range tested
ND: not determinable

Supplementary Table 2. Sequences of PAMP-12-derived peptides and activity in β-arrestin-2 recruitment to ACKR3 and MrgX2

Sequence	Position	Receptor	
		ACKR3	MrgX2
		pEC_{50} ±S.E.M	pEC_{50} ±S.E.M
FRKKWNKWALSR	Ref	6.08 ± 0.03	6.11 ± 0.05
- RKKWNKWALSR	1	4.54 ± 0.06	5.68 ± 0.10
YRKKWNKWALSR	1	5.17 ± 0.10	6.04 ± 0.04
ARKKWNKWALSR	1	4.33 ± 0.23	6.03 ± 0.09
F**K**KKWNKWALSR	2	5.48 ± 0.08	5.84 ± 0.11
F**A**KKWNKWALSR	2	5.03 ± 0.08	6.00 ± 0.14
FR**R**KWNKWALSR	3	6.33 ± 0.06	5.56 ± 0.12
FR**A**KWNKWALSR	3	5.87 ± 0.06	6.01 ± 0.11
FRK**R**WNKWALSR	4	6.12 ± 0.06	5.72 ± 0.09
FRK**A**WNKWALSR	4	6.57 ± 0.06	6.18 ± 0.10
FRKK**F**NKWALSR	5	5.37 ± 0.06	6.23 ± 0.10
FRKK**Y**NKWALSR	5	5.50 ± 0.04	5.61 ± 0.09
FRKK**A**NKWALSR	5	5.12 ± 0.06	5.70 ± 0.09
FRKKW**Q**KWALSR	6	6.11 ± 0.06	5.33 ± 0.05
FRKKW**A**KWALSR	6	6.08 ± 0.06	6.32 ± 0.06
FRKKW**D**KWALSR	6	6.23 ± 0.06	6.32 ± 0.06
FRKKWN**R**WALSR	7	6.48 ± 0.06	5.71 ± 0.06
FRKKWN**A**WALSR	7	5.90 ± 0.05	6.26 ± 0.07
FRKKWNK**Y**ALSR	8	6.11 ± 0.07	6.13 ± 0.08
FRKKWNK**F**ALSR	8	5.94 ± 0.06	5.98 ± 0.06
FRKKWNK**A**ALSR	8	5.65 ± 0.07	5.60 ± 0.06
FRKKWNKW**F**LSR	9	6.29 ± 0.04	6.38 ± 0.06
FRKKWNKW**G**LSR	9	5.80 ± 0.05	5.96 ± 0.06
FRKKWNKWA**A**SR	10	5.54 ± 0.06	5.83 ± 0.05
FRKKWNKWA**F**SR	10	6.02 ± 0.07	6.13 ± 0.04
FRKKWNKWAL**A**R	11	6.04 ± 0.09	6.47 ± 0.05
FRKKWNKWAL**T**R	11	6.24 ± 0.08	6.57 ± 0.04
FRKKWNKWAL**F**R	11	6.24 ± 0.08	6.68 ± 0.07
FRKKWNKWALS**A**	12	4.95 ± 0.08	5.24 ± 0.04
FRKKWNKWALS**K**	12	5.99 ± 0.07	6.10 ± 0.06
FRKKWNKWALS -	12	5.59 ± 0.08	5.81 ± 0.05
AFRKKWNKWALSR	0	5.70 ± 0.05	6.05 ± 0.05
FRKKWNKWALSR-COO^-	C-term	5.80 ± 0.04	5.64 ± 0.04
ARLDVASEFRKKWNKWA COO^-- - -	PAMP 1-17	3.48 ± 1.20	4.02 ± 0.40
WALSR	PAMP 16-20	4.96 ± 0.06	NA
WNKWALSR	PAMP 13-20	4.93 ± 0.06	5.69 0.04
- - - DVASEFRKKWNKWALSR	PAMP 4-20	3.75 ± 1.17	5.48 ± 0.04

Introduced mutations are in bold

Peptides used in Figure 3B-D are shown in red

NA: no activity detected in the concentration range tested

5. Highlights

- ADM is the only member of the CGRP peptide family to display agonist activity towards ACKR3.
- PAMP, released during ADM maturation, and especially PAMP-12 show activity towards ACKR3. While PAMP and PAMP(12-20) show a reduced potency (EC_{50} > 10µM) compared to ADM (EC_{50} approximatively 5 µM), PAMP-12 activates ACKR3 in the high nanomolar range(EC_{50} = 839 nM), a similar potency as towards its so far only described receptor MrgX2.
- ACKR3 is the only receptor that is activated by both ADM and PAMP peptides.
- PAMP-12 structure activity relationship (SAR) assays revealed that MrgX2 shares many peptide interaction determinants with ACKR3, while some peptide residues seem to be exclusively important for one of the receptors.
- Similarly to what we observed for chemokines and opioid peptides, ACKR3 mediates efficient internalization of PAMP-12, without triggering interactions with G-proteins or inducing phosphorylation of ERK.

Together, these data show that similar to chemokines and opioid peptides, ACKR3 efficiently internalizes PAMP-12 without inducing downstream signaling generally observed with classical chemokine receptors or MrgX2, suggesting that it may act as a scavenger regulating PAMP-12 availability for its endogenous signaling receptor(s). Our study further expanded the panel of endogenous ACKR3 ligands to yet another family and suggest, that some conclusions on ADM-ACKR3 crosstalk based on *Adm* gene modulation might rather be attributed to PAMP-ACKR3 interactions or be linked to a simultaneous modification of both ADM and PAMP.

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Chapter 5: Beyond ACKR3:

Extending the network of interaction partners for other ACKRs

Part 1: CCL20 and CCL22 as new ligands of ACKR4

Adapted from: **Meyrath, M**.*, Reynders, N.*, Uchanski, T., Chevigne, A., and Szpakowska, M. (2020) *"Systematic reassessment of chemokine-receptor pairings confirms CCL20 but not CXCL13 and extends the spectrum of ACKR4 agonists to CCL22"*, **J Leukoc Biol.** 109, 373-376 * equal contribution.

After having contributed in extending the variety of ACKR3 ligands beyond its narrow spectrum of two endogenous chemokines, we wondered whether other chemokine receptors, especially other ACKRs might also have other endogenous ligands that might have been overlooked in the early days, where chemokines were not necessarily all available and screening assays less sensitive and sometimes relying on phenotypical observations rather than on quantitative, biochemical readouts[1]. We therefore decided to perform a systemic reassessment of chemokine pairings with ACKR4, a scavenging receptor for the homeostatic chemokines CCL19, CCL21, CCL25 and CXCL13. Screening of all human chemokines on ACKR4 was performed using a highly sensitive β-arrestin recruitment assay based on Nanoluciferase complementation. When communicating our data to collaborators, we realized that our results were in perfect agreement with data from a recently accepted manuscript by Matti *et al*. who could show that CCL20 triggers β-arrestin 1 and 2 recruitment towards ACKR4 and that the receptor is capable of internalizing CCL20[2]. After having contacted the authors and the editor of the journal, we were offered the chance to publish our data hand in hand with Matti *et al.* in the same issue of the journal.

Abstract

Atypical chemokine receptors (ACKRs) have emerged as important regulators or scavengers of homeostatic and inflammatory chemokines. Among these atypical receptors, ACKR4 is reported to bind the homeostatic chemokines CCL19, CCL21, CCL25 and CXCL13. In a recent study by Matti *et al*., the authors show that ACKR4 is also a receptor for CCL20, previously established to bind to CCR6 only. They provide convincing evidence that, just as for its other chemokine ligands, ACKR4 rapidly internalizes CCL20 both *in vitro* and *in vivo*. Independently of this discovery, we undertook a screening program aiming at reassessing the activity of the 43 human chemokines towards ACKR4 using a highly sensitive β-arrestin recruitment assay. This systematic analysis confirmed CCL20 as a new agonist ligand for ACKR4 in addition to CCL19, CCL21 and CCL25. Furthermore, the chemokine CCL22, which plays an important role in both homeostasis and inflammatory responses, and is known as a ligand for CCR4 and ACKR2 only, was found to also act as a potent partial agonist of ACKR4. In contrast, agonist activity of CXCL13 towards ACKR4 was disproved. This independent wide-range systematic study cross-confirms the pairing of CCL20 with ACKR4 newly discovered by Matti and co-authors, and further refines the spectrum of chemokines activating ACKR4.

1. Results and Discussion

Atypical chemokine receptors (ACKRs) form a subfamily of four chemokine receptors unable to trigger G protein-dependent signalling or to directly induce cell migration in response to chemokines [3]. ACKRs play however a crucial role in chemokine biology by capturing, scavenging or transporting chemokines, thereby regulating their availability and signalling through classical chemokine receptors [4, 5]. Among ACKRs, ACKR4, formerly known as CCX-CKR, CCR11 or CCRL1, which is expressed on keratinocytes, astrocytes, lymphatic endothelial cells and on thymic epithelial cells, is an important regulator of homeostatic chemokines [6-8].

Human ACKR4 was deorphanized in 2000 based on competition studies with radiolabeled CCL19 [9]. It was initially proposed to bind CCL19, CCL21, CCL25 and CXCL13, which are the ligands for CCR7, CCR9 and CXCR5, respectively [9, 10]. Of note, CXCL13 interaction with the mouse ACKR4 could not be confirmed [7] and the human CXCL13-ACKR4 pairing inferred from binding competition studies [9] was later re-evaluated and the observations reattributed to co-operative GAG binding rather than direct receptor interactions [11].

By scavenging its chemokine ligands, ACKR4 was shown to regulate mainly the trafficking and positioning of T-cells and dendritic cells (DCs) [8, 12]. ACKR4 is best known for its role in shaping the gradient of CCL19 and CCL21 for CCR7-expressing DCs in the subcapsular sinuses of lymph nodes during

the initiation phase of the adaptive immune response [13, 14]. ACKR4 is also involved in anti-tumor immunity and modulates epithelial-mesenchymal transition and metastasis [15-17], and studies with ACKR4-deficient mice in an experimental autoimmune encephalomyelitis model also demonstrated the receptor implication in autoimmune diseases notably by developing faster and a more severe disease onset attributable to increased Th17 response [6, 18]. Of note, two ACKR4-deficient mouse strains (ACKR4$^{-/-}$ and ACKR4$^{GFP/GFP}$) are available and display distinct phenotypes. While ACKR4$^{-/-}$ mice show a strong accumulation of plasma blasts in mesenteric lymph node and spleen as well as increased B cell proliferation after *in vitro* activation [19], B cells from ACKR4$^{GFP/GFP}$ mice exhibit a phenotype comparable to wild-type cells [20], suggesting that the results from the above-mentioned ACKR4-deficient mouse studies should be interpreted cautiously.

In a recent study, Matti and co-authors reported CCL20, previously established to bind to CCR6 only, as a novel chemokine ligand for ACKR4[2]. The authors had predicted the existence of this interaction based on CCL20 sequence and expression similarities with CCL19 and CCL21. They showed that CCL20 induces β-arrestin-1 and β-arrestin-2 recruitment to ACKR4, and is rapidly internalized and scavenged by ACKR4-expressing cells, both *in vitro* and *in vivo*. They proposed that by scavenging CCL20, ACKR4 regulates its availability for the classical signaling receptor CCR6 and thereby plays a role in the positioning of CCR6-positive leukocytes within secondary lymphoid tissues to initiate effective humoral and memory immune responses[2].

