UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Structural and functional analysis of the constitutively active C-C chemokine receptor type 1 (CCR1)

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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2013

The dissertation of Christian Taylor Gilliland is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2013

# DEDICATION

The work presented herein is dedicated to my parents, Douglas and Deborah Gilliland. From the passion for science instilled within me by my father to the ethic of diligence and persistence exemplified by my mother, I have them to thank for providing the foundation my achievements were built upon.

# EPIGRAPH

The overwhelming complexity of life led me to the conclusion that biology was rather like existential philosophy: it just didn't make sense.

*Francis S. Collins, M.D., Ph.D.*

The answer is never the answer. What's really interesting is the mystery. If you seek the mystery instead of the answer, you'll always be seeking. I've never seen anybody really find the answer – they think they have, so they stop thinking. But the job is to seek mystery, evoke mystery, plant a garden in which strange plants grow and mysteries bloom. The need for mystery is greater than the need for an answer.

*Ken Kesey*

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# LIST OF ABBREVIATIONS

AC ..... adenylyl cyclase

$\beta_2$AR ..... beta 2-adrenergic receptor

βarr ..... beta-arrestin

BRET ..... bioluminescence resonance energy transfer

CAM ..... constitutive activity mutation

CCL ..... chemokine (C-C motif) ligand

CCP ..... clathrin-coated pit

CCR ..... CC-type chemokine receptor

CFP ..... cyan fluorescent protein

COPD ..... chronic obstructive pulmonary disease

CXCR ..... CXC-type chemokine receptor

ECL ..... extracellular loop

F-actin ..... filamentous actin

FRET ..... fluorescence resonance energy transfer

GFP ..... green fluorescent protein

GPCR ..... G protein-coupled receptor

GRK ..... G protein-coupled receptor kinase

GTPγS ..... guanosine 5'-O-[gamma-thio]triphosphate

ICL ..... intracellular loop

L1.2 ..... murine pre-B lymphocyte cell

M3 ..... muscarinic acetylcholine receptor 3

MC4R ..... melanocortin 4 receptor

MS ..... multiple sclerosis

PKA ..... protein kinase A

PKC ..... protein kinase C

PLC..... phospholipase C

PT ..... pertussis toxin

RA..... rheumatoid arthritis

Rluc ..... *Renilla reniformis* luciferase

THP-1 ..... human acute monocytic leukemia cell

TM ..... trans-membrane

YFP..... yellow fluorescent protein

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I would like to thank Irina Kufareva (Abagyan Laboratory, UC San Diego) for contributing the molecular model of CCR1 (**Fig. 3.5A**) as well as helping to identify the original TM3, TM6 and TM7 positions to screen for CCR1 constitutive activity.

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ABSTRACT OF THE DISSERTATION

Structural and functional analysis of the constitutively active C-C chemokine receptor type 1 (CCR1)

by

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Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2013

Professor Tracy Handel, Chair

Chemokine receptors belong to the G protein-coupled receptor (GPCR) family of proteins and are critical mediators of the directed migration of leukocytes in innate and adaptive immune responses. Understanding the

behavior of chemokine receptors under basal and agonist-stimulated conditions is essential to developing effective therapeutics for inflammatory and autoimmune diseases. For the first time, the constitutive activity of the C-C chemokine receptor type 1 (CCR1) is uncovered through ligand-independent cellular migration, constitutive phosphorylation and association with β-arrestin-2, and continual internalization followed by recycling back to the plasma membrane. Initial data suggests that CCR1 can act as a scavenging receptor to sequester chemokines intracellularly without canonical G protein signaling, thereby providing biological relevance to receptor constitutive activity. A Ser/Thr-rich cluster in the distal carboxy-terminal tail of CCR1 is identified as the major site of basal phosphorylation and fulfills a necessary, but not sufficient, role in pre-coupling to β-arrestin-2. Site-directed mutagenesis of receptor transmembrane domains and conserved DRY motif has identified residues important for stabilizing CCR1 in a constitutively active state. Activation of CCR1 primarily leads to a conformational rearrangement with β-arrestin-2, while endogenous chemokines induce this change with differential potency and efficacy. Lastly, small metal ion chelator molecules are able to activate desensitization and down-modulation of CCR1 with similar efficacy to natural ligands. Taken together, the work presented herein underlies the complexity of CCR1 function in the presence and absence of ligand and provides new avenues for therapeutic targeting.

# CHAPTER 1

## OVERVIEW OF THE DISCOVERY, FUNCTION, AND CLINICAL DEVELOPMENT OF THE C-C CHEMOKINE RECEPTOR TYPE 1 (CCR1)

### 1.1 Structure and function of chemokines

The ability of leukocytes to maintain homeostasis in the immune system and respond to physiological insults such as invasion by foreign pathogens and tissue damage is dependent upon their ability to undergo coordinated migration. Specific subsets of leukocytes must be directed to the right place at the right time for proper initiation of the inflammatory cascade leading to pathogen clearance, as well as for development of lymphoid organs (1). Absolutely necessary for this cellular movement are chemotactic cytokines, known as chemokines, and their cognate chemokine receptors expressed on the surface of immune and other related cells. Although the first chemokine was disovered in 1951 (platelet factor 4, now known as CXCL4), this protein family would not be fully appreciated for another 35 years when new members began to be discovered (2). Advances in genetic sequencing and analysis have since led to the identification of approximately 50 chemokines and 23 chemokine receptors in the human genome. First described as potent chemoattractants for immune cells and identified for their role in host defense (3), chemokines also have been shown to function in growth regulation,

hematopoiesis, embryonic development, angiogenesis, and HIV-1 infection (4).

Chemokines are relatively small (8-12 kDa) proteins characterized by a distinctive patterning of conserved cysteine (Cys) residues that are engaged in disulfide bonding. Systematically, the chemokine family can be separated into four different classes based on the number of amino acids separating the first Cys residues on the N-terminus: CC, CXC, CX3C, and XC; where the Cys residues are juxtaposed in CC chemokines, separated by one or three amino acids in CXC and CX3C, respectively, or where there is only one Cys residue present (XC) (5). Virtually all are secreted from the cell following synthesis except for two chemokines (CX3CL1 and CXCL16) that can remain tethered to the plasma membrane via a mucin-like stalk (6). Chemokines can also be divided along functional lines between those with primarily inflammatory or homeostatic properties (7). Inflammatory chemokines play pivotal roles in controlling leukocyte recruitment during innate immune responses and are responsible for recruiting macrophages and neutrophils to sites of tissue insult or injury. Homeostatic chemokines are constitutively expressed by specific cell types for mediating chemotaxis of lymphocytes and dendritic cells in the development of acquired immunity and for defining the cellular organization of the immune system. However, the division between inflammatory and homeostatic is somewhat fluid as certain chemokines can carry out functions of both classes depending upon the biological context (8).

Even though there is highly variable levels of sequence conservation amongst this class of proteins (ranging from 20-90%), chemokines share a remarkably similar tertiary structure (1). Each chemokine possesses a disordered and flexible N-terminus of 6-10 amino acids followed by a long loop (known as the N loop), a $3_{10}$ helix, a three-stranded beta sheet, and, finally, a C-terminal alpha helix. The N-terminus plays a critical role in receptor activation as evidenced by N-terminal truncation that can render chemokines inactive or even convert them into antagonists (9). The chemokine system can be regulated in multiple ways including at the level of gene expression following stimulation with other cytokines, differential patterning of receptor expression on leukocyte subpopulations, proteolytic processing of chemokines that can alter receptor affinity and function, and dual agonist/antagonist behavior of a single chemokine on multiple receptors (10). Currently, a two-step model is proposed for chemokine binding to and activation of chemokine receptors (11, 12). The chemokine core domain binds to the receptor N-terminus and extracellular loops as an initial docking reaction that primarily determines ligand affinity. This initial binding step is dominated by ionic interactions between positively charged residues in the chemokine ligand and negatively charged amino acids in the chemokine receptor. This action then orients the chemokine N-terminal signaling domain into the receptor helical bundle and triggers the requisite conformational change for receptor activation. The two structural regions of the receptor are sequentially involved

in determining ligand affinity and receptor activation and are referred to, respectively, as chemokine recognition site 1 and 2 (CRS1 and CRS2) (12).

### 1.2 Role of chemokines in disease

As mediators of the homeostatic circulation of leukocytes as well as their movement to sites of inflammation and injury, chemokines have been implicated in the pathophysiology of a number of infectious and inflammatory diseases (8). Excessive chemokine-mediated inflammatory responses and continual leukocyte migration leading to tissue damage are hallmarks of multiple diseases including acute inflammation, autoimmunity, organ transplantation rejection, allergic inflammation, cancer growth and metastasis, and infectious disease. The chemokine and chemokine receptor axis is perhaps most well studied for its involvement in autoimmune diseases including rheumatoid arthritis (RA), atherosclerosis, psoriasis, and multiple sclerosis (MS). While the majority of pharmaceutical company efforts to target the chemokine system have focused on developing small molecule inhibitors of chemokine receptors, strategies targeting the chemokines themselves have also been developed. Neutralizing monoclonal chemokine antibodies and structural modification of chemokines for conversion to antagonists have been successful approaches used in the treatment of multiple animal models of inflammatory disease, viral infection, and tissue allograft rejection (8). However, these strategies remain to be clinically verified as the only

therapeutics currently available to target the chemokine system are receptor antagonists.

### 1.3 G protein-coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs) represent the largest protein superfamily in the human genome with over 800 members and mediate the cellular responses to a vast array of extracellular signals ranging from photons and ions to small molecules and peptides. GPCRs are expressed on nearly every cell type and regulate a wide variety of physiological processes including vision and smell, neurotransmission, and thousands of endocrine, autocrine and paracrine functions throughout the body. Because of their role in initiating signal transduction from extracellular signals in nearly all aspects of human biology, GPCRs are the targets of 30-50% of all prescription drugs including those that are targeted for cardiac malfunction, asthma, migraines, and HIV infection (13, 14). GPCRs, also referred to as seven transmembrane (7TM) or serpentine receptors, are structurally characterized by a series of seven membrane-spanning alpha helices that weave through the plasma membrane with an extracellular N-terminus, an intracellular C-terminal tail, and 3 intra-/extra-cellular loops connecting each adjacent pair of alpha helices (15). While the nearly 700 receptors that make up the class A/rhodopsin family of GPCRs typically have greater than 25% sequence identity, considerable variability can exist in their N-terminal domains, extracellular loops, and tips of

transmembrane helices that confers ligand specificity and differential activation (16, 17).

While structural coverage of the GPCR superfamily is expanding at an increasing rate, there still is relatively little known about the precise molecular mechanisms that underlie receptor activation. It is generally accepted that GPCRs exist in a dynamic equilibrium between the inactive and active conformational states with a distinct subset for signaling states in complex with heterotrimeric G protein. The occupancy of a conformational state by a GPCR can vary dramatically depending upon the molecular environment surrounding the receptor, with considerable structural plasticity in the absence of ligand binding that accounts for different levels of basal activities (18). Pharmacological manipulation of GPCRs can shift the equilibrium toward the inactive states in the case of inverse agonists, or shift it toward the active states in the case of agonists, while neutral antagonists merely affect ligand binding without changing the conformational equilibrium in either direction. Activation of a GPCR generally involves a series of significant rearrangements of alpha helices and conserved “micro-switches” of amino acid side chains in the transmembrane region to create a substantial conformational change in the intracellular surface of the receptor exposed to G proteins and other signaling partners (19). Many questions remain, however, including how different ligands are able to stabilize unique conformational states within a receptor and how those correlate with intracellular signaling (*i.e.* what are the

mechanical aspects that form the basis of biased signaling?) and how GPCRs are able to couple to different intracellular effector molecules?

### 1.4 Chemokine receptor structure

Chemokine receptors belong to the class A/rhodopsin-like class of GCPRs and are divided into 4 groups (CCR, CXCR, CX3CR, and XCR) based upon the Cys patterning in the chemokines that serve as their primary ligands. While more than 75 high resolution crystal structures exist for 19 different class A GPCRs (19), only two receptors belong to the chemokine family (CXCR4 and CCR5) (20). The CXCR4 structures represented the first of a peptide-binding GPCR and displayed some notable differences with other receptors whose structures had been solved previously. First, the CXCR4 structure contained a β-hairpin in extracellular loop 2 (ECL2), a region that is critical for the function of CXCR4 as a co-receptor for human immunodeficiency virus (HIV) entry and for chemokine binding (21, 22). Second, it was confirmed that, in addition to the conserved GPCR disulfide bridge between transmembrane helix 3 (TM3) and ECL2, chemokine receptors possess an additional disulfide between the N-terminus and TM7 that constrains the N-terminal region and shapes the entrance to the ligand binding pocket (20, 23). Third, all reported structures of CXCR4 did not possess a putative eighth alpha helix at the onset of the C-terminal region thought to be conserved amongst class A GPCRs. Fourth, and most likely owing to the relatively large size of its chemokine ligand, the ligand-binding pocket of

CXCR4 was significantly larger, more open, and closer to the extracellular surface than previous GPCRs. Lastly, all five crystal structures of CXCR4 bound to either a small molecule antagonist (IT1t) or a cyclic peptide (CVX15) displayed a similar, parallel and symmetric protein dimer that mainly involved TM5 and TM6, compared to TM1 and helix VIII in the β2-adrenergic and κ-opioid receptors (24, 25). Recently, the NMR structure of CXCR1 in phospholipid bilayers has been determined adding to the structural knowledge of this important GPCR subfamily (26); however, much more work needs to be done in order to unravel the structural complexity and molecular underpinnings of their function.

**1.5 Chemokine receptor signaling**

In general, activation of chemokine receptors leads to intracellular signaling through the Gi heterotrimeric G protein complex exemplified by the ability of pertussis toxin to inhibit most chemokine-induced cellular responses (27), although examples exist of coupling to other G protein classes or of an inability to signal through G proteins at all (28, 29). While there is little consensus over canonical signaling pathways, activation of chemokine receptors often results in cellular chemotaxis, adhesion, proliferation or regulation of gene expression (30). Chemotaxis itself is the end result of the integration of multiple signaling pathways in a leukocyte beginning with Gβγ activation and resulting in actin polymerization and cytoskeletal remodeling, cell polarization, increase in integrin binding affinity, firm cellular adhesion to

the endothelium, and transmigration (8, 31, 32). Though it is thought to be independent of cellular migration, a majority of chemokine receptors can also induce calcium flux either by mobilization from intracellular stores or by influx from the extracellular medium (30). This increase in cellular free calcium levels typically occurs via direct activation of phospholipase C by Gβγ subunits causing an increase in inositol trisphosphate, activation of calcium channels, and release of $Ca^{2+}$ from the endoplasmic reticulum. Other downstream signaling events that can follow chemokine receptor activation include inhibition of adenylyl cyclase and reduction in cAMP levels, activation of phosphoinositide 3-kinase (PI3K), mitogen/extracellular signal-related kinase (MEK1) and/or extracellular signal-related kinase (ERK1/2), stimulation of tyrosine phosphorylation of components of the focal adhesion complex, and increases in nuclear factor-κB (NF-κB) and signal transducer and activator of transcription (STAT) 1 and 3 transcriptional activity (31).

**1.6 Discovery of CCR1**

Of the ten human CC-type chemokine receptors - those whose ligands are CC chemokines - the first to be discovered was CCR1 in 1993. Two research groups, the laboratories of Dr. Thomas Schall at Genentech and Dr. Phillip Murphy at the National Institute of Allergy and Infectious Diseases (NIAID), independently and simultaneously cloned and functionally characterized what was initially referred to as the C-C chemokine receptor 1 (C-C CKR-1) (33) or the MIP-1α/RANTES receptor (34). The Schall group,

who published their findings a few months before the Murphy group, utilized a degenerate RT-PCR approach with primers based upon conserved transmembrane domain sequences of the only other known chemoattractant receptors at the time, namely the interleukin-8 receptor A and B (IL-8rA/B), the complement component 5a receptor (C5aR), and formyl peptide (fMLP) receptor, all of which are GPCRs (33). cDNA substrates were isolated from different hematopoietic cell types and lines known to respond to CC chemokines, including primary peripheral blood mononuclear cells (PBMC), U937 lymphoma cells, HL60 promyelocytic leukemia cells, and THP-1 acute monocytic leukemia cells. Previous experiments had demonstrated the signaling responses of U937 cells following stimulation with MIP-1α/CCL3 or RANTES/CCL5 to be pertussis toxin-sensitive, further suggesting that the receptor for these chemokines was indeed a GPCR. Eventually, a nucleotide sequence with an open reading frame of 1,065 bases encoding a protein of 355 amino acids and a predicted molecular weight of 41 kDa was isolated. This gene exhibited relatively high sequence identity to the previously cloned chemokine receptors IL-8rA and IL-8rB (32%, now known as CXCR1 and CXCR2, respectively) and to the newly sequenced but orphan HUMSTR clone (31%, now CXCR4). Transfection of the isolated gene product into HEK293 cells demonstrated that C-C CKR-1 was indeed a functional receptor for MIP-1α/CCL3 and RANTES/CCL5, but not MCP-1/CCL2 nor MIP-1β/CCL4, as measured by calcium mobilization. The Murphy group confirmed this chemokine activity profile following stimulation of *Xenopus* oocytes that had

been injected with the putative receptor gene and further localized the “MIP-1α/RANTES receptor” sequence to the short arm of human chromosome 3 (3p21) (34).