Independently of this discovery, we undertook a systematic screening program aiming at reassessing the agonist activity of the 43 human chemokines (24 CCLs, 16 CXCLs, 2 XCLs and 1 CX3CL) and 2 viral chemokines (vCCL1 and vCCL2) towards ACKR4, by monitoring β-arrestin recruitment to the receptor using a highly sensitive Nanoluciferase complementation-based assay (NanoBiT) (Figure 1A) [21-23]. In agreement with the initial description by Matti and co-authors, our systematic analysis also identified CCL20 as an ACKR4 ligand capable of inducing β-arrestin-1 and β-arrestin-2 recruitment to the receptor and behaving as a partial agonist (80 % efficacy) with a potency (EC_{50} = 42 nM) somewhat lower than potencies observed for CCL19 (EC_{50} = 3 nM), CCL21 (EC_{50} = 5 nM) and CCL25 (EC_{50} = 24 nM) (Figure 1B).

Besides CCL19, CCL20, CCL21 and CCL25, our systematic reassessment of chemokine-receptor interactions revealed that the chemokine CCL22, known to bind to CCR4 and ACKR2, is also a ligand for ACKR4. CCL22 was slightly less potent (EC_{50} = 128 nM) than the other ligands in inducing β-arrestin-1 recruitment to ACKR4 and acted as a partial agonist, displaying about 60 % of the maximum efficacy observed with CCL19. Interestingly, the CCL22 variant lacking the first two N-terminal residues ($CCL22_{3-69}$) and hence mimicking the dipeptidyl peptidase 4 (DPP4 or CD26)-cleaved chemokine, retained significant activity towards ACKR4, which contrasts with its absence of activity towards ACKR2 [24] but is reminiscent of the agonist effect of processed CXC chemokines towards ACKR3 [21, 25] (Figure 1B).

CCL22 was not the only CCR4-related chemokine acting on ACKR4. CCL17, the second ligand of CCR4, showed detectable activity towards ACKR4 but statistical significance was not reached for this pairing. A similar low activity was also observed for several other human CC chemokines including CCL7, CCL13, CCL14, CCL26 and CCL27, which are ligands for CCR1, CCR2, CCR3 and CCR10. Moreover, the viral broad-spectrum antagonist chemokine vCCL2/vMIP-II encoded by the Kaposi sarcoma-associated virus HHV-8 [26], known to bind to ACKR4 [9], and to activate ACKR3 [27], also behaved as a partial agonist of ACKR4 (EC_{50} = 93 nM) inducing 50 % of the maximum efficacy observed with CCL19. In contrast, CXCL13, for which discordant observations have been reported regarding its interaction with ACKR4 [9, 11], did not induce β-arrestin recruitment to ACKR4 in our assay, narrowing down ACKR4 specificity to CC chemokines only. In a similar screening conducted with all 45 chemokines (100 nM) in antagonist mode, *i.e.* in the presence of CCL19 at a concentration equivalent to its EC_{50} (3 nM), no chemokine other than the above-mentioned agonists was able to modulate CCL19-induced β-arrestin recruitment to ACKR4 (data not shown).

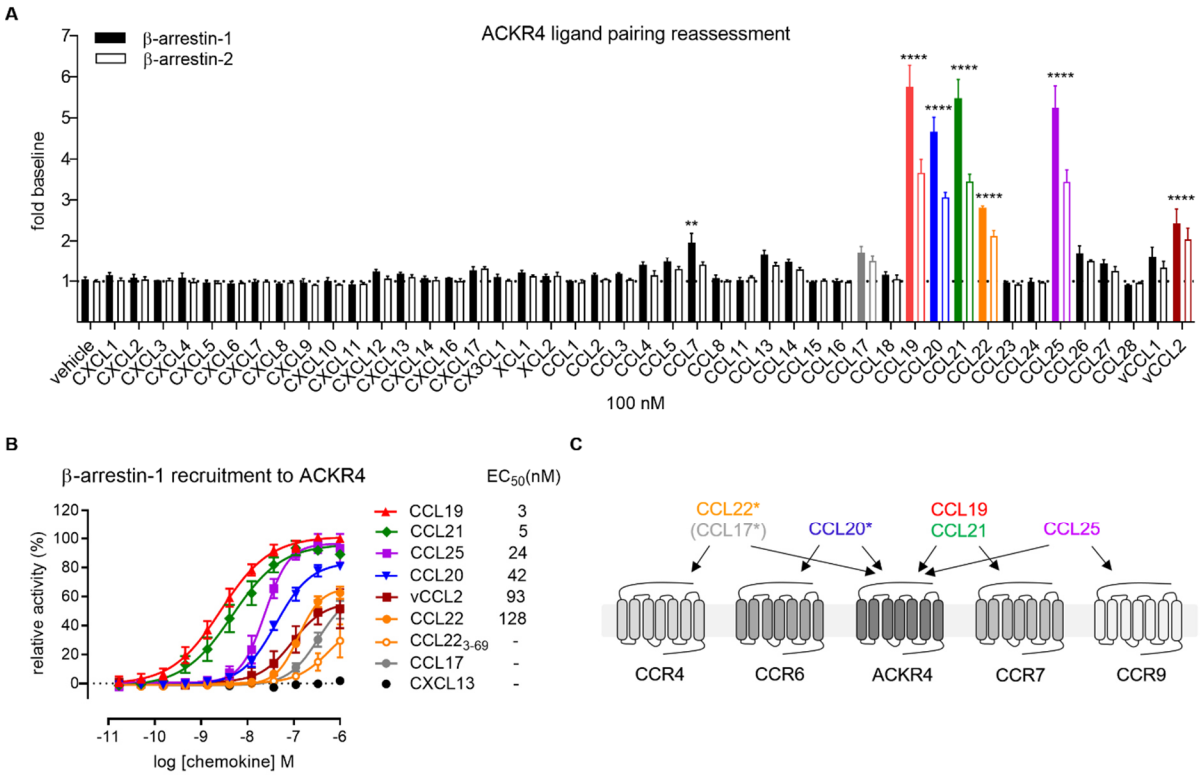

Figure 1. Systematic reassessment of human ACKR4 activation by chemokines using a highly sensitive β-arrestin recruitment assay based on NanoBiT technology. (**A**) β-arrestin-1 and β-arrestin-2 recruitment to ACKR4 in response to all known human and two viral chemokines (100 nM). Experiments were conducted in U87 cells as previously described [21]. For statistical analysis, one-way ANOVA with Dunnett's multiple comparison test with vehicle as reference was performed. ** $p < 0.01$ (β-arrestin 1 only), **** $p < 0.0001$ (in both β-arrestin 1 and β-arrestin 2 recruitment assays). (**B**) β-arrestin-1 recruitment to ACKR4 by the previously reported and newly identified chemokines CCL17, CCL19, CCL20, CCL21, CCL22, CCL25, CXCL13 and vCCL2, showing the concentration-response relationship. EC_{50} values are indicated (nM). (A and B) Data are represented as mean ± SEM of at least three independent experiments. (**C**) Schematic representation of the interactions between ACKR4

and CC receptors CCR4, CCR6, CCR7 and CCR9, and their shared endogenous chemokines. The newly identified ligands are indicated with an asterisk.

A phylogenetic analysis based on amino acid sequences shows that CCL22 clusters together with all other ACKR4 ligands, including CCL20, further supporting its pairing with ACKR4. Moreover, CCL22 is constitutively expressed in lymphoid tissues, the intestine, lung, and skin, where ACKR4 is also expressed [6, 12]. CCL22 acts as a potent agonist for CCR4, primarily expressed on Th2 cells [28, 29] but it has also been described to be involved in self-tolerance and mediate DC-Treg interaction. Indeed, deletion of CCR4 or CCL22 in mice showed a disruption of T-cell immunity, which caused an accumulation of autoreactive T-cells and, as a result, autoimmune diseases [30, 31]. Therefore, together with the reported acceleration of CD4+ T-cell skewing towards Th17 in ACKR4-deficient mice [6], one could postulate the involvement of ACKR4 in the CCR4/CCL22-axis to mediate effective T-cell immunity. The relevance of this newly identified interaction remains nevertheless to be established, especially considering the crosstalk of CCL22 with CCR4 and ACKR2. It also needs to be determined whether the interaction between CCL22 and ACKR4 holds true for the murine homologues.

Altogether, this systematic reassessment of chemokine-receptor interactions confirmed the pairing between CCL20 and ACKR4 in a different cellular background and using a different readout [32], reinforcing the initial description by Matti and co-authors[2]. In addition, it identified another ligand for ACKR4, CCL22. These novel pairings add a level of complexity to ACKR4 interactions within the chemokine-receptor network and extend its regulatory functions to the CCL20/CCR6 and CCL22/CCR4 axes (Figure 1C). Nevertheless, complementary pharmacological experiments, including binding and scavenging or internalization assays, remain to be performed to cross-validate these results. The importance of these novel interacting partners for ACKR4 in pathophysiological context also remains to be elucidated. Finally, our study focused exclusively on chemokine-induced β-arrestin recruitment and thus chemokines with different mode of action towards ACKR4 may have been overlooked [33]. Indeed, although ACKR4-driven chemokine scavenging is generally considered as dependent on β-arrestins, recent studies suggested that they are not essential for ACKR4 scavenging activity, suggesting that further investigations are necessary to fully understand this still enigmatic receptor [10, 34].

2. Highlights

- CCL20 and CCL22 were discovered as novel ACKR4 ligands. Both acted as partial agonists, displaying 80% and 60% maximal efficacy, respectively in a β-arrestin 1 recruitment assay.
- CXCL13, whose interaction with ACKR4 was contradictory, could not be confirmed as an ACKR4 agonist ligand in our β-arrestin recruitment assay.
- Treatment with several other chemokines, like CCL7, CCL13, CCL17 or CCL26, showed low, but detectable signal for β-arrestin recruitment. Although none of them, except CCL7, could reach statistical significance, a clear and consistent trend could be observed for all of them. Physiological relevance of such interactions should be further investigated.
- $CCL22_{3-69}$, mimicking dipeptidyl peptidase 4 (DPP4, or CD26) processed chemokine, retained significant activity towards ACKR4, in contrast to what was described for ACKR2[24].
- All chemokine ligands of ACKR4 were able to trigger recruitment of both β-arrestin 1 and β-arrestin 2 to the receptor.

Our chemokine screening could confirm and strengthen the findings by Matti *et al*. who revealed in parallel to our screen that CCL20 is a novel ligand of ACKR4[2]. Furthermore, our study extended the array of chemokine ligands to CCL22, a chemokine that so far was only described to activate CCR4. Interestingly, the second ligand of CCR4, CCL17, showed also limited, but detectable activity towards ACKR4. However, the regulatory capacity of ACKR4 towards these new ligands and the physiological relevance of these interactions remains to be established, especially considering that CCL22 and CCL17 also interact with the scavenging receptor ACKR2.