In the few years that followed, the CC branch of the chemokine receptor family tree quickly began to be filled in. The second CC-type receptor (MCP-1 receptor, now CCR2) was identified in 1994 and displayed 51% sequence identify to CCR1 (35); however, this receptor was not activated by either of the CCR1 agonists CCL3 or CCL5. The discovery of CCR2 was quickly followed in 1995 by an eosinophil-selective chemokine receptor (CCR3, (36)) and another expressed highly on basophils (CCR4, (37)). ChemR15 (now CCR5) was subsequently cloned in 1996 and was demonstrated, along with CCR1, to be a receptor for both CCL3 and CCL5 (38). However, CCL4, which has since been described as an endogenous antagonist of CCR1 (39), displayed agonistic behavior on CCR5. CCR1 and CCR2 were shown to be expressed jointly on monocytes and exhibited similar signaling responses following stimulation with MCP-3/CCL7 (40). Another study set out to determine the structural domains of CCR1 and CCR2 involved in determining the specificity of chemokine binding (41). Through the systematic creation of chimeric receptors in which the N-terminus or each extracellular loop was swapped, it was demonstrated that CCL2, a potent CCR2 agonist with no efficacy on CCR1, and CCL3, a potent CCR1 agonist with no efficacy on CCR2, had divergent modes of receptor binding and activation, thereby possibly explaining their differential action on two highly homologous chemokine

receptors. By the time a review of chemokines was published in the summer of 1997, the branches of this receptor subfamily were nearly full grown with three more subfamily members added for a total of eight CCR's (only CCR9 and CCR10 remained) (42). These initial studies began to illuminate the complexity of the chemokine:chemokine receptor system wherein multiple chemokines bind to and activate the same receptor, multiple receptors are activated by the same chemokine, and a single chemokine can display a dual identity of an agonist and an antagonist. One of the most promiscuous chemokine receptors, CCR1 is now known to be activated by at least 10 endogenous chemokines: CCL3, CCL3L1, CCL5, CCL7, CCL8, CCL13-16, and CCL23 (**Table 1.1**) (7); however, the precise downstream signaling cascade initiated by each chemokine ligand through CCR1 remains to be determined. Interestingly, a series of bipyridine and phenanthroline metal ion chelator compounds in complex with either $Zn^{2+}$ or $Cu^{2+}$ have also been shown to activate CCR1, albeit at significantly higher concentration levels than the nanomolar affinity chemokines (43).

**Table 1.1. CCR1 endogenous chemokines and their relevance to disease.**

| Systematic Name | Old Name | Disease Implications |
|---|---|---|
| CCL3 | MIP-1α, LD78 | RA, MS, chronic hepatitis, transplant rejection, HIV infection, asthma, inflammatory bowel disease, bacterial meningitis, neuropathic pain |
| CCL3L1 | MIP-1αP, LD78β | glioblastoma, RA, HIV infection |
| CCL5 | RANTES | Atherosclerosis, MS, RA, transplant rejection, HIV infection, asthma, diabetes, obesity, glomerulonephritis |
| CCL7 | MCP-3 | RA, MS, allergy, cancer malignancy |
| CCL8 | MCP-2 | RA, MS, allergy, bacterial meningitis |
| CCL13 | MCP-4 | Atopic dermatitis, allergy, asthma, atherosclerosis |
| CCL14 | HCC-1 | RA, lupus, inflammatory bowel disease |
| CCL15 | HCC-2, Lkn-1, MIP-1δ | hepatic and colon cancer, Alzheimer's disease, pulmonary sarcoidosis, tuberculosis |
| CCL16 | HCC-4, LEC | RA, irritable bowel syndrome, pneumonia, preterm birth |
| CCL23 | MPIF-1, CKβ8 | RA, rhinosinuitis, atherosclerosis |

## 1.7 CCR1 expression and function

Expression of CCR1 was first identified in mature and immature myeloid cell types as well as B cells (34), and has since expanded to both hematopoietic and non-hematopoietic cells. The first use of a clonal antibody against CCR1 to detect cell surface expression was carried out in 1996 and identified positive staining of peripheral blood lymphocytes and monocytes but not neutrophils (44). CCR1 is highly expressed and serves as a key mediator of neutrophil migration in mouse (45); however, its expression in human neutrophils has been more controversial with some evidence suggesting it is dependent upon granulocyte-macrophage colony-stimulating factor (GM-CSF)

stimulation (46). Other leukocytic cell types shown to express CCR1 include memory T cells, basophils, dendritic cells, and $CD34^+$ hematopoietic stem cells (47-49). CCR1 expression has been demonstrated in a wide variety of non-hematopoietic cells, for example: airway smooth muscle cells in the lung suggesting a possible role in asthma (50), normal and dystrophic neurons from patients with Alzheimer's dimensia (51, 52), astrocytes implicating CCR1 in central nervous system inflammation (53), endothelial cells that are chemotaxis-competent in response to CCL23 stimulation suggesting a role in angiogenesis (54), and in vascular smooth muscle cells (55). Additionally, CCR1 is the predominant chemokine receptor on the surface of osteoclasts (56) and has been implicated in the progression of bone cancer following confirmation of CCR1 expression on multiple myeloma cells (57, 58).

The cellular functions associated with CCR1 are as varied as the chemokines it recognizes and the cells it is expressed by. At the simplest level, CCR1 plays an important role in host defense through regulation of leukocyte chemotaxis. More specifically, activation of CCR1 has been shown to result in up-regulation of integrins to promote leukocyte firm adherence to the endothelium during migration (59), enhancement of T cell activation (60), regulation of T helper cell 1 and 2 (Th1 and Th2) polarization (61, 62), and stimulation of macrophage function (63) and secretion of matrix metalloproteinases (64, 65).

The function of CCR1 has been further elucidated by assessing the loss of CCR1 expression on murine physiology through targeted gene knockout

(**Table 1.2**). Mice lacking CCR1 exhibited no developmental abnormalities and no histologic differences in lymphoid organs or mature peripheral blood counts under sterile conditions, suggesting that CCR1 is not essential in normal mouse development in a steady-state environment (61). However, a loss of function in hematopoiesis was identified as spleens from $CCR1^{-/-}$ mice challenged with lipopolysaccharide (LPS) contained fewer granulocyte-macrophage and multipotential progenitor cells than wild-type littermates (61). $CCR1^{-/-}$ mice in this study also displayed increased susceptibility to fungal infection with *Aspercillus fumigatus* and reduced granuloma formation following *Schistosoma mansoni* egg injection, suggesting a role for CCR1 in migration of neutrophils and eosinophils as well as regulation of type 1/type 2 cytokine balance. A similar role for CCR1 was observed following pulmonary *A. fumigatus* (66) and cutaneous *Leishmania major* infection (67). While CCR1 did not appear to be necessary for polymorphonuclear recruitment into the asthmatic airways nor play a role in airway hyperresponsiveness in the prior study, the absence of this receptor did result in a reduction in airway remodeling thought to occur through decreased Th2 cytokine and chemokine levels. In response to *L. major* infection in the latter study, $CCR1^{-/-}$ mice exhibited similar levels of leuckocyte infiltration at the site of infection but developed significantly smaller lesions with fewer parasites correlating with lower IL-4 and IL-10 levels (Th2-associated cytokines). In variation to the pulmonary *A. fumigatis* study, airway hyperresponsiveness and mucus production were significantly reduced in receptor knockout mice following

infection with respiratory syncytial virus (RSV) (68). Another study assessed the impact of CCR1 knockout in a mouse model of nephrotoxic nephritis and found that loss of CCR1 actually aided in disease progression and increased renal injury (69). $CCR1^{-/-}$ mice in this model displayed normal neutrophil accumulation; however, they also displayed an excessive Th1-mediated immune response and increased migration of T cells and macrophages into the kidney that was correlated with the development of more severe glomerulonephritis. Alternatively, in an acute enteritis model induced by *Clostridium difficile* toxin injection, CCR1 knockout resulted in far fewer levels of neutrophil infiltration, ileal fluid accumulation, and epithelial damage (70). Further evidence suggesting inhibition of CCR1 could be clinically advantageous was provided by a study of four models of acute and chronic cardiac allograft rejection (71). In each case, $CCR1^{-/-}$ mice showed significant prolongation of allograft survival compared to wild-type controls. Lastly, the role of CCR1 in bone formation was demonstrated by fewer trabecular bones, lower mineral bone density, reduced osteoblast differentiation, and lowered osteoclastogenesis in mice lacking the receptor (72). In short, the function of CCR1 has been shown to be highly variable and dependent upon the molecular context and disease state the receptor finds itself in.

**Table 1.2. Effect of CCR1 gene deletion in mouse models of disease.** Adapted from (30).

| Mouse Strain | *In vivo* Model | Effect of CCR1 Deletion | Reference |
|---|---|---|---|
| C57Bl/6 | Analysis of bone marrow and spleen myeloid progenitor cell development | Defective migration of progenitors to spleen, reduced cell cycling | 61 |
| C57Bl/6 | *A. fumigatus* intravenous infection | Reduced survival, accelerated mortality | 61 |
| C57Bl/6 | Granuloma formation following *S. mansoni* egg injection | Reduced granuloma formation, excessive Th1 response | 61 |
| Sv129 x C57Bl/6 | Nephrotoxic nephritis | Increased renal injury, excessive Th1 response | 69 |
| Sv129 x C57Bl/6 | *Leishmania* infection | Less Th2 cytokine production, failure of parasite clearance | 67 |
| Balb/c (10 gen) | Pulmonary *A. fumigatus* infection | Reduced airway remodeling | 66 |
| Sv129 x C57Bl/6 | Respiratory syncytial virus (RSV) infection | Reduced pathologic response and tissue damage | 68 |
| Sv129 x C57Bl/6 | *Clostridium difficile* toxin injection | Protective against enteritis | 70 |
| Sv129 x C57Bl/6 | Cardiac allograft | Reduced graft rejection | 71 |
| C57Bl/6 | Analysis of bone formation | Fewer trabecular bones, impaired osteoblast differentiation, defective osteoclastogenesis | 72 |

Upstream of these cellular events, CCR1 is thought to predominantly activate Gi/o-coupled G protein pathways to regulate calcium mobilization and inhibit adenylyl cyclase (73). Activation of CCR1 in monocytes by CCL23 leads to phospholipase C (PLC) and phospholipase A2 (PLA2) activation, subsequent release of intracellular calcium and arachidonic acid, and F-actin polymerization (74). Induction of ERK1/2, Janus kinase (JNK), and p38 MAPK phosphorylation in stimulated HEK293 cells transfected with CCR1 was shown to be pertussis toxin (PT)-sensitive (75, 76). CCR1 is not limited to Gi/o pathways, however, as an early study demonstrated in a heterologous expression system that the receptor is able to signal through $G\alpha_{14}$ to activate $PLC\beta_2$ (28). Additionally, multiple CCR1 chemokine agonists induce phosphorylation of nuclear factor-κB (NF-κB) in THP-1 cells independently of pertussis toxin (PT) treatment (76). In transfected cells, CCR1 was linked to activation of several downstream pathways including protein kinase C (PKC), $Ca^{2+}$/calmodulin dependent protein kinase II, Raf-I, MAP/ERK kinase (MEK1/2) and c-Src (77).

### 1.8 Role of CCR1 in disease

Upregulation of ligand/receptor expression and disregulation of the normal physiological function of CCR1 has been implicated in the progression of multiple inflammatory and autoimmune diseases (78, 79). With an annual prevalence of roughly 800 per 100,000 people in North America, rheumatoid arthritis (RA) is one of the most prevalent maladies that CCR1 has been

associated with (80). RA is a chronic inflammatory autoimmune condition characterized by pain, swelling, stiffness and loss of function in joints, particularly those in the wrist and fingers. The pathology and severity of disease is tied to the infiltration of leukocytes into the inflamed synovial tissue (81) to the extent that the number of monocytes migrating into the synovium can be a strong indicator of severity of joint pain (82). The link between monocytes and RA, coupled with the important role that CCR1 plays in monocyte migration, clearly implicated CCR1 as a potential target for the amelioration of this disease. Indeed, CCR1 ligands CCL3 and CCL5 are elevated in the synovial fluid and tissues of patients with RA (83-85), and the expression of CCR1 on infiltrating leukocytes in diseased synovial tissue has been confirmed by immunohistochemistry (86). The initial work carried out to develop small molecule inhibitors of CCR1 for treatment of RA and other receptor-related diseases will be discussed in a later section; however, the use of a neutralizing antibody against CCR1 was able to inhibit primary human monocyte chemotaxis in response to synovial fluid from RA patients (87).

Multiple sclerosis (MS) is an autoimmune disorder of the central nervous system (CNS) characterized by neuroinflammation, demyelination of nerve fibers, and axonal cell death (88). While the etiology of MS remains unclear, disease onset is thought to begin with infiltration of the CNS by autoreactive T cells and monocytes, and inhibition of this migration process could be beneficial in slowing disease progression and relapse (89). The role of CCR1 in MS pathogenesis has been demonstrated by its expression in

demyelinating lesions (90), through the effect of CCR1 knockout or inhibition in the experimental autoimmune encephalomyelitis (EAE) animal model of the disease (91, 92), and genetic association studies (93). Another CNS pathology that CCR1 has been implicated in is Alzheimer's Disease (AD), which is the most commonly diagnosed form of dementia and is characterized by neuronal cell death in cortical and subcortical regions, β-amyloid (Aβ) peptide plaque deposits, and neurofibrillary tangles in the brain (94). Although the current relevance of CCR1 to AD disease pathology is debatable, one study reported the specific expression of CCR1 on neurons in lesions associated with myeloid plaques of AD patients (52). CCR1 was observed early in disease progression and could be correlated with AD severity; however, this has been the only study yet to demonstrate that CCR1 may be a relevant target. A more recent study set out to test whether CCR1 expression could actually serve as a diagnostic marker for identification of AD lesions (95). The study found that fluorine-18 labeling of the CCR1 antagonist ZK811460 did not show any enhanced binding in brains of AD vs. non-AD patients as measured by PET imaging.

Tumor invasion and metastasis share similarities in cellular mechanisms of action with leukocyte trafficking. Given the role that CCR1 plays in mediating leukocyte recruitment to sites of inflammation, it has been suggested that this receptor could also play a role in the homing of primary tumor cells to metastatic sites throughout the body. A study of human colorectal cancer demonstrated a significant upregulation of CCL15

expression through loss of transcriptional repression that led to a significant increase in the number of $CCR1^+$ myeloid cells in tumor metastases (96). Additionally, a direct correlation was observed between a higher proportion of CCR1-expressing cells in liver metastases and a reduction in disease-free survival rates suggesting that inhibition of CCR1 could be useful in the treatment of metastatic colorectal cancer. Other studies have established a link between CCR1 expression and increased invasion of taxane-resistant prostate cancer cells (97), promotion of tumor growth and metastasis in the liver (98), and an aggressively invasive phenotype of non-small cell lung cancer (NSCLC) cells that could be suppressed through CCR1 knockdown (99). CCR1 function is important for osteoclastogenesis (72), has been found on the surface of multiple myeloma cells (57), and targeted knockdown or inhibition of one of its ligands (CCL3) has been shown to directly reduce bone lesions and tumor burden in mouse models of bone cancer (100). Taken together, these and other reports clearly implicate CCR1 in multiple myeloma. As proof of principle, a small molecule inhibitor of CCR1 impairs osteoclast formation and function and reduces physical interactions between muliple myeloma cells and osteoclasts that leads to a significant decrease in multiple myeloma cell proliferation and survival (101).

The importance of CCR1 in host rejection of organ transplantation has also been well characterized. A common indication of acute cellular rejection of tissue allografts is the infiltration of mononuclear cells, comprised mainly of T cells and macrophages that express chemokine receptors, including CCR1,

into the interstitium (10). While the advantageous effect of CCR1 knockdown on rat cardiac transplantation models was discussed above (71), this effect has been replicated in another rat heart and rabbit kidney models of transplant rejection using pharmacological inhibition of the receptor (102, 103). In both of these cardiac and renal analyses, treatment of the animals with a CCR1 small-molecule inhibitor resulted in significant increases in organ survival and decreases in acute inflammation surrounding the surgical ligation site. Other diseases where a role for CCR1 in etiology or progression has been demonstrated include allergic inflammation (66, 104-106), progressive kidney disease (107), myocarditis (108), endometriosis (109), fibrosis (110), invasive candidiasis (111), oral lichen planus (112), and Chagas disease (113). Recently, a genome association study identified a single nucleotide polymorphism in a potential regulator region of the *Ccr1* gene that correlated with increased risk of Behcet's disease, a complex form of systemic vasculitis characterized by recurrent inflammatory attacks throughout the body (114). Enough evidence existed to propel multiple biotechnology and pharmaceutical companies to include CCR1 in their drug discovery pipelines for inflammatory and autoimmune diseases, the early stages of which are discussed in the next section.