Altogether, such systematic reassessment of ACKR4-chemokine pairings demonstrated how important interactions may have been overlooked so far and prompted us to do a similar screening on other receptors. An obvious choice for the next receptor to screen was ACKR3, but besides the previously described new non-chemokine ligands, the well characterized CXCL12 and CXCL11 chemokines and the viral vCCL2 that was already described by our group[27], a screening of all chemokines on ACKR3 did not reveal any further chemokine induced β-arrestin recruitment (data not shown). Hence, the next atypical chemokine receptor on the list was ACKR2…

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Chapter 6: Beyond ACKR3:

Extending the network of interaction partners for other ACKRs

Part 2: CXCL10 as a new ligand of ACKR2

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ACKR2 is a well-established scavenger for at least 10 different inflammatory CC chemokines, but seems to have no affinity towards homeostatic CC or CXC chemokines. By efficiently internalizing inflammatory CC chemokines, ACKR2 is a key player in the regulation of immune responses and inflammation. A recent publication to which I also contributed furthermore suggests an indirect crosstalk between ACKR2 and CXCL14 with implications in epithelial-to-mesenchymal transition[1], but so far, no direct CXC chemokine ligand was described for ACKR2 (this publication won't be discussed in detail in this thesis but can be found in the appendices). Hence, after having efficiently unraveled novel chemokine ligands of ACKR4, we decided to apply a similar screening on ACKR2, with a focus on CXC chemokine ligands.

Abstract

Atypical chemokine receptors (ACKRs) are important regulators of chemokine functions. Among them the atypical chemokine receptor ACKR2 (also known as D6) has long been considered as a scavenger of inflammatory chemokines exclusively from the CC family. In this study, by using a highly sensitive β-arrestin recruitment assays based on NanoBiT and NanoBRET technologies, we identified the inflammatory CXC chemokine CXCL10, known to play an important role in the infiltration of immune cells into the tumour bed and previously reported to bind to CXCR3 only, as a new strong agonist ligand for ACKR2. We demonstrated that ACKR2 is able to internalize and regulate the availability of CXCL10 in the extracellular space. Moreover, we found that, in contrast to CC chemokines, CXCL10 activity towards ACKR2 was drastically reduced by the dipeptidyl peptidase 4 (DPP4 or CD26) N-terminal processing, pointing to a different receptor binding pocket occupancy by CC and CXC chemokines. Overall, our study sheds new light on the complexity of the chemokine network and the potential role of CXCL10 regulation by ACKR2 in many physiological and pathological processes, including in tumor immunology. Our data also testify that systematic reassessment of chemokine-receptor pairing is critically needed as important interactions may remain unexplored.

1. Introduction

Chemokines are small (8-14 kDa) soluble cytokines that guide directional cell migration and orchestrate many important processes, including leukocyte recruitment during immunosurveillance. They are also involved in numerous inflammatory diseases and the development and spread of many cancers. Based on the presence of specific cysteine motifs in their N termini, chemokines are divided into four classes: CC, CXC, XC and CX3C. Their receptors belong to the G protein-coupled receptor (GPCR) family and are accordingly classified as CCR, CXCR, XCR and CX3CR, depending on the chemokine class they bind. Over the past years, a subfamily of four chemokine receptors has emerged as important regulators of chemokine functions. These receptors are termed atypical chemokine receptors (ACKR1-4) due to their inability to trigger G protein-dependent signaling or directly induce cell migration in response to chemokine binding[2, 3]. Nevertheless, ACKRs do play an important role within the chemokine-receptor network by shaping the gradient of chemokines thereby regulating their effect on cells expressing their respective classical chemokine receptors. Most ACKRs have the ability to constitutively cycle between the cell membrane and the intracellular compartments, internalizing and directing for degradation the chemokines that they bind[2, 4-6]. Although this activity was previously considered to mainly rely on β-arrestins, recent studies showed that alternative mechanisms can drive chemokine scavenging by ACKRs[7-12].

ACKR2 (formerly D6 or CCBP2) has been long reported to bind inflammatory chemokines exclusively from the CC family. ACKR2 main ligands include CCL2-8, CCL11-13, CCL17 and CCL22, which are agonists of the classical receptors CCR1-5[13-16]. By scavenging this large spectrum of inflammatory chemokines, ACKR2 drives the resolution phase of inflammation and prevents exacerbated immune responses[17-22]. ACKR2 is expressed on lymphatic endothelial cells, epithelial cells, trophoblasts in placenta and some subsets of leukocytes, including alveolar macrophages and innate-like B cells[23-25]. Owing to its anti-inflammatory effect, ACKR2-deficient mice show increased number of circulating inflammatory monocytes[26] and neutrophils[27, 28], as well as defects in lymphatic vessel density and function[29]. ACKR2 was also shown as an important regulator of chemokines in inflammatory and autoimmune diseases, notably in psoriasis[19, 30-32]. A scavenging-independent activity of ACKR2 has also been reported in apoptotic neutrophils, where ACKR2 was proposed to present chemokines to macrophages and promote inflammation resolution by shifting their phenotype[33, 34].

Importantly, ACKR2 plays diverse and complex roles in tumor biology from initiation to metastasis[28, 35, 36]. ACKR2-deficient mice were shown to be more prone to tumor development but display increased tumor NK infiltration and circulating neutrophils, while opposing effects were reported regarding ACKR2 involvement in tumor dissemination[28, 35, 37]. Besides CC inflammatory chemokines, several CXC chemokines play important roles in inflammatory responses and are also found as part of tumor-associated inflammatory signatures[38, 39]. In particular, the interferon gamma-induced chemokine CXCL10, also known as IP-10, reported to sustain tumor growth via autocrine loops[40] and to drive T lymphocytes and NK cells through activation of CXCR3[38, 41, 42], is often upregulated in the same manner or simultaneously with CC inflammatory chemokines[43].

In this study, by applying highly sensitive assays monitoring β-arrestin recruitment, we identified CXCL10, previously known to exclusively bind to CXCR3, as a high-affinity agonist for ACKR2. This finding expands the panel of ACKR2 ligands to the CXC chemokine family and at the same time highlights the need for a systematic reassessment of chemokine-receptor pairing, as important interactions may remain unexplored.

2. Materials and methods

Cells and proteins

HEK-ACKR2 cell line stably expressing human or mouse ACKR2 were established by transfection of HEK293T cells (ATCC) with pIRES-puro vector (Addgene) encoding the human or mouse ACKR2 and subsequent puromycin selection (5 µg/mL). Receptor surface expression was verified by flow cytometry using hACKR2-specific mAb (clone 196124, R&D Systems) or polyclonal mACKR2-specific antibody (ab1656, Abcam). The absence of CXCR3 at the cell surface was confirmed using mAb clone

1C6 and the corresponding isotype control (Biologend). The B16.F10 and U87.MG cell lines were purchased from ATCC. Unlabeled chemokines were purchased from PeproTech. CXCL10 was labeled with Cy5 using the Amersham QuickStain Protein Labeling Kit (GE Healthcare Life Sciences). Alexa Fluor 647-labelled CCL2 (CCL2-AF647) was purchased from Almac.

Chemokine processing by dipeptidyl peptidase 4

CCL5, CCL2, CXCL10, CXCL11 and CXCL12 chemokines (9 µM) were incubated with recombinant dipeptidyl peptidase 4 (CD26) (200 U) in Tris/HCl 50 mM pH7.5 + 1 mM EDTA for 1 hour at 37°C. The efficiency of processing was verified by MALDI-TOF analysis using a RapifleX, Bruker Daltonics instrument in positive ion mode and in reflectron mode.

Chemokine-induced β-arrestin recruitment

Chemokine-induced β-arrestin recruitment to receptors was monitored by NanoLuc complementation assay (NanoBiT)[44 45, 46] or by NanoBRET using mNeonGreen as acceptor molecule.

NanoBiT: HEK293T cells were co-transfected with pNBe vectors encoding chemokine receptors C-terminally fused to SmBiT and human β-arrestin-1 N-terminally fused to LgBiT. Twenty-four hours post-transfection cells were harvested, incubated 25 minutes at 37°C with Nano-Glo Live Cell substrate (1:200) and upon addition of chemokines at the indicated concentrations, β-arrestin recruitment was evaluated with a Mithras LB940 luminometer (Berthold Technologies). Each point corresponds to average values acquired for 20 min, represented as percentage of maximum full agonist response.

NanoBRET: HEK293T cells were co-transfected with pNeonGreen and pNLF vectors, encoding ACKR2 C-terminally fused to mNeonGreen and β-arrestin-1 N-terminally fused to Nanoluciferase. Twenty-four hours post-transfection cells were harvested and upon simultaneous addition of Nano-Glo Live Cell substrate (1:200) and chemokines, BRET signal was measured with a Mithras LB940 luminometer (Berthold Technologies) using a 460/70 BP filter for Nanoluciferase and a 515/40 BP filter for mNeonGreen signal.

Chemokine binding

HEK293T and HEK-ACKR2 cells were incubated with CXCL10-Cy5 at indicated concentrations for 45 minutes at 37 °C, then washed twice with FACS buffer (PBS, 1 % BSA, 0.1 % NaN_3). Dead cells were excluded using Zombie Green viability dye (BioLegend). ACKR2-negative HEK293T cells were used to evaluate non-specific binding of CXCL10-Cy5. For binding competition with unlabeled chemokines (50 nM or 10 nM), the signal obtained for CXCL10-Cy5 (100 ng/ml) or CCL2-AF647 (30 ng/ml) in the absence of unlabeled chemokines was used to define 100 % binding. Ligand binding was quantified by mean fluorescence intensity on a BD FACS Fortessa cytometer (BD Biosciences).

Chemokine-induced receptor mobilization to the plasma membrane

Ligand-induced receptor mobilization to the plasma membrane was monitored by NanoBRET. 5×10^6 HEK293T cells were seeded in 10-cm dishes and co-transfected with plasmids encoding ACKR2 C-terminally tagged with Nanoluciferase and mNeonGreen C-terminally tagged with the plasma membrane targeting polybasic sequence and prenylation signal sequence from K-RAS splice variant b[47]. Twenty-four hours post transfection, cells were distributed into black 96-well plates (1×10^5 cells per well) and treated with chemokines (100 nM). After 45-minute incubation at 37°C, coelenterazine H (10 µM) was added and donor emission (460 nm) and acceptor emission (535 nm) were immediately measured on a GloMax plate reader (Promega).

Chemokine-induced receptor-arrestin delivery to endosomes

Ligand-induced receptor-arrestin delivery to early endosomes was monitored by NanoBRET. In brief, 5×10^6 HEK293T cells were seeded in 10-cm dishes and co-transfected with plasmids encoding ACKR2, β-arrestin-2 N-terminally tagged with Nanoluciferase and FYVE domain of endofin interacting with phosphatidylinositol 3-phosphate (PI3P) in early endosomes[47, 48], N-terminally tagged with mNeonGreen. Twenty-four hours post-transfection, cells were distributed into black 96-well plates (1×10^5 cells per well) and treated with full-length or processed chemokines. After 2-hour incubation at 37°C, coelenterazine H (10 µM) was added and donor emission (460 nm) and acceptor emission (535 nm) were immediately measured on a GloMax plate reader (Promega).