### 1.9 Initial CCR1 drug discovery and development

CCR1 was the first chemokine receptor to have a small molecule inhibitor developed against it and, subsequently, the first to be targeted in a

clinical trial. The discovery and development of CCR1 antagonists began with scientists at Berlex BioSciences who set out in the mid-/late-1990's to identify non-peptide compounds that would effectively block the function of this receptor. Peptide-based inhibitors had been previously developed for chemokine receptors; however, they suffered from poor metabolic stability and oral bioavailability (115, 116). Berlex researchers were motivated by previous reports establishing a strong connection between multiple CCR1 chemokine ligands and disease progression in multiple sclerosis (MS) and rheumatoid arthritis (RA); however, a direct causal link to CCR1 differentiating it from other receptors like CCR5 or CCR3 that are activated by many of the same chemokines had not yet been established. At the time, it was shown that significant levels of CCL3 and CCL5, as well as other CC chemokines, could be found in the spinal cords of EAE mice, a murine model of MS (117). Neutralizing antibodies against CCL3, but not CCL2 nor CXCL8, were successful in preventing the development of both acute and relapsing paralytic MS disease as well as leukocyte infiltration into the CNS in EAE mice (118). CCL5 had been shown to be upregulated at both the protein and mRNA levels in cultured synovial fibroblasts that had been isolated from RA patients and then stimulated with TNF-α and IL-1β, suggesting that CCL5 secretion may contribute to chronic inflammation in RA (119). Additionally, high levels of CCL5 were identified in primary human synovial tissue samples taken from rheumatic patients but not in samples from osteoarthritic patients (120). With the clinical relevance of CCR1-binding chemokines in RA and MS established,

researchers at Berlex began to screen their compound libraries for antagonists that could displace radiolabeled CCL3 binding to CCR1 (121).

Through the high-throughput screening ligand displacement assay, a class of 4-hydroxypiperidine analogs was discovered to inhibit CCL3 and, to a lesser extent, CCL5 binding (121). Pharmacological characterization of the most potent member of this series, referred to as compound 1, demonstrated its ability to inhibit intracellular calcium mobilization in transfected HEK293 cells expressing CCR1 as well as reduce the chemotaxis of primary human peripheral blood mononuclear cells (PBMCs) in response to CCL3 or CCL5, but had no effect in either assay on cellular responses to CCL2 or CXCL8. While compound 1 did not bind with high affinity to any other chemokine receptors tested, it did display some inhibitory effect on multiple biogenic amine neurotransmitter receptors due to the structural similarities of the 4-hydroxypiperidine scaffold to typical neuroleptics/anti-depressants. Continued structural-activity relationship analysis of the scaffold led to the development of another series of highly potent CCR1 antagonists with $K_i$ values in the tens of nanomolar exemplified by BX 510 (21 nM) and BX 513 (40 nM) (122). However, these drug candidates for CCR1 still suffered from cross-reactivity with other GPCRs, particularly the aforementioned neurotransmitter receptors, which precluded any further development and optimization. An additional complication that surfaced was species cross-reactivity of these initial compounds; for ease of pre-clinical testing the inhibitors would need to be effective on the rodent homolog of CCR1. The original compound 1 exhibited

poor affinity for mouse CCR1 at concentrations up to 10 μM, thereby significantly limiting the options for CCR1-related disease animal models; however, not all doors to pre-clinical testing were closed as it was a potent and efficacious inhibitor of rabbit and marmoset CCR1 (123). Ultimately, Berlex suspended development of the 4-hydroxypiperidine series and pursued an entirely different class of CCR1 antagonists that did not have the issues of poor species and high GPCR cross-reactivity.

Continued screening led to the discovery of BX 471, a novel, potent and selective functional antagonist of CCR1 (92). BX 471 was able to displace bound CCR1 ligands with high affinity ranging from 1-5.5 nM, and inhibit a series of CCR1 functional responses *in vitro* including calcium mobilization, CD11b expression, and leukocyte migration. Importantly, this compound displayed >10,000-fold binding selectivity for CCR1 compared to 28 other GPCRs and inhibited chemotaxis of human lymphocytes and monocytes in response to CCL3 and CCL5, but not CCL2, CCL4, CXCL4, or CXCL8. Even though BX 471 was approximately 100-fold lower in its affinity for rat CCR1 than human CCR1, it was sufficient enough to effectively reduce the clinical score in a rat EAE model by 50%. This represented the first *in vivo* demonstration of the efficacy of pharmacologically inhibiting CCR1 for the amelioration of disease. These data encouraged Berlex scientists to then test the phamacokinetic properties of BX 471 in dogs and found it had an oral bioavailability of 60% with a half-life of 3 h.

### 1.10 CCR1 inhibitors in clinical trials

Over the next decade, scientists at Berlex and others around the world raced to test whether BX 471 was efficacious in multiple pre-clinical tests of diseases where CCR1 was implicated ranging from MS to organ transplant and from RA to pancreatitis. In the initial report of BX 471 the compound was shown to induce a dose-dependent downward trend in the clinical severity of EAE-associated disease in rats; however, only the highest dose of 50 mg/kg reached statistical significance (92). In the unilateral ureter obstruction (UUO) mouse model of progressive renal fibrosis, BX 471 treatment led to a 40-60% reduction in interstitial macrophage and lymphocyte infiltrate and substantial reduction in renal fibrosis indicating BX 471 could be useful in preventing end-stage renal failure (124). In mouse models of sepsis and acute pancreatitis BX 471 significantly protected mice against further organ injury by attenuating inflammatory leukocyte recruitment (125, 126). Renal ischemia reperfusion injury induces rapid leukocyte recruitment into the kidney; however, in a mouse model, BX 471 led to a reduction in the amount of neutrophil and macrophage recruitment to the site of injury (127). Lastly, this widely-studied inhibitor displayed efficacy in reducing osteolytic lesions in multiple myeloma (128) and extending rat and rabbit organ tissue allografts (92, 102).

In early 2004, BX 471 became the first chemokine receptor antagonist to enter into Phase II clinical trials. The compound had displayed favorable Phase I results and was well tolerated in healthy volunteers with no serious safety concerns (129). In a 16 week randomized placebo-controlled trial of BX

471 in patients with relapsing-remitting MS the drug failed to show a reduction in the cumulative number of newly active inflammatory CNS lesions as assessed by serial magnetic resonance imaging (MRI) of the brain (129). This result suggested that CCR1 does not contribute to the initial leukocyte infiltration in relapsing-remitting MS. A second Phase II clinical trial in patients with relapsing-remitting MS was initiated a few years later and found no effect of BX 471 in reduction of intracellular adhesion molecule (ICAM) expression on the surface of PBMCs, which is expressed by the majority of infiltrating lymphocytes in MS lesions (130). Development of BX 471 was subsequently discontinued.

The clinical failure of BX 471 did not prevent multiple other companies from advancing their lead CCR1 inhibitors into human trials (**Table 1.3**) (78). Pfizer entered into Phase II trials in 2004 with its compound CP-481,715 for treatment of RA (131). Six weeks into the trial there was no demonstration of any efficacy as measured by the proportion of patients who exhibited a 20% improvement in 3 out of 5 core set measures established by the American College of Rheumatology and the effort was stopped. Chemocentryx also took its compound CCX354 into Phase II trials for RA (132). In the CCR1 Antagonist Rheumatoid Arthritis Trial 2 (CARAT-2) the compound displayed a favorable safety profile even at high doses and showed significant signs of clinical utility by modulating synovial inflammation (133). GlaxoSmithKline now has option rights from a license agreement with Chemocentryx to advance CCX354 into a Phase IIb trial. ML3897 passed Phase I trials for the treatment

of RA, MS and psoriasis but further development was terminated in Phase II trials by Milennium Pharmaceuticals when the compound failed to reach its clinical endpoint for RA (134). For treatment of moderate to severe chronic obstructive pulmonary disease (COPD), an inhaled version of AZD4818 was developed by Astra Zeneca and taken into Phase II trials (135). While well tolerated by patients, AZD4818 displayed no effect on lung function and functional capacity. Merck initiated a Phase II trial with C-6448 and C-4462 for MS and RA, respectively, but no reports have been published suggesting their lack of success. Similarly, ZK 811752 was tested by Schering AG for an effect on alleviation of endometriosis-associated pelvic pain but no clinical data is publicly available to assess the trial's outcome.

**Table 1.3. Summary of clinical trials of compounds targeting CCR1**.
N.A. published data not available; *Information accessed from www.clinicaltrials.gov, Identifier NCT00185341. Adapted and modified from (30).

| Drug | Company | Indication | Highest Clinical Phase | Current Status | References |
|---|---|---|---|---|---|
| BX 471 | Berlex/Schering | Multiple sclerosis | Phase II | Failed | 129,130 |
| AZD4818 | AstraZeneca | Chronic obstructive pulmonary disease | Phase II | Failed | 135 |
| MLN3897 | Millennium | Rheumatoid arthritis | Phase II | Failed | 134 |
| CP481,715 | Pfizer | Rheumatoid arthritis | Phase II | Failed | 131 |
| ZK811752 | Bayer Schering | Endometriosis associated pelvic pain | Phase II | Unknown | N.A.* |
| C-4462 | Merck | rheumatoid arthritis | Phase II | Unknown | N.A. |
| C-6448 | Merck | multiple sclerosis | Phase II | Unknown | N.A. |
| CCX354 | Chemocentryx and GSK | Rheumatoid arthritis | Phase II | Successful | 132,133 |
| BB10010 | British Biotech | myeloprotection during chemotherapy | Phase II | Failed | 137,139,140 |

In a departure from the overwhelming trend of searching for small molecule antagonists of CCR1 function for clinical use, one example exists where development of a CCR1 biologic agonist was sought. BB10010, first described in 1995 (136), is a recombinant, active, and non-aggregating mutant of CCL3. Native CCL3 tends to undergo aggregation and form high molecular weight polymers; therefore, a single mutation was introduced (Asp26Ala) to create a more stable and non-oligomerizing mutant. Early studies of BB10010 demonstrated its ability to act as a hematopoietic stem cell proliferation inhibitor and, therefore, could be used as a novel approach for protecting the quality of the stem cell population and its capacity to regenerate after cytotoxic chemotherapy (137). It is well known that each of the steps of hematopoietic stem and progenitor cell (HSPC) mobilization is controlled in part by chemokine-chemokine receptor networks via their stimulation, enhancement, or suppression of the proliferation, differentiation, mobilization and migration of these unique types of cells (138). Specifically, CCL3 has been shown both *in vitro* and *in vivo* to suppress the proliferation and mobilization of hematopoietic stem and immature myeloid progenitor cells (MPCs) while enhancing proliferation and mobilization of more mature and “lineage-committed” MPCs (48). A CCR1 gene knockout study determined that CCR1 is the primary receptor, as opposed to CCR5 or D6, that mediates the proliferation and mobilization of MPCs by CCL3 (48). Considering that cytotoxic chemotherapy for treatment of multiple types of cancer is aimed at rapidly dividing cells, and that HSPCs are rapidly dividing cells that circulate in the blood stream where

they are exposed to chemotherapeutic agents, prevention of the mobilization of HSPCs into the peripheral blood during cancer treatment would maintain a protected pool of these cells in the bone marrow to be released following the cessation of chemotherapy.

With this myeloprotective behavior in mind, British Biotech initiated a dose-escalating Phase I clinical trial of BB10010 in patients with relapsed/refractory breast cancer (138). The purpose of this and subsequent trials, besides demonstrating initial safety of the recombinant CCL3, was to develop a therapeutic agent that could place HSPCs into a slow or non-cycling proliferative state and potentially reduce their exposure to chemotherapy without reducing the efficacy of the cancer treatment itself. Perhaps co-administration of chemotherapy with such a myeloprotective agent would permit higher doses of chemotherapy in the patient as dosage level is primarily limited by its hematological toxicity (139). In this Phase I trial, BB10010 significantly reduced the cycling status of MPCs compared to pre-treatment levels and this effect was reversible. The drug was advanced into a Phase II trial evaluating the effects of a 7-day regimen of BB10010 in 30 patients with locally advanced or metastatic breast cancer who had concurrent administration of chemotherapy (137). Unfortunately, BB10010 displayed no significant myelosuppression and provided no observable benefit to the patients. Two subsequent Phase II trials in patients with malignant lymphoma/breast cancer or newly diagnosed non-Hodgkin's lymphoma demonstrated no effect of BB10010 on hematopoietic recovery in peripheral

blood, nor did it improve the ability of patients to tolerate more intensive chemotherapy (139, 140). The lack of success of BB10010 does not necessarily call for the abandonment of strategies to pursue clinical CCR1 agonists. A recent study reported the potent adjuvantic activity of a *bis*-quinalone compound in a murine model of immunization; however, a direct causal link between the adjuvantic properties of this compound and its CCR1-agonistic properties has yet to be demonstrated (141).

These mostly disappointing clinical failures of CCR1 small molecule antagonists demonstrate the sheer difficulty involved in targeting chemokine receptors; indeed only two chemokine receptor ligands have received US Food and Drug Administration (FDA) approval at the time of writing. Potential reasons why only one CCR1 inhibitor appears to be progressing beyond initial Phase II studies will be discussed in greater detail in a subsequent chapter. Perhaps Richard Horuk, former Director of Immunology and Principal Scientist at Berlex BioSciences during the development of BX 471, described it best when he stated in a review of CCR1 drug development that, "… the roadway of [CCR1] drug discovery has been littered with the corpses of multiple clinical failures." (78) However, companies such as Bristol-Meyers Squibb (BMS) are continuing their CCR1 pipeline programs. BMS scientists recently published a new series of antagonists that inhibit chemokine binding and receptor function with the intent of developing a clinical candidate for RA treatment (142). Additionally, the work presented herein may open new avenues for CCR1 drug discovery.

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# CHAPTER 2

## THE CHEMOKINE RECEPTOR CCR1 IS CONSTITUTIVELY ACTIVE, WHICH LEADS TO G PROTEIN-INDEPENDENT, B-ARRESTIN-MEDIATED INTERNALIZATION

### 2.1 Abstract

Activation of G protein-coupled receptors by their associated ligands has been extensively studied, and increasing structural information about the molecular mechanisms underlying ligand-dependent receptor activation is beginning to emerge with the recent expansion in GPCR crystal structures. However, some GPCRs are also able to adopt active conformations in the absence of agonist binding that result in the initiation of signal transduction and receptor down-modulation. In this report we show that the CC-type chemokine receptor 1 (CCR1) exhibits significant constitutive activity leading to a variety of cellular responses. CCR1 expression is sufficient to induce inhibition of cAMP formation, increased F-actin content, and basal migration of human and murine leukocytes. The constitutive activity leads to basal phosphorylation of the receptor, recruitment of β-arrestin-2 and subsequent receptor internalization. CCR1 concurrently engages $G\alpha_i$ and β-arrestin-2 in a multi-protein complex, which may be accommodated by homo-oligomerization or receptor clustering. The data suggests the presence of two functional states for CCR1; whereas receptor coupled to $G\alpha_i$ functions as a canonical GPCR

albeit with high constitutive activity, the CCR1:β-arrestin-2 complex is required for G protein independent constitutive receptor internalization. The pertussis toxin-insensitive uptake of chemokine by the receptor suggests that the CCR1:β-arrestin-2 complex may be related to a potential scavenging function of the receptor, which may be important for maintenance of chemokine gradients and receptor responsiveness in complex fields of chemokines during inflammation.

## 2.2 Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of cell surface molecules involved in signal transduction and mediate physiological responses to extraordinarily diverse extracellular stimuli including light, odorants, neurotransmitters, chemoattractants, and peptides (1). They are one of the most therapeutically important family of receptors in the human genome and constitute the targets of roughly half of all drugs in clinical use (2).The historical paradigm of GPCR signaling suggests that receptors activate G proteins only upon agonist binding (3). However, this paradigm has significantly shifted since the seminal 1989 discovery by Costa and Herz of negative intrinsic efficacy of δ opioid receptor inhibitors (4). In the roughly two decades since, increasing evidence has shown that many GPCRs exhibit some level of ligand-independent signaling and that constitutive activity can play an important role in both normal and diseased tissues and cells (5). Spontaneous receptor isomerization from an inactive to an active state,

resulting in elevated basal signaling to effector proteins and consequent cellular responses, is a hallmark of receptor constitutive activity (6). Dozens of studies have demonstrated mutations capable of inducing basal signaling by GPCRs (7); however, constitutive activity occurs amongst many wild-type endogenous receptors as well. For example the ghrelin receptor (8), melanocortin 4 receptor (MC4R) (9), histamine H4 receptor (10), and multiple orphan receptors (11, 12) exhibit variable levels of ligand-independent G protein coupling or effector signaling. A review of constitutive GPCR activity documented more than 60 naturally-occurring GPCRs from multiple receptor families that displayed this behavior (5). While constitutive activity may be an intrinsic feature of many GPCRs, it can be modulated by receptor expression, cell type and microenvironment, as well as endogenous ligands that shift the equilibrium between the active and inactive conformational states. Its functional implications are, however, poorly understood, as are the molecular interactions that promote or regulate such behavior. Nevertheless, the physiological significance of this phenomenon is underscored by the key role that loss of GPCR constitutive activity can play in human disease. Examples include mutations that reduce ligand-independent activity in the ghrelin receptor and MC4R resulting in familial short stature syndrome and obesity, respectively (13).

Chemokine receptors belong to the class A rhodopsin-like family of GPCRs. As mediators of directional migration and localization of leukocytes, chemokine receptors are essential to the development, maintenance and

proper functioning of the immune system (14). This subfamily of GPCRs has proven difficult for drug development with only two FDA-approved compounds on the market (Selzentry® targeting CCR5 in HIV/AIDS treatment and Mozobil® targeting CXCR4 for hematopoietc stem cell mobilization) despite significant pharmaceutical industry investment (15). Among the chemokine family of GPCRs, CCR1 is one of the most prevalent targets for drug development according to the distribution of patents for small-molecule inhibitors of chemokine receptors (16). CCR1 was originally cloned in 1993 and was shown to be expressed by neutrophils, T cells, B lymphocytes, natural killer (NK) cells, monocytes, and $CD34^+$ bone marrow cells. It has ten known human ligands and, like most chemokine receptors, is a $Ga_i$-coupled receptor (17). Gene deletion of CCR1 in mice is not lethal; however, knockout of the receptor revealed both beneficial and detrimental effects dependent on the cellular context. In some studies of immune system challenge, mice lacking CCR1 exhibited an increased rate of pathogen clearance (18), attenuation of an excessive inflammatory response (19), and suppression of tissue allograft rejection (20). In non-challenged mice CCR1 has been shown to play an important role in osteoclastogenesis (21) and in mobilization of bone marrow progenitor cells to the spleen (22). CCR1 has been demonstrated to play a key role in diseases associated with inappropriate leukocyte infiltration and activation such as multiple sclerosis (23, 24), rheumatoid arthritis (25, 26), progressive kidney disease (27-29), and transplant rejection (20, 30, 31). Many attempts have been made to develop small-molecule drugs that

effectively inhibit receptor signaling, but thus far all have failed during clinical trials primarily due to lack of efficacy (32-34). Despite its biomedical relevance, relatively little has been reported on the molecular pharmacology of the receptor in its apo (i.e. basal, non-ligand bound) state.

With the clinical relevance of CCR1 clearly established, our laboratory set out to study the behavior of the receptor in its basal state, following initial observations that cells expressing CCR1 showed significant basal migration compared to cells expressing other chemokine receptors. The purpose of this study was to validate and explore the extent of CCR1 constitutive activity and to investigate the effect of agonist-independent signaling on the interactions between the receptor and intracellular proteins as well as its functional consequences. Previous studies have demonstrated constitutive activity amongst both wild type (35-39) and mutant (40-43) CC- and CXC-type chemokine receptors, but not for CCR1 to the best of our knowledge. This report demonstrates for the first time that CCR1 expression is sufficient to induce ligand-independent inhibition of cAMP (consistent with $Ga_i$ stimulation) and migration of both murine and human leukocytes. Constitutive phosphorylation, β-arrestin association, and pertussis toxin (PT)-independent internalization were also demonstrated, as well as the ability of CCR1 to oligomerize and assemble a preformed CCR1/G protein/β-arrestin-2 complex. Preliminary data suggests that the β-arrestin mediated constitutive internalization may be related to a chemokine scavenging activity, which in

turn may be important for the responsiveness of CCR1 expressing cells to chemokine gradients.

**2.3 Experimental procedures**

*Cell culture and transfections* — HEK293, HeLa, and COS-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with Glutamax (Invitrogen) supplemented with 10% fetal bovine serum (FBS). The THP-1 human acute monocytic leukemia cell line (ATCC) was maintained in RPMI-1640 media (Invitrogen) supplemented with 10% FBS and the L1.2 murine pre-B lymphoma cell line (kind gift of Brian Zabel, Palo Alto Institute for Research and Education, Palo Alto, CA) was maintained in RPMI-1640 media (Invitrogen) supplemented with 10% FBS, 1% non-essential amino acids (Invitrogen), 1% sodium pyruvate, and 0.1% β-mercaptoethanol. Lastly, wild-type and β-arrestin-2$^{-/-}$ mouse embryonic fibroblasts (MEFs, kind gifts of Robert Lefkowitz, Duke University, Durham, NC) were cultured in DMEM with Glutamax supplemented with 10% FBS. All cells were maintained at 37°C and 5% $CO_2$. Transfection of HEK293 cells was carried out in 6-well plates at 50-60% confluency using TransIT-LT1 reagent (Mirus Bio) per the manufacturer's protocol. HeLa, COS-7 and MEF cells were transfected on glass coverslips at 80-90% confluency using Lipofectamine™2000 (Invitrogen) per the manufacturer's protocol. Stable L1.2 lines were generated by electroporation of 10 μg of CCR1, CCR2, CCR5, or CCR10 in pcDNA3.1 plasmid into 1x10$^7$ cells, followed by G418 selection and identification of high expressers through

limiting dilution. Stable CCR1 expression in the inducible pACMV-TetO/HEK293 vector/cell system was generated as described previously (44, 45).

*In vitro migration and actin polymerization* — Migration assays were performed using 24-well transwell plates with 5 μm pore size filter inserts (Corning). Prior to migration assays, L1.2 or THP-1 cells were pretreated with either 100 μM BX-471 (kind gift of Richard Horuk, UC Davis, Davis, CA), 0.2 μg/mL pertussis toxin (List Biological Laboratories) or DMSO as a control for 1 h at 37°C. Cells were resuspended at a concentration of $2x10^6$ cells/mL in RPMI-1640 + 10% FBS, and 100 μL of cells were distributed in the upper part of each well with 600 μL of RPMI-1640 + 10% FBS in the bottom well. Cells were allowed to migrate for 2 h at 37°C and 5% $CO_2$ after which time cells that migrated to the bottom chamber were counted on a Guava® EasyCyte™ Flow Cytometer (Millipore). Wells with cells only (no filter) were used to quantify maximal migration. Migration was plotted as the percent of cells migrated to the total number of cells (± SD). For the actin polymerization assay, L1.2 cells were resuspended in assay buffer (1X HBSS, 0.5% BSA, 10 mM HEPES pH 7.4) at a concentration of $6x10^6$ cells/mL and fixed with 4% paraformaldehyde (PFA). Cells were then permeabilized with 0.1% TritonX100 in assay buffer and stained with 2.5 units/mL Alexa Fluor® 488 phalloidin (Life Technologies). Extent of F-actin staining was measured using the Guava® EasyCyte™ Flow Cytometer (Millipore).

*GloSensor™ cAMP assay* — The GloSensor™ cAMP assay (Promega) uses a genetically encoded biosensor with cAMP binding domains fused to a mutant form of *P. pyralis* luciferase. Upon binding to cAMP, conformational changes occur that yield large increases in luminescence. HEK293 cells stably expressing the pGloSensor™-22F plasmid were seeded in 6-well plates and transiently transfected with 1 µg HA-CCR1, HA-M3 or empty pcDNA3.1 vector for 24 h. Cells were then seeded at $2x10^5$ cells/well into 96-well white assay plates (BD Biosciences) in Opti-MEM™ media (Invitrogen) and incubated with 4% (v/v) GloSensor™ substrate for 1 h at 37°C. Upon maintaining equilibrium at room temperature, the luminescent signal following stimulation with 10 µM forskolin was measured using a VictorX Light multilabel plate reader (Perkin-Elmer).

*Flow cytometry and receptor internalization* — For receptor internalization assays, HEK293 cells stably expressing FLAG-CCR1 were cultured in 6-well plates. Cell surface receptor was labeled with mouse anti-CCR1 antibody (clone 53504, R&D Systems) for 30 min on ice in wash buffer (DMEM, 0.5% BSA, 10 mM HEPES pH 7.4), unbound antibody was washed away with cold wash buffer, and then the media was replaced with pre-warmed wash buffer for specified periods of time. Cells were washed with PBS + 0.5% BSA and the remaining cell surface receptor was labeled with anti-mouse antibody conjugated to phycoerythrin (PE) (R&D Systems). The relative amount of receptor remaining on the surface at each time point was quantified using a Guava® EasyCyte™ Flow Cytometer (Millipore) and

analyzed using FlowJo software (Tree Star). A similar approach was taken to measure transfected or endogenous CCR1 expression and subsequent internalization from the surface of L1.2 and THP-1 cells, respectively, grown in suspension.

*Chemokine scavenging* — For chemokine scavenging experiments, L1.2 cells stably expressing CCR1 were cultured at $2x10^6$ cells/mL in the absence or presence of 0.2 μg/mL pertussis toxin or 100 μM BX-471 for 1 h at 37°C in serum-free RPMI-1640 supplemented with 0.5% BSA and 10 mM HEPES, pH 7.4. Wild-type and CCR5-expressing L1.2 cells were included as controls. Cells were then incubated with 100 nM CCL7 conjugated to Cy3B maleimide (manuscript in preparation) for up to 30 minutes. Non-internalized CCL7-Cy3B was removed by repeated washing with cold PBS supplemented with 0.5% BSA. The relative amount of internalized CCL7-Cy3B was quantified using a Guava® EasyCyte™ Flow Cytometer and analyzed using FlowJo software.

*CCR1 phosphorylation* — HEK293t cells were transiently transfected in 6-well plates as described above with vector containing FLAG-CCR1. Forty-eight hours post-transfection, cells were labeled with 250 μCi [$^{32}$P] orthophosphate (Perkin Elmer) in phosphate-free DMEM for 2 h at 37°C. Cells were incubated with or without 1 μM CCL14 for up to 6 min and then lysed in RIPA buffer supplemented with protease inhibitor mixture (Sigma) and phosphatase inhibitor cocktail II and III (Sigma) for 1 h at 4°C. Cell lysates were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma) and

immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membrane. Membranes were analyzed by autoradiography and then probed with anti-FLAG (Sigma) antibody to detect FLAG-CCR1 expression by Western blotting.

*Bioluminescence resonance energy transfer (BRET) assay* — The BRET assay protocol has been described extensively elsewhere (46-48). In brief, the chemokine receptors CCR1, CCR2, CCR5 and CXCR4 and the $\beta_2$-adrenergic receptor ($\beta_2$AR) were genetically fused to *A. victoria* YFP on the receptor C-terminus followed by a short linker in pcDNA3.1 (receptor-pYFP3.1). The *R. reniformis* luciferase (Rluc) genetic sequence was fused to the C-terminus of β-arrestin-2 in the phRluc-N2 vector. YFP (energy acceptor) and Rluc (energy donor) constructs were generous gifts from M. Bouvier, University of Montréal, Québec, Canada. HEK293t cells were transiently transfected in defined stoichiometric ratios of each vector, usually 1.5-2 µg of a receptor-YFP construct and 0.05-0.1 µg of β-arrestin-2-Rluc. Forty-eight hours post-transfection, cells were washed, suspended in phosphate buffered saline (PBS) supplemented with 0.5% (w/v) glucose, aliquoted at $1x10^5$ cells/well in triplicate into a white, clear bottom 96-well plate (BD Biosciences) and incubated for 1 h at 37°C prior to BRET measurement. YFP-tagged receptor expression was quantified by measuring fluorescence of the wells at 485 nm excitation and 538 nm emission wavelengths on a SpectraMax fluorescence spectrometer (Molecular Devices). The luciferase substrate coelenterazine-*h* (Biotium) was added to a final concentration of 50 µM in

each well 10 min prior to the beginning of the BRET assay. Luminescence and fluorescence measurements were collected at room temperature with 1 sec exposure times using a VictorX Light multilabel plate reader (Perkin-Elmer) at repeating time intervals. The BRET signal was calculated as the ratio of YFP emission (550±40 nm) to Rluc emission (470±30 nm). The $BRET_{net}$ signal is calculated by subtracting the background BRET ratio of cells expressing only the Rluc fusion from the BRET ratio of cells expressing both the YFP- and Rluc-fused proteins. CCL14 was prepared as previously described (49) and diluted in PBS and added following incubation with coelenterazine-*h* but prior to BRET measurement. BRET saturation curve experiments were carried out in which the levels of β-arrestin-2-Rluc were kept constant while increasing amounts of CCR1-YFP were co-transfected. The BRET signal was then plotted against the acceptor/donor ratio; a hyperbolic curve is indicative of a specific interaction as opposed to random collisions within the cell that would yield a quasi-linear relationship (46).

*Co-immunoprecipitation and western blotting* — HEK293t cells were transfected as above in 6-well plates with FLAG-CCR1 and β-arrestin2-HA constructs in pcDNA3.1 vectors. Forty-eight h after transfection, cell media was replaced with serum-free DMEM with or without 1 μM CCL14 for specified periods of time at 37°C. Cells were then washed with ice-cold PBS and lysed in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.4, 300 mM NaCl, 1% igepal/NP40, 0.25% deoxycholate, 0.1% SDS) with protease inhibitor mixture and phosphatase inhibitor cocktail II and III (Sigma-Aldrich)

for 1 h at 4°C. The supernatant fraction of the cell lysate was collected after 10 min of centrifugation at 20,000 x g. Anti-FLAG M2 affinity gel (Sigma-Aldrich) was added to the sample and incubated overnight at 4°C on a rotating platform. The affinity gel was then washed 3-4 times in RIPA buffer, and elution performed with 3X-FLAG peptide (Sigma-Aldrich). Eluted proteins were mixed with 5X Laemmli buffer and separated via SDS-PAGE in a 10% polyacrylamide gel. The presence of FLAG-CCR1 and β-arrestin-2-HA was measured by Western blotting and ECL Plus chemiluminescent detection (GE Healthcare) using anti-FLAG (Sigma-Aldrich), anti-mouse-HRP (horseradish peroxidase, Promega) and anti-HA-HRP (Roche) monoclonal antibodies.

*Confocal fluorescence microscopy* — HeLa, COS-7, or MEF cells were cultured on glass coverslips coated with 10 μg/mL human plasma fibronectin (Millipore) and transiently transfected with HA-CCR1, CCR1-mCherry and/or β-arrestin-2-GFP using Lipofectamine™2000 (Invitrogen) as per the manufacturer's protocol. For cells transfected with CCR1-mCherry, 24 h post-transfection the media was replaced with serum-free DMEM, with or without 100 nM CCL14 for specified periods of time. For cells transfected with HA-CCR1, receptor was pre-labeled with anti-HA antibody directly conjugated to Alexa Fluor® 594 (Life Technologies) for 30 min on ice in wash buffer (DMEM, 0.5% FBS, 10 mM HEPES pH 7.4), washed with cold wash buffer, and then the media was replaced with pre-warmed wash buffer with or without 1 μM CCL14 for specified periods of time. The cells were then fixed with 4% paraformaldehyde (PFA) for 5 min at room temperature. The coverslips with

fixed cells were mounted onto microscope slides with FluoroSave (Calbiochem). Images were collected using an Olympus DSU spinning disk confocal microscope.

## 2.4 Results

*CCR1 is a constitutively active receptor* — While testing the chemotactic ability of various mutants of CCR1 activating chemokines, it was observed that CCR1 expressing L1.2 cells consistently displayed a significant level of basal migration in transwell migration assays (**Fig. 2.1A**). Since constitutive activity had not previously been reported despite the extensive literature devoted to studies of CCR1, we set out to further characterize this phenomenon. The high levels of basal migration was specific to CCR1 as L1.2 cells expressing CCR2, CCR5 or CCR10 at comparable levels as CCR1 did not exhibit similar ligand-independent migration (**Fig. 2.1A and 2.2A**). Each receptor cell line remained capable of responding to chemokine agonist in a transwell migration assay (data not shown). The essential role of CCR1 in this constitutive process was demonstrated by the ability of the CCR1-specific antagonist BX-471 (50), a potent and competitive inhibitor of chemokine-mediated CCR1 activation, to ablate the basal migration (**Fig. 2.1A**). Treatment with pertussis toxin (PT) also blocked migration, suggesting that it is driven by $G\alpha_{i/o}$ activation. To rule out the possibility that the constitutive activity is an artifact of heterologous overexpression of the receptor, the effect of endogenous CCR1 in untransfected cells was characterized. Human acute

monocytic leukemia (THP-1) cells have been shown to express CCR1 and to respond to CCR1 chemokine agonists in various functional assays (51, 52). While not as exaggerated as in L1.2 cells, THP-1 cells exhibited ligand-independent migration that represented a substantial fraction of the ligand induced migration (data not shown), and could be attributed to a $G\alpha_{i/o}$-coupled receptor identified as CCR1 by the inhibitory effects of PT and BX-471, respectively (**Fig. 2.1B**). These observations were further supported by the effect of CCR1 expression on basal filamentous actin (F-actin) content in the L1.2 cells. These experiments showed elevated actin polymerization in cells expressing CCR1 compared to those expressing CCR2, CCR5 or CCR10 (**Fig. 2.1C**). One might hypothesize that the constitutive activity was a consequence of some unidentified agonist in the culture media. However, basal migration was also observed in serum-free media albeit at a lower level (data not shown). Furthermore, additional experiments carried out in the absence of serum (described below) were consistent with constitutive activity. Autocrine secretion of chemokine can also be ruled out as contributing to the observed constitutive activity because of the ten chemokine ligands known to activate CCR1, eight are also known agonists of CCR2 and/or CCR5 (CCL3, 5, 7, 8, 13, 14, 16) leaving it highly unlikely that any potentially-secreted chemokine would singularly activate CCR1.