Chemokine scavenging

Chemokine depletion from the extracellular space was quantified by ELISA. HEK293T and HEK-ACKR2 cells were incubated 8 hours at 37 °C with chemokines at 0.3 and 30 nM. Chemokine scavenging by ACKR2 was evaluated by quantifying the concentration of chemokines remaining in the supernatant using commercially available ELISA kits (CXCL10 R&D Systems, CCL5 BioLegend and CXCL11 Peprotech) and was expressed as the percentage of input chemokine concentrations.

Chemokine internalization

Chemokine internalization using labeled CXCL10 or CCL2 was visualized by imaging flow cytometry as previously described[8]. HEK.293T or HEK-ACKR2 cells were incubated 15 minutes at 37 °C in the presence or absence of unlabeled chemokines (200 nM) after which Cy5-labelled CXCL10 (100 nM) or AF647-labelled CCL2 (100 ng/ml) was added for 45 minutes at 37 °C. Cells were washed twice with FACS buffer. Dead cells were excluded using Zombie Green viability dye (BioLegend). Images of 1×10^4 in-focus living single cells were acquired with an ImageStream MKII imaging flow cytometer

(Amnis) using 60x magnification. Samples were analyzed using Ideas6.2 software. The number of spots per cell was determined using a mask-based software wizard.

For confocal microscopy, 4×10^4 HEK-ACKR2 cells/well were seeded on poly-L-lysine coated 8-well chamber slides (µ-Slide 8 well, Ibidi). After 36 hours, cells were incubated 2 hours at 37 °C with 100 nM Cy5-labeled chemokines (CXCL10, CXCL11 or CCL2) and co-incubated one additional hour with 750 nM LysoTracker™ Red DND-99 (ThermoFisher). Cells were then washed twice with PBS, fixed with 3.5% (w/v) paraformaldehyde for 20 minutes at room temperature and washed again twice with PBS. Nuclear staining was performed with Hoechst 33342 dye (1 µg/mL) for 20 minutes at room temperature and cells were washed 3 times with PBS. Images were acquired on a Zeiss LSM880 confocal microscope using a 63x oil-immersion objective and Zen Black 2.3 SP1 software (Zeiss). Representative cells from 12 image acquisitions of three independent experiments are shown.

Inhibition of chemokine uptake by anti-mACKR2 antibodies

HEK-mACKR2 or B16-F10 cells were incubated 45 minutes at 37°C with Cy5-labeled mCXCL10 (100 nM) in the presence or absence of the polyclonal goat anti-mACKR2 antibody (50 µg/ml) (ab1656, Abcam,) or goat IgG control antibody (ab37373, Abcam) and the secondary donkey anti-goat-AF647 antibody (Jackson ImmunoResearch). Dead cells were excluded using Zombie Green viability dye (BioLegend). Ligand uptake was quantified by mean fluorescence intensity on a BD FACS Fortessa cytometer (BD Biosciences). Inhibition of mCXCL10 scavenging by anti-mACKR2 was expressed as the percentage relative to conditions where the antibody was absent.

Data and statistical analysis

Concentration-response curves were fitted to the four-parameter Hill equation using an iterative, least-squares method (GraphPad Prism version 8.0.1) to provide EC_{50} values and standard errors of the mean. All curves were fitted to data points generated from the mean of at least three independent experiments. All statistical tests, *i.e.* t-tests, ordinary one way ANOVA, and post hoc analysis were performed with GraphPad Prism 8.0.1. P-values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, **** p<0.0001.

3. Results and discussion

The pairing of ACKR2 with CC chemokines dates back to when many chemokines, especially the CXC chemokines, had not yet been known or available[13, 16, 49]. Recent identification of CCL20 and CCL22 as ligands for ACKR4[50, 51], demonstrates that some pairings within the complex chemokine-receptor interaction network may have been overlooked. Several reports point to increased CXC chemokine levels in ACKR2-deficient mice[52, 53] and an indirect crosstalk between the orphan CXCL14 and ACKR2

has recently been described[1]. These observations prompted us to re-evaluate the ability of ACKR2 to scavenge chemokines also from the CXC family.

First, we assessed the activity of the 16 human CXC chemokines (100 nM) towards ACKR2 by monitoring their ability to induce β-arrestin-1 recruitment using Nanoluciferase complementation-based assay (NanoBiT). Our screening revealed that at least three CXC chemokines, namely CXCL2, CXCL10 and CXCL12 are capable of inducing β-arrestin-1 recruitment to ACKR2. However, only CXCL10 reached statistical significance in this assay (Figure 1A).

To evaluate the functional relevance of the interactions between these chemokines and ACKR2, especially in light of a possible scavenging function, we next performed an in-depth analysis of intracellular events and monitored the fate of the chemokines and receptor following their interactions.

CXCL2 and CXCL12 consistently showed reduced potency and efficacy in β-arrestin recruitment towards ACKR2 compared to CXCL10 or to the activity they display towards their already known receptors[46, 54-56] (Figure 1B, E, H and I). Given this limited activity, they were not further investigated. CXCL10 however, showed a strong potency towards ACKR2 (EC_{50} = 8.2 nM, pEC_{50} = 8.08 ± 0.14) and induced approximately half of the maximal response compared to the full agonist CCL5 (Figure 1B). This partial agonist behavior of CXCL10 was reminiscent of the activity towards its long-established signaling receptor CXCR3 relative to the full agonist CXCL11 (Figure 1C, F and G)[57, 58]. The potency of CXCL10 towards ACKR2 appears approximately 3 times stronger than towards CXCR3 (EC_{50}= 24.9 nM, pEC_{50} = 7.60 ± 0.12), consistent with a potential scavenging role of ACKR2. In NanoBRET, the potency of CXCL10 towards ACKR2 (EC_{50} = 5.1 nM, pEC_{50} = 8.29 ± 0.11) was close to that of CCL2 and approximately 20 fold stronger than towards CXCR3. The efficacy of CXCL10 in this assay reached approximately 70% of the maximal signal measured with CCL5 (Figure 1E and F). This was further confirmed in a different cellular background and with a similar assay monitoring the recruitment of β-arrestin-2 (Figure 1H and I). Moreover, the screening of CXCL10 on 23 chemokine receptors showed that CXCR3 and ACKR2 are the only human receptors activated by CXCL10 (Figure 1D). Fluorescently labeled CXCL10 also strongly and specifically bound to HEK293T cells expressing ACKR2 (IC_{50} = 5.4, pIC_{50}= 8.27 ± 0.09) (Figure 1J and K) and was only displaced by ACKR2-related chemokines CCL5, CCL2 and by CXCL10 itself (Figure 1K inset). Inversely, binding competition studies showed that CXCL10 was able to fully displace fluorescently labeled CCL2 from the receptor with an IC_{50} of 2.1 nM (pIC_{50} = 8.68 ± 0.03) (Figure 1L).

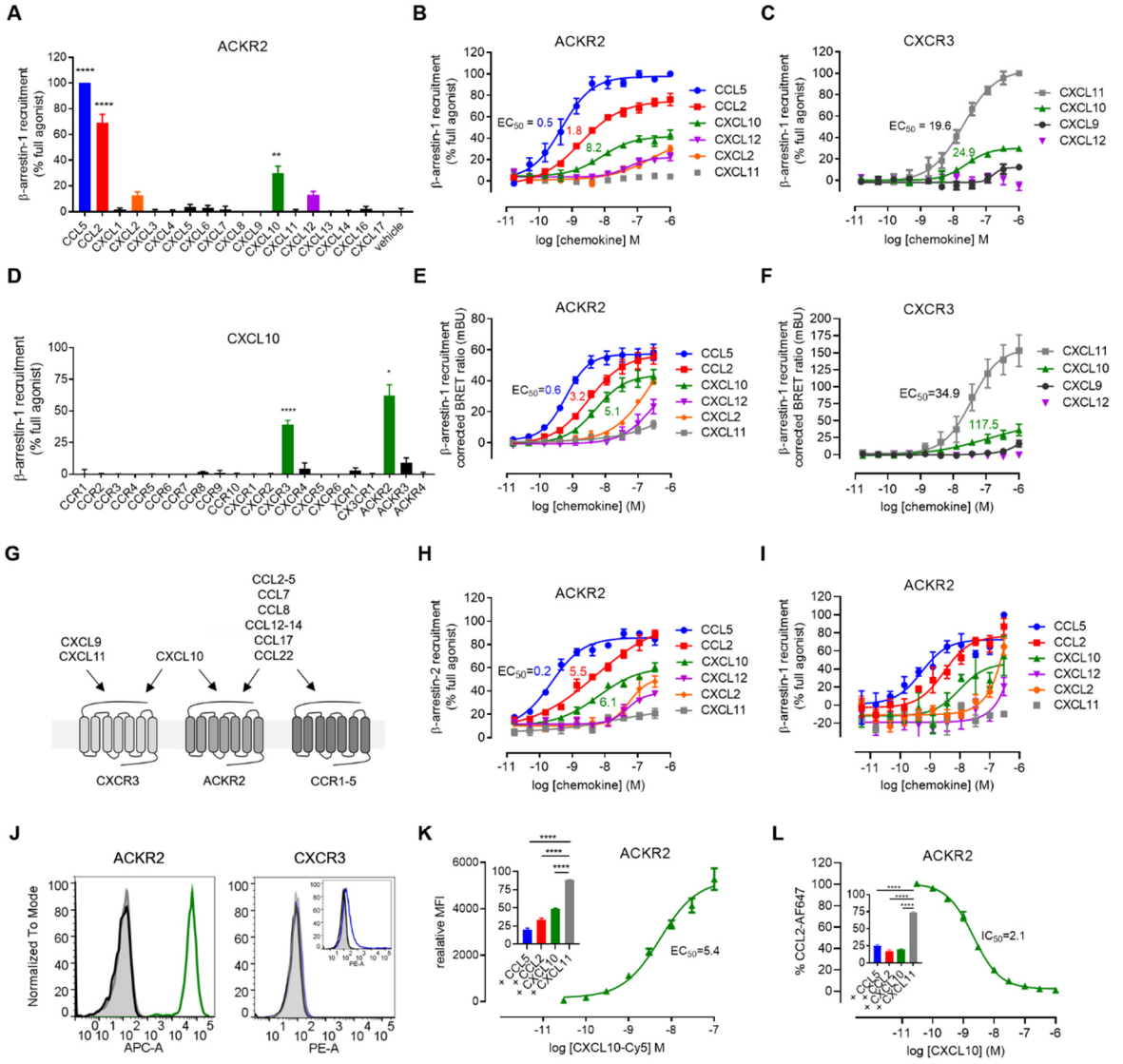

Figure 1. ACKR2 activation by CXCL10. (**A**) β-arrestin-1 recruitment to ACKR2 in response to all known human CXC chemokines (100 nM) monitored by NanoBiT-based assay. CCL2 and CCL5 were used as positive control chemokines. (**B)** β-arrestin-1 recruitment to ACKR2 by the CXC chemokines CXCL2, CXCL10 and CXCL12 monitored by NanoBiT, showing the concentration-response relationship. CXCL11 was used as negative control (**C)** β-arrestin-1 recruitment to CXCR3 induced by its cognate ligands CXCL9, CXCL10 and CXCL11 monitored by NanoBiT. CXCL12 was used as negative control. (**D**) β-arrestin-1 recruitment to all known chemokine receptors in response to CXCL10 (100 nM). (**E-F**) β-arrestin-1 recruitment to ACKR2 (E) and CXCR3 (F) monitored by NanoBRET. (**G**) Schematic representation of chemokine-receptor interactions between ACKR2, CXCR3 and the CC receptors CCR1, CCR2, CCR3, CCR4 and CCR5, including the newly identified pairing between CXCL10 and ACKR2. (**H**) β-arrestin-2 recruitment to ACKR2 by the CXC chemokines CXCL2, CXCL10 and CXCL12 monitored by NanoBiT. (**I**) β-arrestin-1 recruitment to ACKR2 by the CXC chemokines CXCL2, CXCL10 and CXCL12 monitored by NanoBiT in U87.MG cells. (**J**) Flow cytometry analysis of cells used in the binding studies, left panel: ACKR2 surface expression in HEK-ACKR2 (green histogram) and the parental HEK293T cell line (grey filled histogram) evaluated using the ACKR2-specific mAb (clone 196124) or the corresponding isotype control (black histogram); right panel: CXCR3 surface expression in HEK-ACKR2 evaluated using the CXCR3-specific mAb (clone 1C6) (blue histogram) and the corresponding isotype control (black histogram). Unstained cells are represented as grey filled histogram. (**inset**) Positive control surface expression staining for CXCR3 in HEK293T cells transiently transfected with a