To further demonstrate constitutive CCR1/$G\alpha_{i/o}$ signaling, the GloSensor™ assay (Promega) was used to measure forskolin-stimulated levels of intracellular cAMP in HEK293 cells stably expressing the

pGloSensor™-22F construct and either CCR1, M3 muscarinic cholinergic receptor or empty pcDNA3.1 vector control. M3 is known to couple to $G\alpha_{q/11}$ and activate phospholipase C (PLC) (53); therefore, it serves as a negative control for the inhibition of adenylyl cyclase (AC) resulting from activation of a $G\alpha_{i/o}$-coupled receptor. CCR1 expression was shown to significantly lower the maximal forskolin-stimulated cAMP production compared to M3 expressed at similar levels or vector control (**Fig. 2.1D**). This effect was further demonstrated in a gene-dosage experiment in which increasing levels of CCR1 expression resulted in significant reduction in maximal cAMP production (**Fig. 2.1E**). Expression of CCR1 was lower than that of M3 (**Fig. 2.2B**) yet it still had a significant inhibitory effect on AC activity. Together these data suggest that CCR1 is a constitutively active receptor that can activate G proteins and stimulate cell migration in an agonist-independent manner.

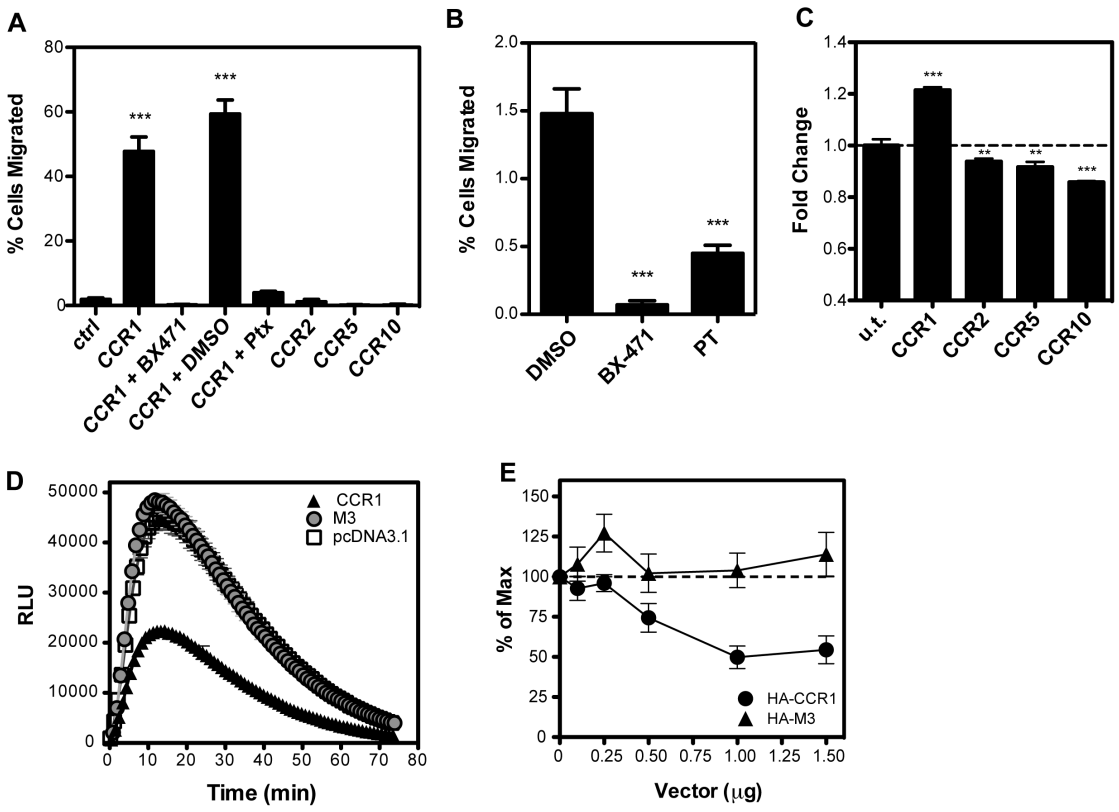


**Figure 2.1. CCR1 expression is sufficient to induce basal migration and inhibit cAMP formation**. **A.** Murine L1.2 cells stably transfected with either CCR1, CCR2, CCR5 or CCR10 or **B.** human THP-1 cells endogenously expressing CCR1 were placed in a micro-chemotaxis chamber and the number of cells that spontaneously migrated into the lower chamber after 2h at 37°C were measured. The effect of the CCR1-specific inhibitor BX-471 and pertussis toxin (PT) was also determined. Percent of cells migrated was calculated as the ratio of cells in the lower chamber in the microchemotaxis well to the number of cells initially added to the upper chamber. Data are the mean +/- S.D. **C.** Basal F-actin content of L1.2 cells stably transfected with chemokine receptors or left untransfected (u.t.). Cells were permeabilized and stained with AlexaFluor 488-phalloidin; results of a representative experiment performed in triplicate are plotted as fold-change over u.t. control. **D.** Luminescence of pGloSensor™-22F/HEK293 cells transiently transfected with 1.0 μg CCR1 (▲), M3 (◍) or empty pcDNA3.1 (□) vector. The signal from a representative experiment measured in triplicate following incubation of cells with 4% GloSensor™ cAMP reagent for 1 h at 37°C and stimulation with 10 μM forskolin. **E.** Gene-dosage experiment performed in triplicate in which increasing amounts of HA-CCR1 or HA-M3 in pcDNA3.1 vectors were transfected into pGloSensor™-22F/HEK293 cells and assayed as in C. Data were plotted as the mean +/- S.D. using GraphPad Prism® (GraphPad Software) and the statistical significance calculated using an unpaired t test or one-way ANOVA with Dunnett post-test: **p<0.01, ***p<0.0001.

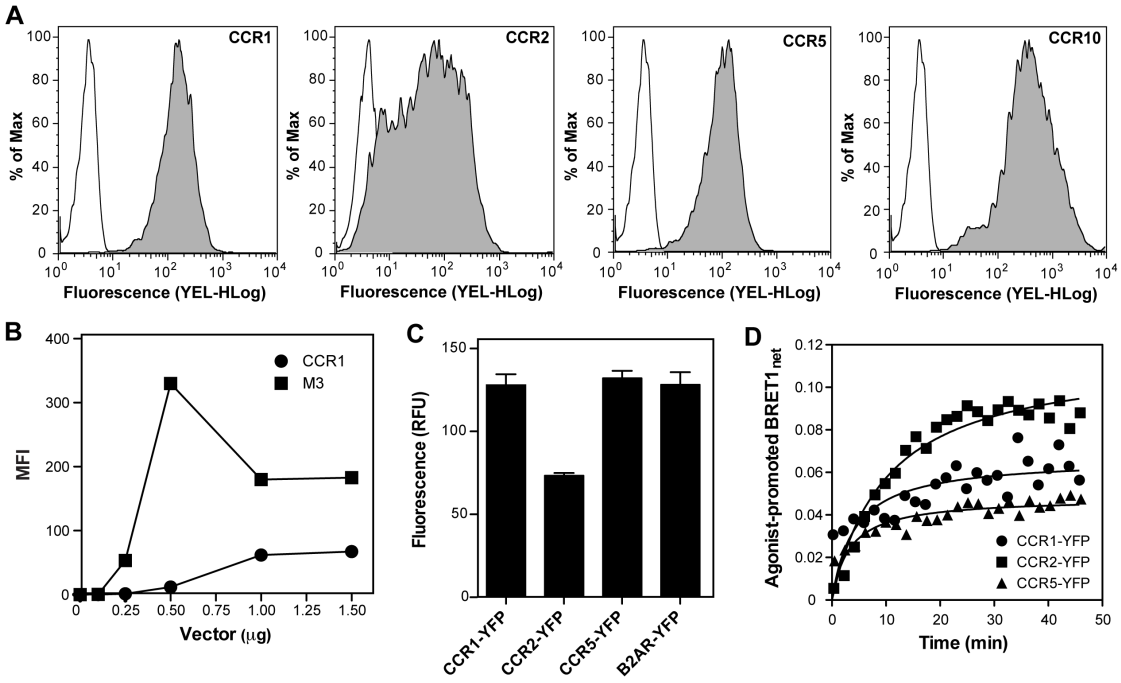


**Figure 2.2. Expression of chemokine receptors in various cell lines. A**. Expression of CCR1, CCR2, CCR5, and CCR10 in stably transfected L1.2 cells. Cell surface receptor expression was determined using receptor specific antibodies conjugated to phycoerythrin (PE) and analyzed via flow cytometry. Tinted lines demonstrate receptor expression, unfilled lines demonstrate isotype control antibody binding. **B**. Expression of HA-CCR1 and HA-M3 transiently transfected into HEK293 cells stably expressing the pGloSensor-22F construct. Cell surface receptor levels were detecting using an antibody directed against the HA epitope tag conjugated to PE and analyzed via flow cytometry. Data shown from a representative experiment in triplicate as median fluorescence intensity (MFI). **C**. Mean relative fluorescence values +/- S.D. of HEK293t cells transiently transfected in triplicate with CCR1-YFP, CCR2-YFP, CCR5-YFP or β2AR-YFP in the basal β-arrestin-2 BRET association assay. **D.** Effect of chemokine agonist on the $BRET1_{net}$ signal over time between β-arrestin-2-Rluc and either CCR1-YFP (●), CCR2-YFP (■), or CCR5-YFP (▲). CCR1- and CCR5-expressing HEK293t cells were treated with 100 nM CCL14 whereas CCR2-expressing cells were treated with 100 nM CCL7. Data shown from a representative experiment performed in triplicate.

*CCR1 is constitutively internalized in multiple cell types* — Agonist binding to GPCRs typically results in G protein activation followed by receptor desensitization mediated by phosphorylation of intracellular domains and recruitment of arrestins. The arrestin proteins sterically occlude the receptor from further G protein coupling and connect the receptor to the internalization machinery of the cell to initiate endocytosis (54). Here it was tested whether CCR1 is internalized in the absence of agonist in various cell systems. HEK293 and L1.2 cells stably expressing CCR1 were labeled on ice with anti-CCR1 antibody, warmed to 37°C for 1 h in the absence of exogenous agonist, and the subsequent amount of receptor remaining at the surface was determined by flow cytometry. In both cell types, CCR1 was constitutively internalized as a marked reduction in the cell surface levels of the receptor after 1 h of incubation was observed (**Fig. 2.3A**, left and middle panels). To confirm that constitutive internalization was not an artifact of heterologous overexpression of the receptor, the experiment was repeated with THP-1 cells, which endogenously express CCR1. A similar result was obtained demonstrating extensive down-regulation of CCR1 to the point where cell surface receptor was barely detectable (**Fig. 2.3A**, right panel). It remained possible, however, that CCR1 was being activated by secreted chemokine agonists in some autocrine fashion thereby explaining the significant internalization of the receptor. To rule this out, the effect of treating the CCR1-expressing L1.2 cells with BX-471 in the constitutive internalization assay was

tested. BX-471 was shown to have no effect on constitutive internalization of the receptor, making it highly improbable that a secreted agonist was responsible (**Fig. 2.3B**). Additionally, CCR1 constitutive internalization was independent of $G\alpha_{i/o}$ protein activation, as PT treatment did not inhibit basal down-regulation of the receptor (**Fig. 2.3B**), in line with previous studies of other chemokine receptors (55-57).

The effect of agonist-mediated CCR1 internalization over time in comparison to constitutive internalization was additionally measured. HEK293 cells expressing HA-CCR1 were pre-labeled with CCR1 antibody and warmed as above in the presence or absence of 100 nM CCL14 (**Fig. 2.3C**). While CCR1 exhibited rapid ligand-independent internalization, CCL14 stimulation significantly increased the extent of receptor removal from the cell surface. The same constitutive internalization assay was carried out without antibody pre-labeling to measure whether CCR1 that is internalized in the absence or presence of agonist is recycled back to the cell surface. Over the time course of the assay, the amount of CCR1 on the cell surface at any given time point remained relatively constant suggesting that CCR1 undergoes constitutive recycling to maintain consistent expression at the plasma membrane (**Fig. 2.3C**). However, overall levels of cell surface receptor were significantly down-regulated following CCL14 stimulation (**Fig. 2.3C**), suggesting the receptor is being sent along a different internalization pathway following agonist-binding compared to constitutively internalized receptor and is not being recycled, which is in agreement with a previous report (58).

In order to observe localization of the receptor following constitutive internalization, the fate of CCR1 was measured via confocal immunofluorescence microscopy. HeLa and COS-7 cells were transiently transfected with HA-tagged CCR1, pre-labeled with anti-HA antibody conjugated to Alexa Fluor 594®, and incubated for 1 h at 37°C in the presence or absence of 1 μM CCL14. Use of an antibody against the N-terminal HA-tag served as a control to ensure that the anti-CCR1 antibody utilized in the flow cytometry experiments above was not acting as an agonist and inducing internalization. Additionally, data from many internalization assays demonstrate that the anti-HA and anti-CCR1 antibodies do not preclude CCR1 activation by chemokines. Surface staining of HA-CCR1 was observed in HeLa and COS-7 cells followed by significant internalization in both unstimulated and agonist-stimulated experimental conditions; however, CCL14-mediated activation of CCR1 appeared to induce more rapid receptor internalization compared to those cells left untreated (**Fig. 2.3D**). The majority of surface-stained HA-CCR1 was localized into discrete intracellular puncta in both cell types. These data again demonstrate that CCR1 is a constitutively active receptor and undergoes agonist-independent internalization in a variety of cell types from human, simian and murine sources.

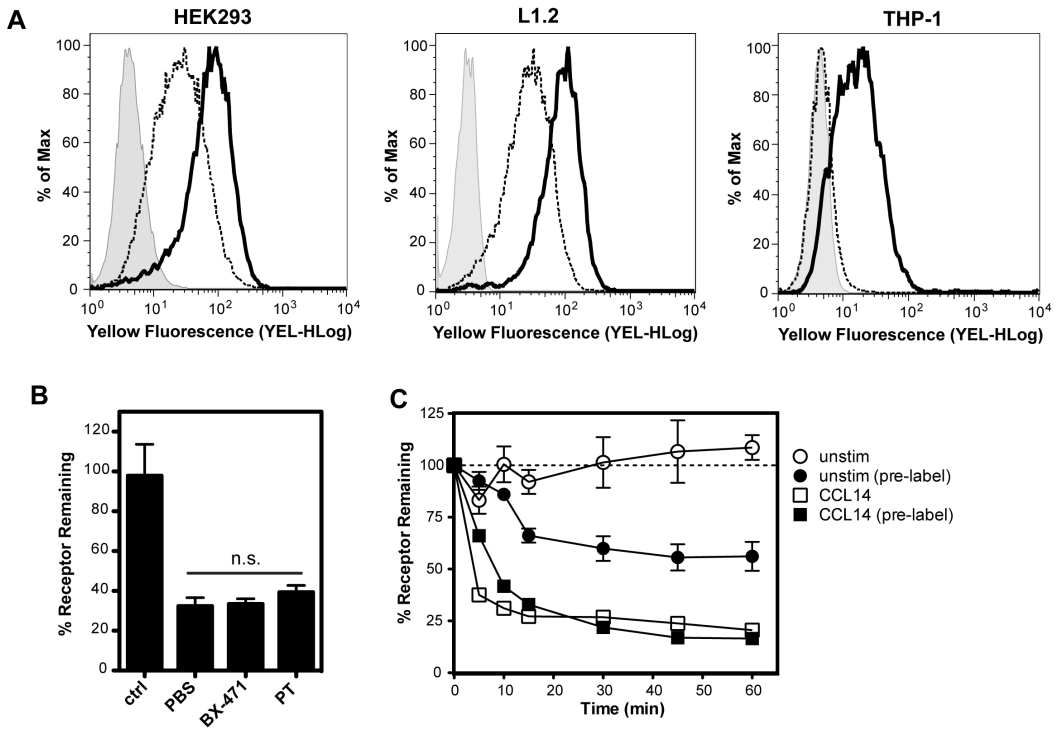


**Figure 2.3. CCR1 undergoes constitutive internalization in multiple cell types. A.** CCR1 constitutive internalization was measured in HEK293 cells stably expressing FLAG-CCR1 (left panel), L1.2 cells stably expressing CCR1 (middle panel), and THP-1 cells endogenously expressing CCR1 (right panel). Receptor initially present at the cell surface was labeled with CCR1 antibody on ice and the amount of receptor remaining after 1 h of warming to 37°C in serum-free media was measured using a phycoerythrin(PE)-conjugated secondary antibody and analyzed on a Guava® easyCyte™ Flow Cytometer (Millipore). Representative flow cytometry plots from experiments conducted in triplicate normalized to the percent maximal fluorescent value are shown with initial cell surface CCR1 levels (solid black line), receptor remaining after 1 h of agonist-independent internalization (dotted black line), and unstained control cells (gray tinted line). **B.** The effect of 1 h pre-treatment with 1 μM BX-471 and 200 ng/mL pertussis toxin (PT) on CCR1 constitutive internalization in L1.2 cells. The data are shown as mean +/- S.D. of the percent receptor remaining compared to control cells at 0 h timepoint. The variance between the PBS, BX-471 and PT treated cells was shown to be not significant (n.s.) by one-way ANOVA with Tukey's post-test using GraphPad Prism® (GraphPad Software). **C.** Constitutive and agonist-induced internalization and recycling of HA-CCR1 in stably expressing HEK293 cells over time. Cells were either pre-labeled (closed symbols) or not (open symbols) with CCR1 antibody and stimulated with 100 nM CCL14 (squares) or left unstimulated (circles). The amount of receptor remaining on the cell surface after was measured at each time point in triplicate as described above.

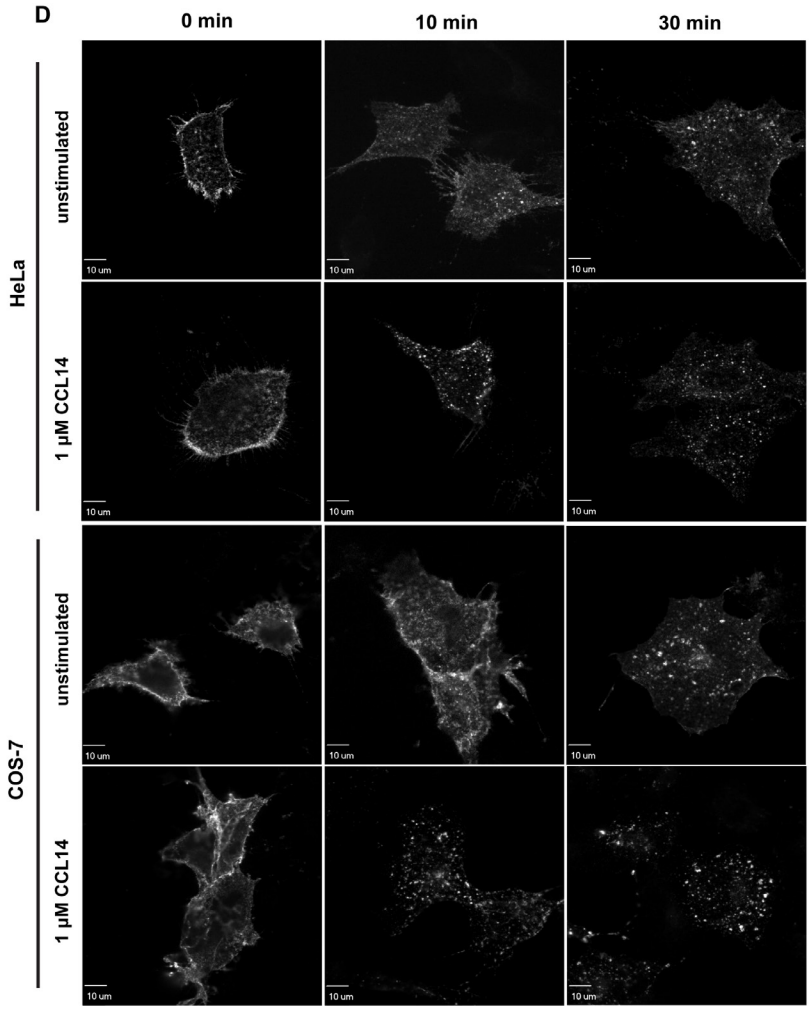


**Figure 2.3. CCR1 undergoes constitutive internalization in multiple cell types, Continued.** D. Constitutive internalization of HA-CCR1 transiently transfected in HeLa (upper panels) and COS-7 (lower panels) cells. Cells with HA-CCR1 at their surface were pre-labeled with anti-HA antibody conjugated to Alexa Fluor® 594 and then warmed in serum-free cell culture media with or without 1 μM CCL14 for 1 h. Cells were fixed and imaged using an Olympus DSU spinning disk confocal microscope.

*CCR1 is constitutively phosphorylated leading to basal association with β-arrestin-2* — The findings above on the constitutive signaling and internalization of CCR1 suggest that the receptor may be basally phosphorylated, as phosphorylation is a canonical event that follows GPCR activation and mediates down-regulation. To determine whether CCR1 is basally phosphorylated, HEK293t cells expressing FLAG-tagged CCR1 were labeled with $^{32}$P orthophosphate and either left untreated or stimulated with 1 μM CCL14. FLAG-CCR1 was shown to be extensively labeled prior to agonist treatment compared to the untransfected control, with only a minor increase in phosphorylation following agonist addition (**Fig. 2.4)**. Previous studies monitoring phosphorylation of related chemokine receptors CCR2 and CCR5 did not demonstrate this basal phosphorylation behavior (59, 60). The observation that CCR1 is constitutively phosphorylated conforms well with the data above indicating basal signaling activity and receptor down-regulation, and suggests the involvement of β-arrestins in CCR1 constitutive internalization.

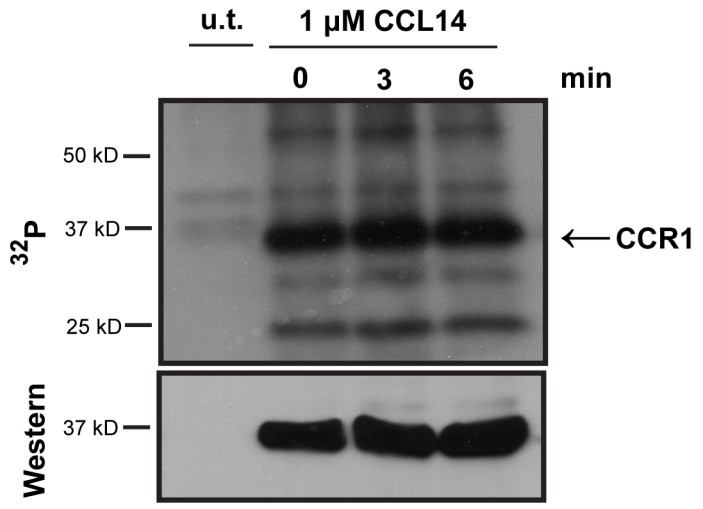


**Figure 2.4. CCR1 is constitutively phosphorylated.** HEK293t cells expressing FLAG-CCR1 were orthotopically labeled with the $^{32}P$ radioisotope and either left unstimulated or stimulated with 1 μM CCL14 for 3 or 6 min (upper panel). The arrow indicates the band corresponding to the correct molecular weight for CCR1. Receptor levels were measured by Western blot using anti-FLAG-HRP (Sigma) and chemiluminescent detection (lower panel).

To investigate the agonist-independent association of CCR1 with β-arrestins, a bioluminescence resonance energy transfer (BRET) assay was initially employed. BRET has been extensively used to measure GPCR oligomerization as well as to monitor receptor interactions with intracellular proteins, including arrestins (47, 61, 62). HEK293t cells were transiently transfected with CCR1-YFP (energy acceptor) and β-arrestin-2-Rluc (energy donor) or with β-arrestin-2-Rluc alone as a control. The BRET signal was measured following addition of the luciferase substrate coelenterazine-*h*. In the absence of CCR1 agonist, cells co-expressing CCR1-YFP and β-arrestin-

2-Rluc exhibited a significantly higher BRET signal than cells expressing β-arrestin-2-Rluc alone, indicative of constitutive interaction **(Fig. 2.5A)**. As with its effect on CCR1 constitutive internalization, treatment with BX-471 had no effect in preventing basal association between CCR1 and β-arrestin-2 (**Fig. 2.5B**). In order to determine if this observation was unique to CCR1 or if it is common amongst other chemokine receptors, the BRET signal was evaluated between β-arrestin-2-Rluc and YFP-tagged chemokine receptors CCR2 and CCR5 as well as the $\beta_2$-adrenergic receptor ($\beta_2$AR). Under basal conditions and with similar levels of receptor expression (measured by YFP fluorescence, **Fig. 2.2C**), CCR1 was the only receptor tested that exhibited a basal $BRET1_{net}$ signal significantly above baseline, indicative of constitutive association with β-arrestin-2-Rluc **(Fig. 2.5B)**. This finding is also consistent with the observation that neither CCR2 nor CCR5 promote constitutive migration *in vitro* (**Fig. 2.1A**). To ensure that the lack of basal association observed with CCR2 and CCR5 was not an artifact of non-functional receptors, the ability of the YFP-tagged chemokine receptors to form agonist-induced interactions with β-arrestin-2-Rluc was confirmed (**Fig. 2.2D**). Stimulation of CCR1 and CCR5 with CCL14 and CCR2 with CCL7 each led to a significant and time-dependent increase in the BRET signal, suggesting these receptors are functional and capable of engaging β-arrestin-2.

To determine if CCR1 exhibits preferential association with a particular arrestin isoform, the BRET assay was repeated with β-arrestin-1. Under non-stimulating conditions, CCR1-YFP exhibits a significantly higher $BRET1_{net}$

signal for β-arrestin-2-Rluc compared to that when β-arrestin-1-Rluc was used as the BRET donor (**Fig. 2.5C**). Importantly, CCR5-YFP did not exhibit preferential pre-coupling with either β-arrestin isoform under basal conditions. The specificity of the basal interaction between CCR1 and β-arrestin-2 was further demonstrated by co-transfecting increasing levels of β-arrestin-2-GFP into cells co-expressing CCR1-YFP and β-arrestin-2-Rluc and measuring the impact on the basal BRET signal. In the BRET1 assay, GFP expression does not significantly interfere with the resonance energy transfer between Rluc and YFP as there is little spectral overlap between Rluc emission and GFP excitation. Thus the dose-dependent decrease in the basal $BRET_{net}$ signal with addition of β-arrestin-2-GFP is indicative of competition between β-arrestin-2-Rluc and β-arrestin-2-GFP for interaction with CCR1-YFP and provides further evidence that the constitutive interaction with CCR1 is specific to β-arrestin-2 (**Fig. 2.5D**).

The BRET measurements reflect interactions averaged throughout the cell. Therefore, to determine the subcellular localization of the interaction between CCR1 and β-arrestin-2, HeLa cells transiently transfected with CCR1-mCherry and β-arrestin-2-GFP were imaged by confocal fluorescence microscopy. CCR1 and β-arrestin-2 were shown to co-localize both at the cell surface and within intracellular compartments under basal conditions (**Fig. 2.5E**). Co-expression of CCR1-mCherry with β-arrestin-2-GFP brought about a significant translocation of β-arrestin-2-GFP from a homogenous distribution within the cell in the absence of CCR1 to localization within discrete puncta

when co-expressed with the receptor (**Fig. 2.5E**), even in the absence of ligand stimulation.

To further confirm the observation from both the BRET and fluorescence microscopy experiments that CCR1 is constitutively associated with β-arrestin-2, the ability of β-arrestin-2 to co-immunoprecipitate with CCR1 was tested. HEK293t cells were transiently transfected with FLAG-CCR1 and increasing levels of β-arrestin-2-HA in order to ensure that a sufficient signal from β-arrestin-2 was obtained and that the interaction was not an artifact of overexpression. β-arrestin-2-HA was shown to co-immunoprecipitate with FLAG-CCR1 at each level of detectable β-arrestin-2-HA expression (**Fig. 2.5F)**. Interestingly, treatment with CCL14 did not appear to significantly increase the amount of β-arrestin-2-HA pulled down by the receptor, possibly in agreement with the previous data showing only a minor increase in CCR1 phosphorylation (**Fig. 2.4**). While the BRET signal between CCR1-YFP and β-arrestin-2 is significantly increased upon addition of CCL14 (**Fig. 2.2D**), this is likely due to an intermolecular conformational change between CCR1 and β-arrestin-2 that leads to a more favorable orientation for energy transfer between luciferase and YFP. To our knowledge, the data above represent the first demonstration of a wild-type G protein-signaling chemokine receptor that forms a stable and constitutive association with β-arrestin.

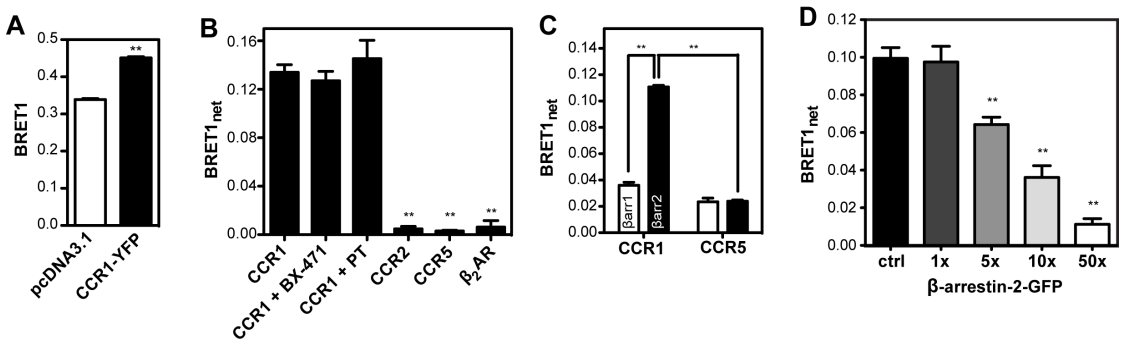


**Figure 2.5. CCR1 is constitutively associated with β-arrestin-2. A.** HEK293t cells were transiently transfected with β-arrestin-2-Rluc and pcDNA3.1 (white bar) or β-arrestin-2-Rluc and CCR1-YFP (black bar) and the BRET1 ratio was measured 48 h later at room temperature in the absence of ligand stimulation. **B.** Comparison of the basal $BRET1_{net}$ signal from HEK293t cells co-expressing β-arrestin-2-Rluc and CCR1-, CCR2-, CCR5- or beta 2-adrenergic receptor ($b_2$AR)-YFP. The effect of CCR1 inhibition with 1 μM BX-471 and blockade of Gi/o signaling with 200 ng/mL pertussis toxin (PT) on the basal $BRET1_{net}$ signal is also shown. **C.** Comparison of the basal $BRET1_{net}$ signal between β-arrestin-1-Rluc (white bar) and β-arrestin-2-Rluc (black bar) with either CCR1-YFP or CCR5-YFP. **D.** The basal $BRET1_{net}$ signal between CCR1-YFP and β-arrestin-2-Rluc with increasing levels of β-arrestin-2-GFP expression. The fold increase in β-arrestin-2-GFP levels on the x-axis is the ratio of the micrograms of β-arrestin-2-GFP vector to β-arrestin-2-Rluc vector transfected into cells co-expressing CCR1-YFP. The data above (**A.-D.**) are plotted as the mean ± S.D. of a representative experiment done in triplicate. Statistical significance was calculated using an unpaired t-test (**A**.) or one-way ANOVA with Dunnetts multiple comparisons test (**B., C., D**.) (*, $p<0.05$; **, $p<0.01$).