CXCR3-encoding vector, using CXCR3-specific mAb (clone 1C6) (blue histogram) and the corresponding isotype control (black histogram) (**K**) Binding of Cy5-labeled CXCL10 to HEK-ACKR2 cells (**inset**) Binding competition (100 ng/ml CXCL10-Cy5) with unlabelled chemokines (50 nM). (**L**) Binding competition of unlabeled CXCL10 with Alexa Fluor 647-labelled CCL2 (30 ng/ml) on HEK-ACKR2 cells (**inset**) Binding competition with unlabeled chemokines (10 nM). EC_{50} and IC_{50} values for concentration-response curves (B-L) are indicated (nM). All NanoBiT and NanoBRET assays were conducted in HEK293T cells except for (I) for which U87.MG cells were used. Data points represent mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001 by one-way ANOVA with Dunnett (A and D) and Bonferroni (K and L) post hoc test.

The ability of ACKR2 to mediate CXCL10 scavenging and control its extracellular concentration was then analyzed. CXCL10 stimulation resulted in rapid mobilization of intracellular ACKR2 to the plasma membrane reminiscent of the activity of CC chemokines[59, 60] (Figure 2A). The CXCL10-induced receptor mobilization was followed by its delivery to the endosomes with an EC_{50} of 6.0 nM (pEC_{50} = 8.22 ± 0.06) (Figure 2B and C). Imaging flow cytometry also revealed specific and efficient uptake of labeled CXCL10 by ACKR2-expressing cells. A notably higher number of distinguishable intracellular vesicle-like structures and mean fluorescent intensity were observed compared to HEK293T cells or HEK-ACKR2 cells pre-treated with CCL5 (Figure 2D and 2F). Confocal microscopy further confirmed CXCL10 uptake and in addition showed its distribution within acidic intracellular vesicles (Figure 2E). Moreover, the uptake of CXCL10 by ACKR2 was more efficient compared to that by CXCR3, consistent with the stronger potency of CXCL10 towards ACKR2 and the possible scavenging function (Figure 2G). As an additional selectivity control, CXCL10—just like CCL5 and CCL2—was able to compete with the uptake of fluorescently labeled CCL2 by ACKR2-expressing cells in imaging flow cytometry (Figure 2H). Importantly, the ACKR2-driven intracellular accumulation of CXCL10 was also associated with a reduction of its availability in the extracellular space as demonstrated by ELISA quantification. The efficiency of ACKR2-driven CXCL10 scavenging was similar at high (30 nM) and low (0.3 nM) chemokine concentrations (Figure 2I) and was comparable to the depletion of CCL5, while no reduction was observed for CXCL11. The interaction between CXCL10 and ACKR2 was also observed with the murine counterparts, as illustrated by the uptake of labelled murine CXCL10 (mCXCL10) by HEK-mACKR2 cells or the mouse melanoma cell line B16.F10, which was partially inhibited by mACKR2-specific polyclonal antibody but not the isotype control (Figure 2J).

Similar to many other CC and CXC chemokines, CXCL10 was shown to be subject to post-translational modification by proteolytic enzymes[61]. In particular, N-terminal cleavage by the dipeptidyl peptidase 4 (DPP4 or CD26) was demonstrated to turn CXCL10 from CXCR3 agonist to antagonist[62]. Based on recent reports demonstrating that, in contrast to CXCR3, ACKR3 is responsive to DPP4-inactivated CXCL11[46], the impact of the CXCL10 N-terminal processing on ACKR2 activation was evaluated and compared to CXCR3. We observed that, in contrast to CC chemokines, truncation of CXCL10 drastically reduced its ability to induce β-arrestin-1 recruitment to ACKR2 (Figure 2K and 2L) and abolished its

targeting to the early endosomes (Figure 2M) indicating that CXCL10 N-terminal residues are critical for its activity towards ACKR2[60, 63]. These results, in addition to partial agonist behaviour of CXCL10, point to distinct ACKR2 interaction and activation modes compared to CC chemokines. This may be attributed to notable differences in the N terminus orientation and occupation of the receptor binding pockets of CXC and CC chemokines[64].

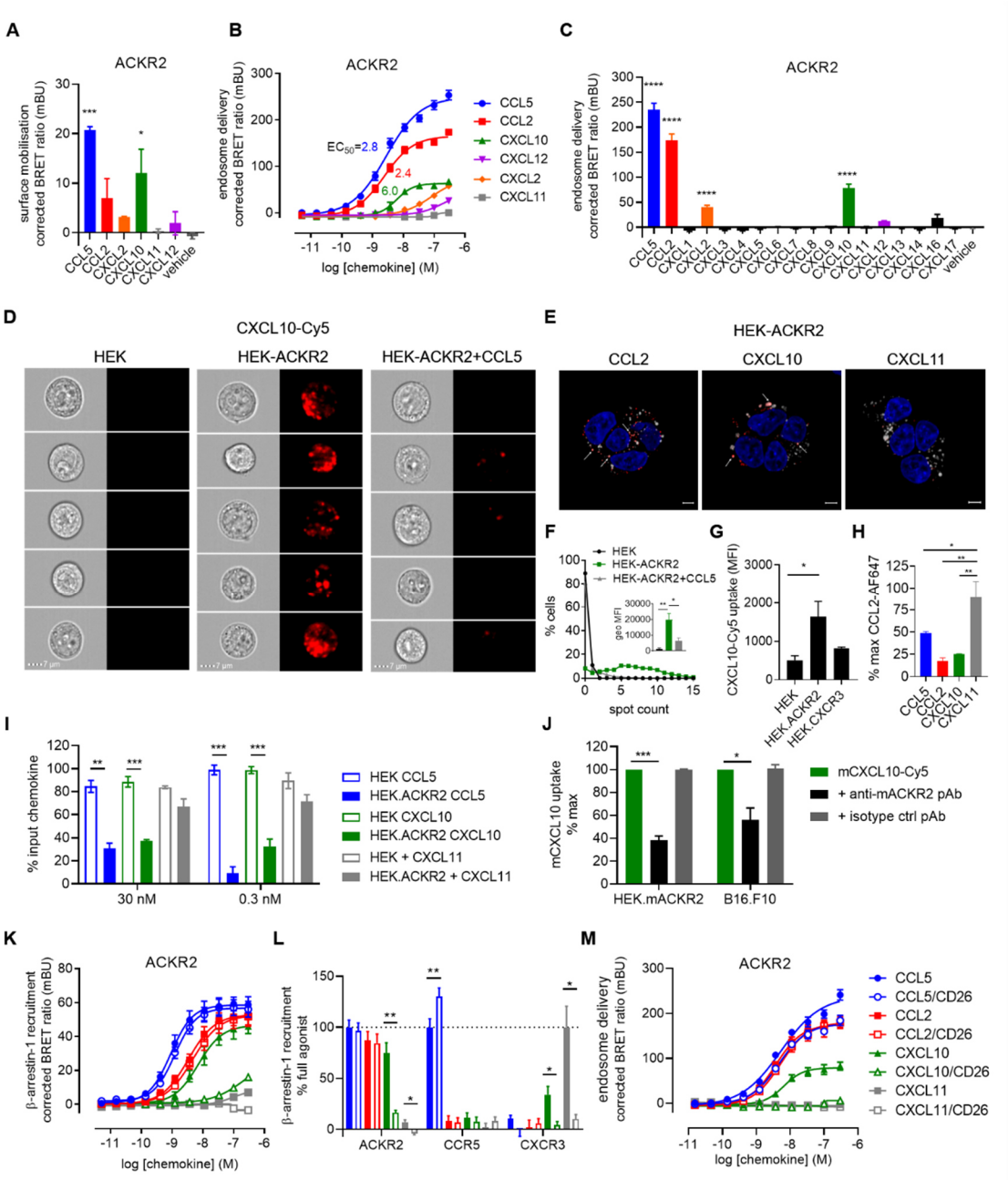

Figure 2. CXCL10 scavenging by ACKR2. (**A**) ACKR2 mobilization to the plasma membrane in response to chemokines (100 nM) monitored by NanoBRET-based assay (**B-C**) β-arrestin-1/ACKR2 complex delivery to the early endosomes in response to the CXC chemokines CXCL2, CXCL10 and CXCL12 (B) or all known human CXC chemokines (100 nM) (C) monitored by NanoBRET-based assay. CCL2 and CCL5 were used as positive control chemokines. (**D-F**) Uptake of fluorescently labelled CXCL10 by ACKR2-expressing cells visualized by imaging flow