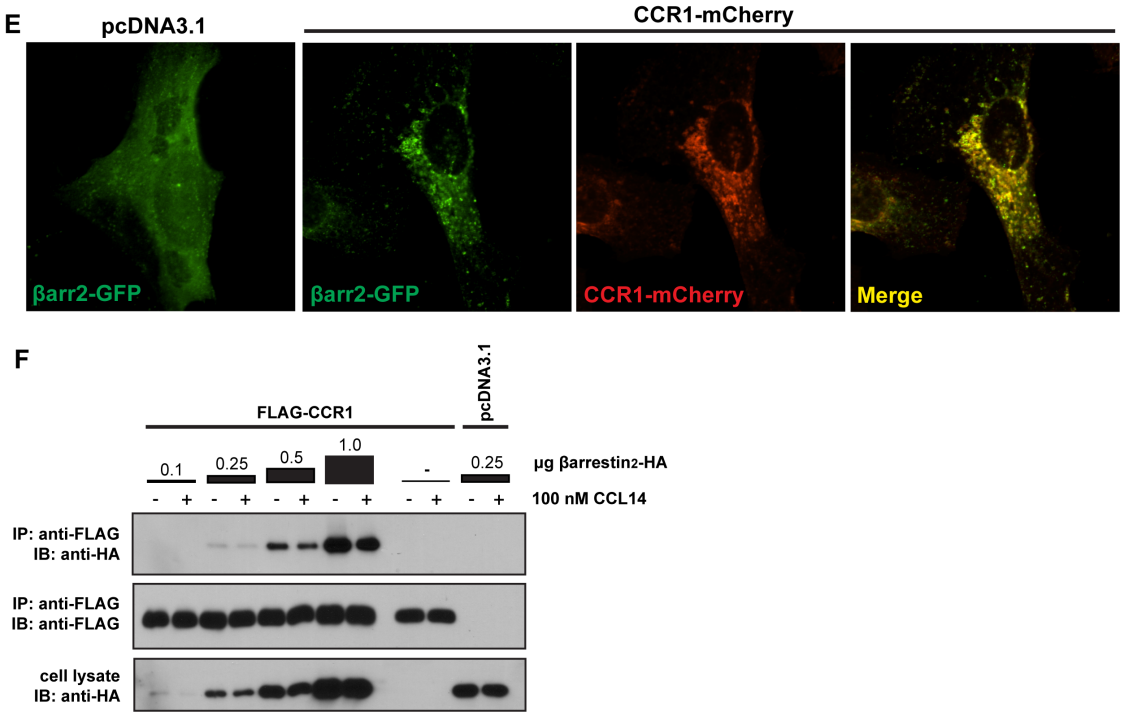


**Figure 2.5. CCR1 is constitutively associated with β-arrestin-2, Continued. E.** HeLa cells were cultured on fibronectin-coated coverslips and transfected with β-arrestin-2-GFP alone or with co-transfected with CCR1-mCherry. Cells were washed, fixed and imaged using a confocal microscope. Co-localization is indicated as yellow in the merged image. The bottom panel is a comparison of HeLa cells expressing β-arrestin-2-GFP and co-transfected with empty pcDNA3.1 vector (left) or CCR1-mCherry (right). **F.** HEK293t cells were transiently transfected with FLAG-CCR1 or pcDNA3.1 (far right lanes) and increasing levels of β-arrestin-2-HA and treated with PBS or 100 nM CCL14 for 15 min at 37°C. CCR1 and any associated proteins were immunoprecipitated and the presence of receptor and β-arrestin-2-HA was measured by Western blot.

*Constitutive internalization of CCR1 is mediated by β-arrestin-2* — To investigate the role of β-arrestin-2 in the constitutive internalization of CCR1, HA-tagged receptor was expressed in mouse embryonic fibroblasts (MEFs) isolated from wild-type and β-arrestin-2$^{-/-}$ mice. Cell surface receptor was pre-labeled with anti-HA-Alexa Fluor 594® and incubated at 37°C as described above. CCR1 underwent constitutive internalization in wild-type MEF cells as shown by the formation of discrete intracellular puncta after incubation for 30 min compared to the plasma membrane localization of the receptor at the 0 min timepoint (**Fig. 2.6**, top panels). By contrast, HA-CCR1 expressed in β-arrestin-2-deficient MEF cells was significantly impaired in its ability to be basally down-regulated (**Fig. 2.6**, bottom panels). These data, together with the previous observations of constitutive association, demonstrate that β-arrestin-2 is an important mediator of agonist-independent CCR1 internalization.

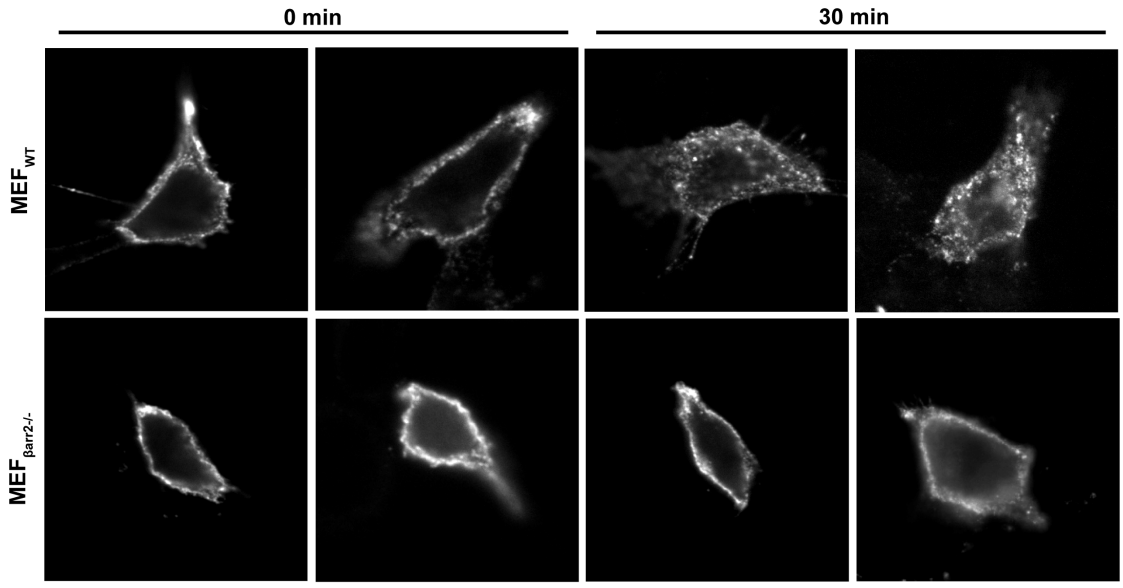


**FIGURE 2.6**. Constitutive internalization of CCR1 is mediated by β-arrestin-2. Wild-type and β-arrestin-2-deficient mouse embryonic fibroblasts (MEFs) were transiently transfected on coverslips with HA-CCR1 and cell surface receptor was pre-labeled with anti-HA Alexa Fluor® 594. Cells were then warmed with serum-free media without agonist for 30 min and the extent of receptor internalization was observed using a confocal microscope.

*CCR1 constitutively interacts with both $G\alpha_i$ and β-arrestin-2* — The observation of constitutive G protein-mediated migration and PT-insensitive β-arrestin-2-mediated internalization suggests the possibility that CCR1 can coordinate both signaling and regulatory molecules. The BRET assay was therefore used to demonstrate whether CCR1 exhibited ligand-independent coupling with $G\alpha_i$. HEK293 cells expressing CCR1-YFP and one of two variants of $G\alpha_i$-Rluc (with the Rluc sequence inserted into one of two loops of $G\alpha_i$ at positions 60 or 122, neither of which affects G protein function (63))

exhibited a basal $BRET1_{net}$ signal significantly above background (**Fig. 2.7A**). Both $G\alpha_i$-60-Rluc and $G\alpha_i$-122-Rluc were tested to ensure the positioning of the luciferase did not yield an artificially positive signal. M3-YFP, which does not couple to $G\alpha_i$, did not yield an appreciable $BRET1_{net}$ signal when expressed at similar levels as CCR1-YFP in cells with either $G\alpha_i$-Rluc construct. To further confirm the specificity of the basal $G\alpha_i$ association with CCR1, a BRET saturation assay was carried out in which CCR1-YFP expression was steadily increased in cells expressing a constant level of $G\alpha_i$-Rluc or $G\alpha_{12/13}$-Rluc, which was included as a negative control. When the $BRET1_{net}$ signal is plotted against the YFP:Rluc expression level, and the result is a hyperbolic saturation curve as opposed to a quasi-linear and non-saturable one, the interaction is believed to be specific (46). Indeed, the result of the BRET saturation curve between CCR1-YFP and $G\alpha_i$-Rluc indicates the interaction is specific with a slightly higher signal resulting from the CCR1-YFP/$G\alpha_i$-122-Rluc pair compared to CCR1-YFP/$G\alpha_i$-60-Rluc (**Fig. 2.7B**). As a negative control, CCR1-YFP and $G\alpha_{12/13}$-Rluc were examined and exhibited a linear, non-saturable BRET curve indicative of a non-specific interaction (**Fig. 2.7B**).

Recent reports involving the intermolecular interactions of constitutively active GCPRs have brought into question the dogmatic idea that G protein coupling to and β-arrestin association with the same receptor are mutually exclusive (64). Given the data that CCR1 is basally associated with both $G\alpha_i$ and β-arrestin-2, the question arose whether it associates with $G\alpha_i$ and β-

arrestin-2 in a multi-protein assembly or as functionally distinct CCR1 populations. Accordingly, the effect of CCR1 expression on the interaction between $G\alpha_i$ and β-arrestin-2 was examined. HEK293 cells stably transfected with CCR1 under a doxycycline inducible expression system were co-transfected with $G\alpha_i$-122-Rluc and β-arrestin-2-YFP. In the absence of significant CCR1 expression, $G\alpha_i$ and β-arrestin-2 exhibited a small basal $BRET1_{net}$ signal (**Fig. 2.7C**). However, when CCR1 expression was upregulated by exogenous doxycycline addition, the agonist-independent $BRET1_{net}$ signal between $G\alpha_i$ and β-arrestin-2 was significantly increased. Additionally, activation of these cells with 1 μM CCL14 resulted in a relatively rapid and stable increase in the $BRET1_{net}$ signal suggesting either closer proximity of the two proteins or a conformational change that places the YFP and Rluc into a more permissive orientation for energy transfer (**Fig. 2.7D**). Together, these data suggest the constitutive formation of a receptor:G protein:β-arrestin complex that remains stably associated upon receptor activation. However, one cannot exclude the presence of functionally distinct subpopulations of receptor as well (e.g. CCR1:G protein and CCR1:β-arrestin-2).

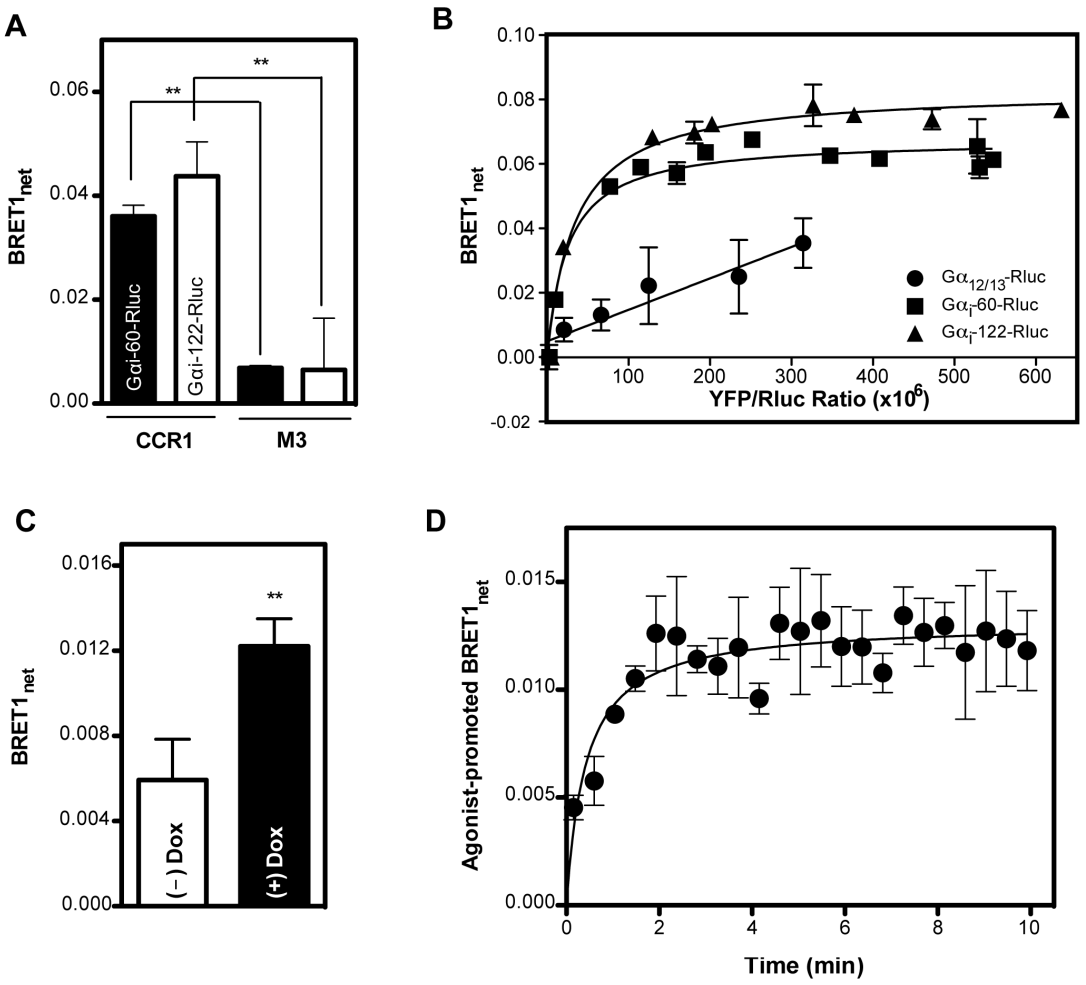


**Figure 2.7. CCR1 is constitutively associated with $G\alpha_i$ and forms a basal complex with G protein and β-arrestin. A.** HEK293 cells were transiently co-transfected with CCR1-YFP or M3-YFP and $G\alpha_i$-Rluc with insertion of the luciferase at position 60 ($G\alpha_i$-60-Rluc, black bar) or 122 ($G\alpha_i$-122-Rluc, white bar) to test for effects of Rluc orientation. Fourty eight hours post transfection the basal $BRET1_{net}$ value was measured. **B.** BRET saturation assay in which the expression level of $G\alpha_i$-60-Rluc (■), $G\alpha_i$-122-Rluc (▲), or $G\alpha_{12/13}$-Rluc (●) is kept constant while the expression of CCR1-YFP is continually increased. The respective curves indicate a specific association between CCR1-YFP and either of the $G\alpha_i$-Rluc constructs but not for $G\alpha_{12/13}$-Rluc . **C.** FLAG-CCR1/HEK293/TetO cells in which CCR1 expression can be induced with doxycycline were transfected with both $G\alpha_i$-122-Rluc and β-arrestin-2-YFP. The change in the $BRET1_{net}$ signal between $G\alpha_i$ and β-arrestin-2 in the absence (white bar) and presence (black bar) of 2 μg/mL doxycycline is shown. The statistical significance was calculated using an unpaired t test (**, $p$<0.01)(GraphPad Prism®). **D.** The same cells as in C were stimulated with 1 μM CCL14 and the effect of the agonist on the $BRET1_{net}$ signal over time is shown.

*CCR1 forms a constitutive homo-oligomer* — A question immediately arises as to how CCR1 is able to structurally accommodate concurrent association with G protein and β-arrestin. Previous studies have suggested GPCR oligomerization may account for the formation of multi-protein complexes at the cytoplasmic surfaces of the receptors (27, 65, 66), and indeed, many chemokine receptors have been shown to homo- and hetero-oligomerize in cells (67-69). Furthermore, five crystal structures of CXCR4 from different space groups all revealed similar dimers (70). However, the propensity of CCR1 to homo- or hetero-oligomerize has not yet been reported. In a BRET saturation assay with cells expressing CCR1-Rluc and CCR1-YFP, M3-YFP, or γ-aminobutyric acid B2 receptor-YFP (GABA-YFP), only the CCR1-Rluc/CCR1-YFP pair yielded a hyperbolic saturation curve indicative of a specific BRET signal whereas M3 and GABA yielded quasi-linear association curves indicating non-specific associations (**Fig. 2.8A**). To further confirm the specificity of the homo-oligomerization of CCR1, a variant of the BRET saturation assay was used in which the expression ratio between the YFP- and Rluc-tagged proteins is kept constant while the overall density of the receptors in the cell is increased. In this assay, a specific interaction is demonstrated by a linear curve with a non-zero Y-axis intercept (71), as was observed for the CCR1-Rluc/CCR1-YFP interaction as opposed to CCR1-Rluc/M3-YFP (**Fig. 2.8B**). Therefore, it seems that CCR1 is capable of forming either receptor clusters or more well-defined oligomeric complexes, minimally

containing two subunits. While the role of oligomerization in GPCR signaling and regulation remains unclear, such complexes may provide the surface area needed to overcome the steric hindrance of concurrent G protein and β-arrestin binding to CCR1 (72, 73).