cytometry (D and F) and confocal microscopy (E). (D) HEK, HEK-ACKR2 or HEK-ACKR2 cells pre-treated with CCL5 at saturating concentration (200 nM) were stimulated for 45 minutes at 37 °C with 100 nM (Cy5)-labeled CXCL10 (CXCL10-Cy5, red channel). Five representative cells for each condition are shown (10.000 events recorded). Scale bar: 7 µm. (F) Percentage of cells from (D) with a given number of distinguishable vesicle-like structures (spots), as well as the geometrical mean fluorescence intensity (MFI) for the red channel were determined (inset). Data shown are representative of three independent experiments and for inset, mean ± SEM of three independent experiments. (E) Cellular localization of Cy5-labeled chemokine (red) following HEK-ACKR2 stimulation (100 nM) for 2 hours monitored by fluorescent confocal microscopy. Lysosomes and nucleic DNA were stained using LysoTracker™ Red DND-99 (white) and Hoechst 33342 (blue), respectively. Pictures are representative of 12 acquired images from three independent experiments. Scale bar: 5 µm. Arrows highlight colocalization of Lysotracker and chemokine-Cy5 signal. (**G**) Uptake of Cy5-labeled chemokine (100 nM) by HEK cells transfected or not with equal amounts of ACKR2 or CXCR3 vectors analyzed by imaging flow cytometry as described in (D). (**H**) Binding competition between Alexa Fluor 647-labeled CCL2 (100 ng/ml) and unlabeled chemokines (100 nM) in HEK-ACKR2 analyzed by imaging flow cytometry. (**I**) ACKR2-mediated depletion of extracellular CXCL10 monitored by ELISA. Chemokines in the supernatant of HEK293T cells expressing or not ACKR2 were quantified after 8-hour stimulation, and expressed as percentage of the input concentrations (30 nM and 0.3 nM). CCL5 and CXCL11 were used as positive and negative controls, respectively. Data points represent mean ± SEM of three independent experiments. (**J**) Inhibition of mACKR2-mediated mCXCL10 uptake by neutralizing antibodies. Cy5-labeled mouse CXCL10 (mCXCL10-Cy5) (100 nM) was incubated with HEK-mACKR2 or B16.F10 in the presence of mACKR2 neutralizing polyclonal antibody (Ab1656) or corresponding isotype control (Ab37373) for 45 minutes at 37 °C and analyzed by flow cytometry. (**K-M**) Impact of chemokine N-terminal processing by dipeptidyl peptidase 4 (DPP4/CD26) on the activation of ACKR2 and related receptors CXCR3 and CCR5 and ACKR2 delivery to the endosomes. (K-L) β-arrestin-1 recruitment to ACKR2 by processed chemokines monitored by NanoBRET. (L) Comparison of the impact of N-terminal processing on the ability of CXC and CC chemokines (100 nM) to induce β-arrestin-1 recruitment to ACKR2, CXCR3 and CCR5. (M) β-arrestin-1/ACKR2 complex delivery to the early endosomes in response to processed chemokines monitored by NanoBRET. *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA with Dunnet (A, C and G) and Bonferroni (H) post hoc test or repeated measures one-way ANOVA with Bonferroni post hoc test (J) and two-tailed unpaired Student's t-test (I).

In conclusion, our study shows that CXCL10 is a novel ACKR2 ligand. CXCL10 is one of the most important inflammatory CXC chemokines and is involved in many physiological and pathological processes such as angiogenesis, chronic inflammation, immune dysfunction, tumor development and dissemination, in which ACKR2 has also been shown to play critical roles[36]. Together with CCL5, CXCL10 is a key player in driving NK cells and CD8 cells into the tumor bed[38, 39, 41, 42]. This novel pairing consequently adds an unforeseen level of complexity to ACKR2 functions and a new level of CXCL10 regulation and could thus encourage to re-examine previous studies taking into account CXCL10-ACKR2 interactions (Figure 1G)[28, 52, 53, 65, 66].

The ability to bind and respond to both CXC and CC chemokines has already been reported for ACKR1[67] ACKR3[68] and ACKR4[69], although this property has recently been challenged for the latter. Here, we identified an agonist CXC ligand for ACKR2, which until now had been recognized for binding to inflammatory CC chemokines only. Therefore, such cross-family spectrum of chemokine ligands, uncommon among the classical chemokine receptors, seems to represent an additional functional property of ACKRs[3] besides their inability to trigger G protein signaling. Overall, this study highlights

that a systematic reassessment of chemokine-receptor pairings for both long-established and recently deorphanized receptors may be necessary, as important interactions may have been overlooked.

4. Highlights

- CXCL10 is as partial agonist of ACKR2, with an apparent stronger potency than to its so far only described receptor CXCR3, towards which CXCL10 also behaves as partial agonist. Other CXC chemokine CXCL2 and CXCL12 also showed limited but detectable activity towards ACKR2.
- A screening of CXCL10 on all chemokine receptors revealed that ACKR2 and CXCR3 are the only receptors that recruit β-arrestin in response to CXCL10 stimulation.
- CXCL10 is internalized by cells expressing ACKR2 leading to a progressive depletion from the extracellular milieu.
- In contrast to what was shown for CC chemokines[60, 63], CXCL10 N-terminal residues are critical for ACKR2 activation, since N-terminal cleavage by dipeptidyl peptidase 4 (CD26) abolished CXCL10 driven β-arrestin recruitment towards ACKR2. This indicates that CXC chemokines have a different ACKR2 binding mode and/or ligand pocket occupancy than CC chemokines.

As for ACKR4, our systemic screen could unravel new pairings for ACKR2, with CXCL10 being the most promising candidate. CXCL10 was so far only described to interact with CXCR3, yet, considering its important functions as inflammatory chemokine involved in many physiological and pathological processes like angiogenesis, chronic inflammation or tumor development, it is coherent that its availability is tightly regulated by an atypical scavenging receptor[70, 71]. Noteworthy, besides CXCL10, our study could show that CXCL2 and CXCL12 also show low, but detectable activity towards ACKR2. However, especially for CXCL12, it is unclear whether this interaction is of physiological relevance, considering that ACKR3 is already an extremely potent CXCL12 scavenger.

Besides these direct interactions of CXC chemokines with ACKR2, a more indirect link between the orphan CXC chemokine CXCL14 and ACKR2 also seems to exist. Indeed, we were fortunate to collaborate with Prof A. Östman from Karolinska Institutet, Sweden, and could reveal in a series of experiments that ACKR2 is mediating the effects of CXCL14 promoting tumor growth and metastasis[1]. However, β-arrestin recruitment assays as well as binding assays with fluorescently labeled CXCL14 could not reveal a direct CXCL14-ACKR2 interaction, suggesting an indirect, maybe synergistic influence of both partners.

A similar indirect influence of CXCL14 on several other receptors could also recently be shown in a collaborative effort with the group of Prof. B. Moser, where we could show that, although CXCL14 is not inducing β-arrestin recruitment towards any known chemokine receptor, it synergizes with several

homeostatic chemokines, increasing drastically their potency, signaling properties and physiological effects[72].

The interested reader can find an additional paragraph about CXCL14 in the discussion section of this thesis and both papers on CXCL14 (which won't be discussed in more detail in this thesis) in the appendices.

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Discussion and Outlook

1. Atypical activation mode and atypical ligands of ACKR3

The concept of atypical chemokine receptors gained significant interest in the last years. However, although their reclassification dates back to 2014, many research groups still tend to use the "traditional" receptor names, like CXCR7 instead of ACKR3, showing that it takes time for the community to fully accept this new family of receptors. When I started my PhD thesis in 2016, work on ACKR3 was one of the top priorities of our research group. It is why a lot of tools, plasmids and cell lines were already set up upon my arrival, which extremely facilitated my initiation into the different ACKR projects. I hereby want to especially acknowledge the work of Martyna Szpakowska and Manuel Counson, who paved the way for the success of this PhD thesis, especially the first two chapters dealing with ligand and receptor determinants for efficient binding and activation; two projects, that were already ongoing upon my arrival and which I could help to bring to conclusion in my first years of the thesis.

The data described in these first two papers, together with reports from other groups[1-3], significantly enlarged our understanding of chemokine ligand interactions with ACKR3. It showed that ACKR3 is not only atypical in regard to its signaling behavior and function, but also its high propensity for beta-arrestin recruitment and its tolerance to disulphide bridge breakages when compared to the classical receptors CXCR4 and CXCR3, with which it shares chemokine ligands. This highlights that the molecular determinants of ACKR3 required for activation are less stringent compared to classical receptors, which is in line with what was shown in other studies[1, 2]. This concept was later also confirmed for other ACKR3 ligands, especially opioid peptides (e.g. Dynorphin 2-17 or [Phe]-BAM22).

The discovery that ACKR3 is a broad-spectrum scavenger for opioid peptides became the center of my PhD thesis for almost three years. The initiative to screen a larger opioid peptide library on ACKR3 inferred from the initial observation that proenkephalin-derived peptides trigger arrestin recruitment to ACKR3. In turn, this modulates circadian glucocorticoid oscillation and induces anxiolytic-like effects in mice synergistically with ACTH[4]. This important paper, although paving the way for the discovery of ACKR3 as a broad-spectrum scavenger for opioid peptides in the CNS, significantly differs from our study in regard to the interpretation of the results. Even though Ikeda *et al*. never directly claimed that ACKR3 ligands (both endogenous and synthetic) have a stimulatory effect on glucocorticoid secretion per se, they deduced from their observations that stimulation of ACKR3 in the adrenal glands induces β-arrestin-mediated ERK phosphorylation, which enhances the effect of ACTH, the agonist of the melanocortin receptor 2 (MC2R). This in turn modifies glucocorticoid oscillation and consequently has an anxiolytic-like effect on the animal behavior. On the other hand, we could never detect any direct ACKR3-mediated signaling, neither via canonical G proteins, nor β-arrestin-mediated ERK phosphorylation, despite using several experimental setups. Hence, our findings rather suggest that

ACKR3's function in the opioid system is to scavenge opioid peptides in order to regulate their abundance for signaling receptors, which is still in agreement with the data generated by Ikeda *et al*.. Further studies will show whether both hypotheses hold true, depending on the type of the ACKR3-expressing cells, or whether ACKR3 rather has only one dominant role in opioid peptide biology. Independent of its mode of action, Ikeda *et al.* clearly demonstrated that activating ACKR3 has a synergistic impact on the ACTH-MC2R signaling axis to modulate glucocorticoid levels. Hence, a promising route to pursue is investigating the exact role of ACKR3 in the hypothalamic-pituitary-adrenal (HPA) axis to get deeper insights into the functions of ACKR3 in glucocorticoid modulation and its synergistic effect on ACTH-MC2R. In analogy to the mechanism we describe for ACKR3 in the CNS, it is tempting to speculate that modulating ACKR3 in the adrenal glands might increase the availability of particular ligands for their signaling receptors, for example of ACTH for its receptor MC2R.

Another question that remains open is whether ACKR3, besides directly scavenging opioid peptides in the extracellular space, has also a direct influence on classical opioid receptor in the same cell. The concept of heterologous desensitization between chemokine and opioid receptors for instance is well established in the meantime, but so far only focused on few chemokine receptors[5, 6]. Chen *et al*. showed for example, that CXCL12 or CCL5, administered 30 minutes before the prototypical opioid agonist morphine, blocked the antinociceptive effect of the drug, and interpreted these observations as heterologous desensitization of MOR by CXCR4 and CCR5[7]. However, preliminary NanoBRET results from our group indicate that ACKR3 can heterodimerize as well with each of the four other opioid receptors, with so far unknown outcomes. A second hypothesis of how ACKR3 could have a direct influence on opioid receptors in the same cell, is the concept of "β-arrestin pirating". ACKR3, being a highly activation-prone receptor, whose activity relies mainly on β-arrestin recruitment, might deplete a large part of intracellular β-arrestin pools once activated, which could slow down the desensitization and internalization of classical opioid receptors, potentiating or prolonging their response to ligands. Even though no solid proof for such arrestin-pirating exists yet, the concept was already suggested for cells expressing both ACKR3 and CXCR4[8].

Even the final experiment performed in our study on ACKR3 as an opioid scavenger, where rat locus coeruleus slices were perfused with dynorphin A, pretreated or not with the specific ACKR3 agonist LIH383, cannot clearly answer this question. We cannot distinguish whether the increase in apparent dynorphin A potency after treatment with LIH383 mainly results from saturating ACKR3 and its scavenging function, or from the pre-activation of ACKR3, influencing intracellularly opioid receptor activity. Investigating whether ACKR3, besides scavenging opioid peptides also directly influences opioid receptor activity remains thus of crucial importance for the development of novel ACKR3 modulators for the treatment of pain or depression. The recently developed first ACKR3 antagonist

(ACT-1004-1239) might be very useful to distinguish between a functional outcome originating from truly blocking ACKR3 activity and simply saturating it with a selective agonist like LIH383[9-11]. First in human studies with this antagonist showed that the drug was well tolerated and increased CXCL12 plasma concentrations dose-dependently to more than twice the baseline[10]. It would thus be highly interesting to analyze opioid peptide availability as well as classical opioid receptor activity after administration of this antagonist. Along these lines, in a joint effort with the Center for Drug Discovery, RTI International, our team was also able to show that the natural analgesic alkaloid conolidine targets ACKR3 (published in Signal Transduction and Targeted Therapy: "The natural analgesic conolidine targets the newly identified opioid scavenger ACKR3/CXCR7", *in press*, and a patent is pending on "Novel selective ACKR3 modulators"). We could reveal that conolidine acted as an agonist of ACKR3 and was able to restrain BAM22 uptake by ACKR3. Hence, similar to LIH383, saturating ACKR3 with conolidine might inhibit its scavenging function, leading thus to an increase of pain relief-inducing opioid peptides for classical opioid receptors. But again, as previously discussed, we cannot distinguish whether conolidine, acting as an agonist of ACKR3, prevents other ACKR3 ligands from binding through simple competition or whether it also influences the activity of classical opioid receptors by for example depleting intracellular arrestin pools. Even though I won't be able to continue my work on the ACKR3-opioid interplay, I am glad that a very talented new AFR fellow, Christie Palmer, will take over this project to shed light on these yet unanswered questions.

With the discovery of proadrenomedullin-derived PAMP peptides as another family of ligands that activate ACKR3, we further enlarged the panel of endogenous ACKR3 agonists and added another level of complexity to the regulatory roles of this receptor. Noteworthy, we again observed no ACKR3-mediated G protein or ERK signaling for this family of ligands. The phylogenetically rather distant receptor MrgX2 is besides ACKR3 so far the only other receptor described to bind and become activated by PAMP peptides[12]. A quite peculiar observation since it also shares with ACKR3 ligands form another family, the endogenous opioid peptides[13]. Given that MrgX2 was already described to become activated by several other peptides from different families, like cortistatin-14[14] or antimicrobial peptides like LL-37[15], beta-defensins[16], PMX-53[17] and complanadine A[18], it might be worth scrutinizing these peptide families in search for other ACKR3 ligands.

All the above observations and open questions undoubtedly stress that ACKR3 well deserves its designation as an "atypical" receptor. A part from its atypical signaling behavior, ACKR3 is quite unique in regard to its high activation propensity, meaning that most of the ligands able to bind this receptor induce arrestin recruitment to it. Moreover, ACKR3 is one of few examples in GPCR biology with an extremely diverse cross-family ligand selectivity: from a narrow spectrum of chemokines to a broad

spectrum of endogenous opioid peptides to yet another unrelated family of ligands, namely proadrenomedullin-derived peptides. This diversity of ligands is also in compliance with the broad expression profile of ACKR3, ranging from endothelial cells, cardiomyocytes and immune cells, to different regions of the central nervous system and the adrenal glands[19, 20]. Considering that ACKR3 is interacting with various cellular partners (i.e. RAMPs, arrestins, Connexin43 or other GPCRs like CXCR4), it is likely that ACKR3 functions are largely dependent on the expression profile of its interaction partners[21-23]. This tissue bias was recently highlighted again by a study confirming functional coupling between ACKR3 and G_i proteins in astrocytes, an observation initially described almost a decade ago[22, 24]. In light of the diversity of already described ligands and the plethora of different biological roles that were in the meantime attributed to ACKR3, it would not be surprising if other ligand families and functions of ACKR3 are discovered in the near future.

The fact that we could considerably enlarge the panel of endogenous ACKR3 ligands made us wonder, whether other endogenous ligands for chemokine receptors, especially in the family of atypical chemokine receptors, might have been overlooked in the past. Thus, in an attempt to further enlarge the chemokine ligand-receptor interactome, we applied a chemokine screening approach building on state-of-the-art nanoluciferase complementation-based β-arrestin recruitment. Even though more and more studies suggest that β-arrestins are dispensable for some of the receptor functions[25-29], agonist induced β-arrestin recruitment assays remain a powerful tool to screen large panels of ligands for their activity towards a receptor. Still, the assays have some limitations as will be further discussed in the next paragraphs. While no new chemokine ligand could be identified for ACKR3, CXCL10 was uncovered as the first CXC chemokine ligand for ACKR2 and CCL20 and CCL22 were found as novel ACKR4 agonists. This easy and efficient way of how we enlarged the panel of ACKR ligands might encourage the chemokine community to undergo a systemic reassessment of all chemokine receptors, since many other interactions may still remain uncovered.

2. Challenges and considerations to fully unscramble the chemokine network

Noteworthy, in line with previous observations[30], our β-arrestin recruitment assay did not lead to any ligand-induced signal for ACKR1, likely due to a lack of specific interactions of this receptor with β-arrestins. No matter how efficient our ligand screenings have been, this also underlines their biggest weakness: they only rely on ligand-induced β-arrestin recruitment. Certainly, the different hits were later confirmed with various additional approaches like binding competition or internalization assays, but we cannot exclude that we overlooked important ligands that bind to these receptors but do not induce β-arrestin recruitment. This drawback was recently highlighted with the deorphanization of a new atypical chemokine receptor GPR182, not triggering any β-arrestin recruitment upon ligand

binding[31]. Furthermore, some ligand interactions with the receptor might only be possible in the presence of appropriate adaptor proteins, like RAMPs for example (i.e. tissue bias)[21, 32]. An interesting observation for this claim is that we only detect a strong specific signal for XCL2 induced β-arrestin recruitment towards XCR1 in U87 cells, but not in HEK cells. Although we never pursued this observation, it shows the limitation of applying screening assays in only one cell line. Additionally, some chemokine ligands might only show their physiological activity towards a receptor if properly posttranslationally modified or proteolytically processed[33, 34]. Interestingly, such modifications might already occur during the synthesis process of chemokines, since we observe for example an almost 10-fold difference in potency and efficacy of CCL1 towards CCR8, depending on the chemokine supplier (i.e. Peptrotech or R&D). These issues might partly explain why convincing published data can sometimes not be reproduced by other research groups and why the proper deorphanization of novel ligands or receptors can be contradictory for a long time[35]. For instance, whether the orphan chemokine CXCL17 is a ligand for the putative chemokine receptor CXCR8 is still under debate[36, 37]. Similarly, several receptors have been proposed for the orphan CXCL14, but no robust pharmacological proof of a direct CXCL14-receptor interaction could be provided so far[38-42].

Hence, although these nanoluciferase complementation-based β-arrestin recruitment assays are an extremely efficient way to identify new GPCR ligands, they have their limitations and the choice of cell line and chemokine supplier should be carefully defined. Additionally, other complementary tests have to be used to fully unravel the chemokine-receptor network. These additional tests could be extended to direct ligand binding or binding competition assays, receptor internalization and recycling (e.g. HiBIT technology), interaction with different G proteins and GRKs (e.g. similar nanoluciferase complementation assays) as well as various functional assays, ideally for all receptors with all available chemokine ligands. Although requiring substantial financial and human resources, such an undertaking would most likely reveal several new interactions and signaling pathways and would dramatically increase our knowledge on this promiscuous interactome.

Although extremely challenging to take into account in *in vitro* assays, chemokine homo- and heterodimers were described to have altered binding and activation properties with respect to monomeric chemokines, which adds yet another level of complexity to the chemokine network[43, 44]. Generally, chemokines can dimerize in a CC-type or CXC-type interaction mode, relying either their N termini or the first beta-strand, respectively[45]. Depending on the chemokines, mixed CC-CXC interactions may either engage in CC-type or CXC-type interactions and all interaction modes can confer either synergistic or inhibitory properties to the dimer. Functional assays could show for example that CC-type heterodimers CCL5 with CCL17 acted synergistically and enhanced chemotaxis, whereas CXC-type heterodimers CCL5 with CXCL12 had inhibitory effects on cell migration[43]. Such

dimerization events further impede a thorough understanding of the chemokine interactome and allow to speculate that some chemokine-receptor interactions are only possible, once the chemokine is in its proper dimerized state.

Of particular interest for this might be the orphan chemokine CXCL14 which has been of some importance throughout my thesis due to different and independent collaborations but without being the focus of one specific PhD project. CXCL14 is constitutively expressed by various epithelial tissues, fibroblasts and macrophages[46]. Even though the receptor of CXCL14 could not be clearly identified so far, a variety of biological functions can be directly attributed to CXCL14 such as immune surveillance, cell differentiation or angiogenesis, but also cancer progression[38, 47, 48]. CXCL14 was recently proposed to act on the receptor GPR85[39], but a solid pharmacological proof for this interaction is missing and we could not detect any CXCL14-induced β-arrestin recruitment towards GPR85. A further receptor candidate is CXCR4, but it seems that its modulation by CXCL14 is rather indirect [41, 42]. A last candidate is ACKR2, since a link between CXCL14 and ACKR2-dependent epithelial-to-mesenchymal transition was revealed. However, we contributed to showing that, as for CXCR4, these effects do not seem to be linked to a direct chemokine-receptor interplay, since ligand binding assays and β-arrestin recruitment assays could not show such an ACKR2-CXCL14 interaction[40] (This study was performed in collaboration with Karoliska Institutet, Sweden. Although not a focus of this thesis, the full manuscript can be found in the appendices).

Interestingly, CXCL14 encodes for an unusually short N terminus (Ser-Lys) before its conserved CXC motif. Given that the N terminus of chemokines is considered to interact with the transmembrane segments of the receptor, triggering its activation, the interaction mode of CXCL14 with its receptor is either quite distinct compared to other chemokines, or limited to few amino acids and hence probably of low affinity. Considering that despite tremendous efforts, CXCL14 could still not be convincingly deorphanized since its discovery in 1999[49], the hypothesis emerged that CXCL14' main mode of action relies on modulation of other chemokines rather than on interaction with its bona fide receptor. This might either happen via heterodimerization, influencing the signaling properties of other chemokines, or via binding to glycosaminoglycans (GAGs), to which most chemokines bind rather nonspecifically[50, 51]. Saturation of GAGs by CXCL14 could either increase the concentration of freely available chemokines to their receptors, but could also promote diffusion and degradation of chemokines by preventing their accumulation on GAGs. Hence, CXCL14 might generally act as a synergistic chemokine to modulate the activity of other chemokines in various tissues, as we recently suggested for various homeostatic chemokines[52]. Other examples of non-chemokine proteins influencing chemokine signaling behavior have already been described, like the High Mobility Group Box 1 protein (HMGB1),

which was shown to form a complex with CXCL12 and enhance monocyte recruitment in a mouse model of tissue damage[53].

Undoubtedly, the chemokine receptor-ligand network is highly complex. With this thesis, I aimed to increase our understanding of this intertwined network by characterizing the until recently unknown ligand interactions with atypical chemokine receptors, especially with ACKR3 (Figure 1). However, despite tremendous efforts and technological improvements, a thorough understanding of the entire chemokine interactome remains an ambitious goal. To achieve this, it is indispensable to confirm interactions with multiple biochemical readouts and to take into account the correct choice of cell lines and ligand supplier, as well as proteolytic processing, posttranslational modifications and heteromerization of ligands and receptors, which all can have dramatic influences on ligand-receptor interactions.

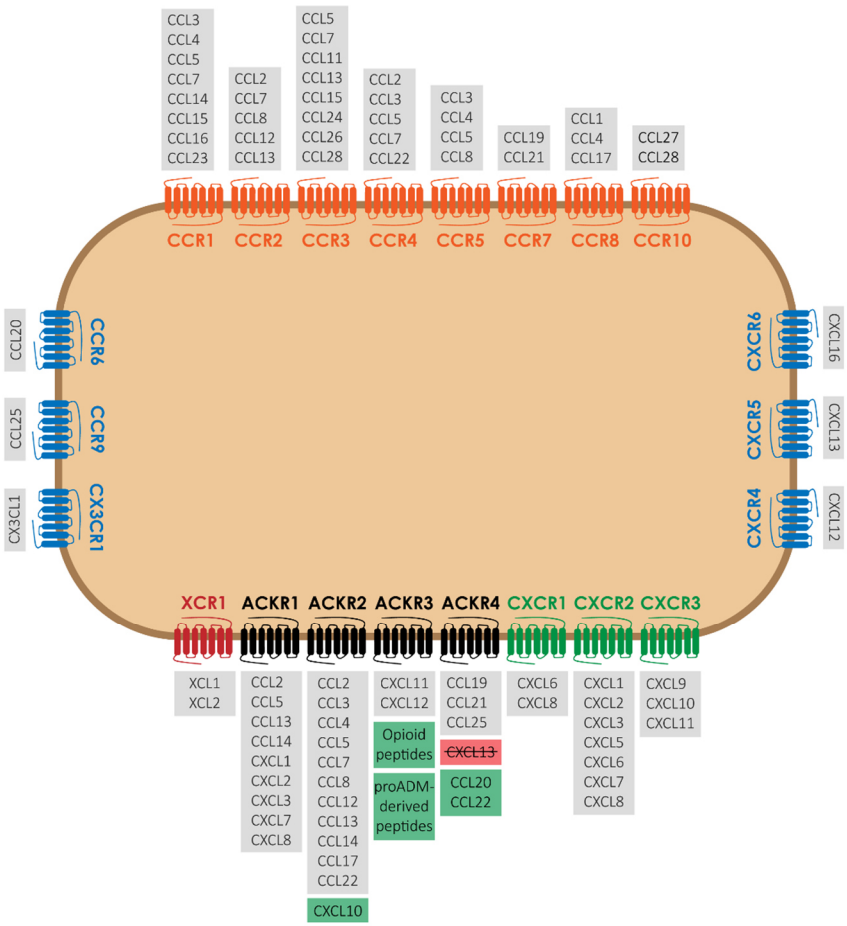

Figure 1. Overview of an updated chemokine receptor–ligand network after this thesis. Overview of the different chemokine receptors and their ligands as presented in the introductory part of the thesis. Newly identified interaction partners with ACKR2, ACKR3 and ACKR4 are highlighted in green. The previously described interaction of CXCL13 with ACKR4 that could not be confirmed in this thesis is crossed out and in red. Adapted from Lazannec & Richmond [54].

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Appendices

Accepted PhD Thesis Related Publications

1. Szpakowska, M., Decker, AM., **Meyrath, M**., Palmer, CB., Blough, BE., Namjoshi, OA., Chevigne, A., The natural analgesic conolidine targets the newly identified opioid scavenger ACKR3/CXCR7. **Signal Transduct Target Ther**. 6, 209, (2021)

2. **Meyrath, M**., Palmer, CB., Reynders, N., Vanderplasschen, A., Ollert, M., Bouvier, M., Szpakowska, M., Chevigne, A., Proadrenomedullin N-Terminal 20 Peptides (PAMPs) are agonists of the chemokine scavenger receptor ACKR3/CXCR7. **ACS Pharmacol Transl Sci,** 4 (2), 813-823. (2021)

3. Chevigne, A., Janji, B., **Meyrath, M**., Reynders, N., D'Uonnolo, G., Uchanski, T., Xiao, M., Berchem, G., Ollert, M., Kwon, Y. J., Noman, MZ., Szpakowska, M., CXCL10 Is an Agonist of the CC Family Chemokine Scavenger Receptor ACKR2/D6. **Cancers (Basel),** *13* (5) (2021)

4. **Meyrath**, **M.*,** Reynders, N.*, Uchański, T., Chevigné, A., Szpakowska, M. Systematic reassessment of chemokine-receptor pairings confirms CCL20 but not CXCL13 and extends the spectrum of ACKR4 agonists to CCL22. **J Leukoc Biol,** 109 (2), 373-376 (2021) * equal contribution

5. Kouzeli, A., Collins, P. J., Metzemaekers, M., **Meyrath, M.**, Szpakowska, M., Artinger, M., Struyf, S., Proost, P., Chevigne, A., Legler, D. F., Eberl, M., Moser, B., CXCL14 Preferentially Synergizes With Homeostatic Chemokine Receptor Systems. **Front Immunol**, 11, 561404 (2020)

6. **Meyrath, M.***, Szpakowska, M.*, Zeiner, J., Massotte, L., Merz, MP., Benkel, T., Simon, K., Ohnmacht, J., Turner, JD., Krüger, R., et al.. The atypical chemokine receptor ACKR3/CXCR7 is a broad-spectrum scavenger for opioid peptides. **Nat Commun.** 11, 3033 (2020), *equal contribution

7. Sjoberg, E.*, **Meyrath, M.,*** Chevigne, A., Ostman, A., Augsten, M., and Szpakowska, M. The diverse and complex roles of atypical chemokine receptors in cancer: From molecular biology to clinical relevance and therapy. **Adv Cancer Res** 145, 99-138. (2020) *equal contribution

8. Wouters, E., Walraed, J., Robertson, M.J., **Meyrath, M.,** Szpakowska, M., Chevigne, A., Skiniotis, G., and Stove, C. Assessment of Biased Agonism among Distinct Synthetic Cannabinoid Receptor Agonist Scaffolds. **ACS Pharmacol Transl Sci,** 3, 285-295 (2020).

9. Sjoberg, E., **Meyrath, M.**, Milde, L., Herrera, M., Lovrot, J., Hagerstrand, D., Frings, O., Bartish, M., Rolny, C., Sonnhammer, E.*, et al.* A Novel ACKR2-Dependent Role of Fibroblast-Derived CXCL14 in Epithelial-to-Mesenchymal Transition and Metastasis of Breast Cancer. **Clin Cancer Res.** 25, 3702-3717 (2019).

10. Neirinckx, V., Hau, AC., Schuster, A., Fritah, S., Tiemann, K., Klein, E., Nazarov, P., Matagne, A., Szpakowska, M., **Meyrath, M.**, Chevigné, A., Schmidt, MHH., Niclou, SP., The soluble form of pan-RTK inhibitor and tumor suppressor LRIG1 mediates downregulation of AXL through direct protein–protein interaction in glioblastoma. **Neurooncol. Adv.** 1, vdz024 (2019)

11. Szpakowska, M.*, **Meyrath, M.***, Reynders, N., Counson, M., Hanson, J., Steyaert, J., and Chevigne, A.. Mutational analysis of the extracellular disulphide bridges of the atypical chemokine receptor ACKR3/CXCR7 uncovers multiple binding and activation modes for its

chemokine and endogenous non-chemokine agonists. **Biochem Pharmacol** 153, 299-309 (2018). *equally contributed

12. Szpakowska, M., Nevins, A.M., **Meyrath, M**., Rhainds, D., D'Huys, T., Guite-Vinet, F., Dupuis, N., Gauthier, P.A., Counson, M., Kleist, A.*, et al*. Different contributions of chemokine N-terminal features attest to a different ligand binding mode and a bias towards activation of ACKR3/CXCR7 compared with CXCR4 and CXCR3. **Br J Pharmacol** *175*, 1419-1438 (2018).

Unrelated Publications

13. Schenk , C., **Meyrath, M**., Warnken, U., Schnolzer, M., Mier, W., Harak, C., and Lohmann, V. Characterization of a Threonine-Rich Cluster in Hepatitis C Virus Nonstructural Protein 5A and Its Contribution to Hyperphosphorylation**. J Virol** *92* (2018)

14. Harak, C., **Meyrath, M**., Romero-Brey, I., Schenk, C., Gondeau, C., Schult, P., Esser-Nobis, K., Saeed, M., Neddermann, P., Schnitzler, P.*, et al.*. Tuning a cellular lipid kinase activity adapts hepatitis C virus to replication in cell culture. **Nat Microbiol** *2*, 16247 (2016)

15. Bellanca, S., Summers, R.L., **Meyrath, M**., Dave, A., Nash, M.N., Dittmer, M., Sanchez, C.P., Stein, W.D., Martin, R.E., and Lanzer, M. Multiple drugs compete for transport via the Plasmodium falciparum chloroquine resistance transporter at distinct but interdependent sites. **J Biol Chem** *289*, 36336-36351 (2014)

Patents

Inventors: **Max Meyrath**, Andy Chevigné and Martyna Szpakowska: Novel Selective ACKR3 Modulators And Uses Thereof., Publication number: WO/2020/225070

International Conferences

April 2021	4th ERNEST (European Research network on Signal Transduction) Conference (Virtual Conference) **Oral Presentation:** The atypical chemokine receptor ACKR3/CXCR7 is a broad-spectrum scavenger for opioid peptides
June 2019	3rd European Chemokine and Cell Migration Conference (Salamanca, Spain) **Poster Presentation:** NanoLuX: a network-wide Nanoluciferase-based platform to monitor activation, modulation and bias of classical and atypical chemokine receptors
June 2018:	Gordon Research Seminar followed by Gordon Research Conference, Chemotactic Cytokines (Newry, ME, USA) **Oral presentation and Poster presentation**: Atypical Ligand Binding and Activation Modes of ACKR3/CXCR7
September 2017	2nd European Chemokine and Cell Migration Conference (Cardiff, Wales) No active contribution
August 2016	28th Conference of European Comparative Endocrinologists (Leuven, Belgium) No active contribution

Some might say that this was nothing more
Than another footnote on a postcard from nowhere,
Another chapter in the handbook for exercises in futility...
But let me tell you:
The struggle itself toward the heights is enough to fill a man's heart.
One must imagine Sisyphus happy...

Loosely based on Mgła– Exercises in Futility and Albert Camus - The Myth of Sisyphus