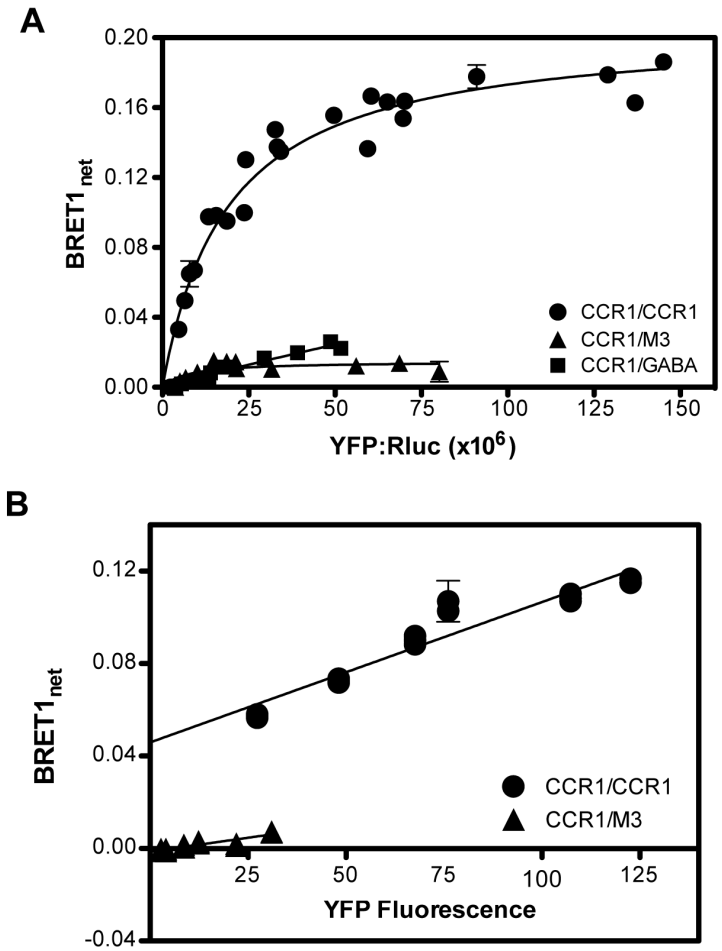


**Figure 2.8. CCR1 forms a specific homo-oligomer. A.** BRET saturation assay in which energy donor (CCR1-Rluc) expression levels are kept constant while the expression of energy acceptor (receptor-YFP) is continually increased in order to compare the homo-oligomerization of CCR1 (●) with hetero-oligomerization with the M3 (▲) or GABA(B2) (■) receptor. A non-linear and saturable curve is indicative of a specific interaction between the two proteins being studied. **B.** Type II BRET saturation assay in which the expression ratio between the energy donor (CCR1-Rluc) and energy acceptor (receptor-YFP) is kept constant while the overall expression of both proteins is continually increased in order to compare homo-oligomerization of CCR1 (●) with hetero-oligomerization with the M3 (▲) receptor. A linear relationship with a non-zero intercept is indicative of a specific interaction.

*CCR1 internalizes chemokine independently of G protein activation —* Based on the above data, it appears that CCR1 possesses significant constitutive activity. However, as for many other constitutive GPCRs, the functional relevance of this behavior is not known. Several atypical chemokine receptors (e.g. D6, DARC and CXCR7), classified as such because they do not couple to G proteins, have been shown to exhibit chemokine scavenging activity defined as receptor-mediated internalization of chemokine without G protein-mediated signaling (74-76). Scavenging activity of G protein-coupled chemokine receptors has also been suggested on the basis of elevated levels of ligands in the serum and central nervous system tissue of chemokine receptor knockout mice (77). Finally, a direct demonstration of the ability of CCR2 on human monocytes to scavenge CCL2 in the presence of PT was recently reported (55). To test whether CCR1 could be acting in a similar manner, CCR1/L1.2 cells were tested for their ability to internalize Cy3B-labeled CCL7 in the presence and absence of PT. The effect of CCR1 inhibition with BX-471 was also tested and the results with CCR1/L1.2 cells compared to untransfected and CCR5-expressing L1.2 cells. Non-treated CCR1/L1.2 cells showed significant internalization of CCL7-Cy3B over time while CCR5/L1.2 and untransfected L1.2 cells exhibited only minimal background binding to the chemokine, suggesting specificity of the CCL7-Cy3B interaction with CCR1 (**Fig. 2.9**). The specificity was further evidenced by the significant reduction of CCL7-Cy3B uptake by CCR1/L1.2 cells in the

presence of BX-471. Critically, CCL7-Cy3B uptake was only minimally affected by PT treatment, demonstrating that CCR1 is capable of internalizing chemokine independent of G protein activation and consistent with a potential scavenging behavior.

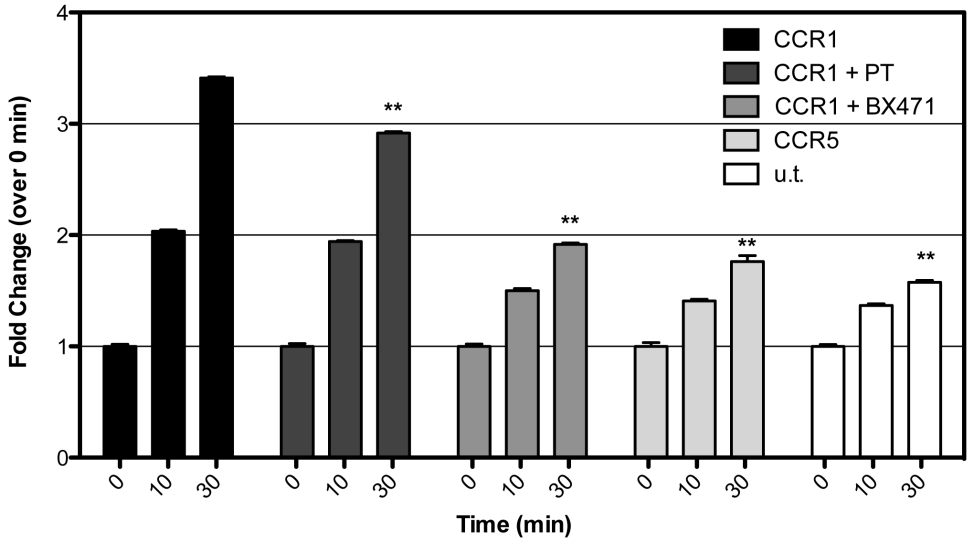


**Figure 2.9. CCR1-mediated internalization of CCL7-Cy3B and effect of G protein inactivation.** CCR1/L1.2 cells were treated in the absence or presence of 0.2 μg/mL pertussis toxin (PT) or 100 μM BX-471 for 1 h prior to incubation with CCL7-Cy3B for up to 30 min at 37°C. CCR5/L1.2 and untransfected (u.t.)/L1.2 cells were included as controls. Internalization of CCL7-Cy3B was measured in triplicates as the median fluorescence intensity (MFI) of cells analyzed on a Guava® EasyCyte™ Flow Cytometer. The data are displayed as fold change of MFI over un-stimulated cells. The statistical significance of the difference in fold-change MFI is displayed for the 30 min timepoint and compared to untreated CCR1/L1.2 using a two-way ANOVA with Bonferroni post-tests, ** $p<0.01$ (GraphPad Prism®).

## 2.5 DISCUSSION

In this report we demonstrated that heterologous or endogenous expression of CCR1 is sufficient to induce basal migration of both murine and human leukocytic cell lines and agonist-independent G protein signaling in HEK293 cells. Constitutive activity amongst members of the chemokine receptor family has been reported previously; however, the data is limited and this study represents the first to identify CCR1 as a constitutively active receptor. The wild-type sequence of the related CCR3 receptor has been shown to exhibit constitutive activity as measured by GTPγS binding in CCR3-expressing CHO membranes (35). In this study the CCR3-specific small molecule inhibitor Banyu (I), whose previous inverse agonist efficacy was not known, inhibited basal GTPγS binding to membranes. Similarly in the present study, the CCR1-specific inhibitor BX-471, also previously thought to be a competitive antagonist, acted as an inverse agonist to inhibit the basal migration activity of CCR1. CCR4 also showed evidence of constitutive activity on the basis of ligand-independent increases in F-actin content correlating directly with increasing CCR4 expression in primary human $CD4^+$ T cells (36). Interestingly, in this case none of the several CCR4 inhibitors tested were able to reduce the constitutive activity of the receptor. Amongst the CXC-class of chemokine receptors only CXCR1 has been shown to possess constitutive activity (39); an analysis of the intensely studied CXCR4 in Sf9 insect cell membranes demonstrated no basal signaling (78). While GPCR constitutive

activity is sometimes criticized as being dependent upon the cell line being used, the expression level of the receptor, or the functional assay employed to identify constitutive activity, it is noteworthy that CCR1 exhibited considerable basal activity in the context of multiple cell lines from different mammalian species, in all signaling and physical interaction assays tested, and at endogenous levels of expression.

The observation that CCR1 is constitutively active motivated subsequent studies on the regulation and trafficking of the receptor, and its interactions with intracellular proteins. Recent studies have suggested that careful regulation of chemokine receptor activity may be more important than the activation of the receptor itself (14). Classically, agonist activation of a GPCR initiates a G protein-signaling cascade followed by phosphorylation of receptor intracellular loops by GPCR kinases (GRKs) or second messenger kinases (such as PKA or PKC) (79). Phosphorylation uncouples the receptor from G proteins and allows recruitment of β-arrestin. In turn, β-arrestin sterically occludes the receptor from further coupling to G proteins and facilitates its association with clathrin-coated pits, leading to internalization (80). Many subsequent factors determine whether the receptor is recycled back to the membrane or destined for degradation. The regulation of constitutively active receptors in the absence of agonist is less clear. Beginning with the discovery that a constitutively active $\beta_2$ adrenergic receptor mutant is basally phosphorylated and continually internalized (81), a variety of scenarios have since been reported. For example, a constitutively active

mutant of CXCR4 was shown to be basally phosphorylated and internalized (43, 82), while the D6 decoy chemokine receptor is constitutively internalized in a phosphorylation-independent manner (83). Additionally, the dopamine D4 receptor is constitutively phosphorylated but not internalized (84). Therefore, whether or not the constitutively active CCR1 was continually internalized was addressed first, followed by whether basal phosphorylation was evident.

In multiple cell systems using either heterologously or endogenously expressed protein, CCR1 exhibited significant constitutive internalization. These systems included human HEK293, HeLa, and THP-1, simian COS-7, and murine L1.2 cells labeled with antibodies directed against the receptor itself or an epitope tag on the N-terminus. Therefore, CCR1 constitutive activity appears to be an intrinsic property allowing the receptor to adopt an internalization-competent conformation or set thereof in multiple cellular environments. Agonist stimulation increased the rate of internalization as evidenced by more rapid removal of CCR1 from the cell surface in HEK293 cells and increased localization of CCR1 within intracellular vesicles in HeLa and COS-7 cells. Interestingly, inhibition of CCR1 with the specific antagonist BX-471 had no effect on constitutive internalization, suggesting that it is a neutral antagonist, whereas it acted as an inverse agonist towards constitutive cell migration. Constitutive internalization was also shown to be independent of $G\alpha_{i/o}$ activation as PT treatment did not affect the amount of CCR1 remaining at the cell surface. This result is similar to previous studies of CCR2 where $G\alpha_i$ activation was necessary for leukocyte migration but not for β-

arrestin recruitment and receptor internalization (55, 85). Finally, it was demonstrated that constitutively internalized CCR1 is recycled back to the plasma membrane to maintain a constant level of receptor at the cell surface.

Consistent with its propensity to constitutively internalize, CCR1 exhibited significant basal phosphorylation. In fact, only a minor increase in phosphorylation was observed following CCL14 addition. Furthermore, the constitutive phosphorylation was correlated with the ability of CCR1 to constitutively associate with β-arrestins as demonstrated in multiple assays (BRET, co-immunoprecipitation and confocal microscopy). Similar to its phosphorylation status little additional β-arrestin was recruited after CCL14 stimulation. Basal phosphorylation has been shown to occur in many constitutively active wild-type and mutant GPCRs including CXCR4 (43), the HCMV-encoded GPCR US28 (86), lutropin/choriogonadotropin (LH) receptor (87), and vasopressin 2 receptor (V2R) (88), among others. While receptor phosphorylation is not absolutely necessary for recruiting β-arrestins (62), many of these studies demonstrated a reliance upon Ser/Thr phosphorylation for basal or agonist-induced β-arrestin association as seems to be the case for CCR1. However, while atypical chemokine receptors that do not exhibit the capacity to signal through G proteins have previously been shown to form agonist-independent complexes with β-arrestins (27, 83); this behavior has not previously been observed for any other G protein-coupled chemokine receptor. This non-canonical interaction was specific for CCR1 compared to the other chemokine receptors tested in this study, suggesting that CCR1 has

unique structural traits that set it apart from other receptors that require agonist binding to drive β-arrestin association. As with constitutive internalization, BX-471 had no effect on the agonist-independent association between CCR1 and β-arrestin-2**,** suggesting that it is a functionally selective ligand permissive to basal phosphorylation of CCR1 and β-arrestin-2 association while antagonistic to G protein activation. The pre-coupling of CCR1 and β-arrestin-2 was also shown to be required for constitutive internalization as CCR1 expressed in MEFs lacking β-arrestin-2 remained at the cell surface while receptor expressed in wild-type MEFs exhibited significant constitutive internalization. Thus in the absence of agonist, CCR1 adopts a conformation (or set thereof) resulting in basal phosphorylation of the receptor, interaction with β-arrestin-2 and removal of CCR1 from the plasma membrane by a β-arrestin-2-dependent, G protein-independent mechanism.

The question remained as to how CCR1 could apparently induce cell migration while being phosphorylated and associated with β-arrestins? A recent study on the signaling and regulatory proteins associated with the constitutively active relaxin family peptide 1 (RXFP-1) receptor challenged the canonical view that G protein coupling and binding of β-arrestin are mutually exclusive (64, 89). RXFP1 was shown to constitutively form a multi-protein "signalsome" complex at its intracellular surface that contained both positive and negative modulators of cAMP production including G protein, β-arrestin, a protein kinase, a phosphodiesterase, and a scaffolding protein. This complex

was hypothesized to allow for rapid and fine-tuned regulation of RXFP1 signaling and led our laboratory to consider the possibility that CCR1 could functionally coordinate more than one protein at a time. Using the BRET assay, CCR1 was shown to exhibit significant basal pre-coupling to $G\alpha_i$ in a specific manner. Thus, taken together with its constitutive coupling with β-arrestin-2, it appears that CCR1 associates (or is in close proximity to) both $G\alpha_i$ and β-arrestin-2. Furthermore, direct proximity between $G\alpha_i$-122-Rluc and β-arrestin-2-YFP was demonstrated via BRET in cells with or without co-expression of unlabeled CCR1. While a small basal signal was observed in cells lacking CCR1, induction of CCR1 expression significantly increased the BRET signal between $G\alpha_i$ and β-arrestin-2. The specificity of this CCR1-mediated complex was further demonstrated by activation with CCL14, which further increased the BRET signal over time. Since co-immunoprecipitation experiments suggested that CCL14 does not induce additional β-arrestin-2 recruitment, this result likely represents either a decrease in the distance separating $G\alpha_i$ and β-arrestin-2 or a conformational rearrangement of YFP and luciferase that places them into a more favorable orientation for energy transfer. Either way, the signal reached a plateau approximately 2 min after CCL14 stimulation and remained stable for the remainder of the assay suggesting that $G\alpha_i$ and β-arrestin-2 do not dissociate following receptor activation but instead remain complexed with CCR1 as it presumably undergoes internalization. This finding is not unprecedented as other GPCRs (e.g. dopamine D4, calcium sensing receptor, and mutant V2R) have exhibited

constitutive association with β-arrestins that is undiminished following receptor activation despite clear evidence of G protein signaling (90-92) indicating stable complex formation.

How CCR1 is physically able to interact with both G protein and β-arrestin was then considered. Structural analyses and experimental modeling of β-arrestin binding to the intracellular domains of a GPCR indicate an extensive surface area of contact that would effectively preclude G protein coupling (93). On the other hand, the crystal structure of the β2 adrenergic receptor in complex with Gs did not reveal contacts between helices VII and VIII (the latter previously implicated in β-arrestin binding) opening the possibility for association with other proteins (94). While it remains unclear whether a monomeric GPCR can accommodate concurrent association with multiple proteins, receptor oligomerization could provide sufficient intracellular domain surface area to support the formation of a multi-protein complex. Indeed dimeric receptors have been reported as functional units for β-arrestin binding, including the CXCR4/CXCR7 heterodimer recently shown to constitutively recruit β-arrestin-2 (27). Therefore, the propensity for CCR1 to form homo-oligomers was tested. Using two types of BRET saturation assays CCR1 was shown to clearly form homo-oligomeric complexes, thereby providing a plausible explanation for concurrent G protein/β-arrestin association with the receptor. Despite their apparent proximity in a multiprotein complex it is still unclear whether CCR1:G protein and CCR1:β-arrestin or CCR1:β-arrestin:G protein complexes act as functionally distinct units. For

example there may be CCR1 populations that are only coupled to β-arrestin and involved in constitutive internalization and recycling and other assemblies of receptor:G protein:β-arrestin that are involved in canonical agonist-dependent signaling and internalization.

The functional relevance and mechanism of CCR1 constitutive activity remains to be more thoroughly investigated. However, the data presented here suggests that constitutive internalization and recycling of CCR1 could be associated with a chemokine scavenging function of the receptor. Mechanistically, internalization of CCL7 was not inhibited by PT providing evidence that it is capable of internalizing ligand separate from activation of canonical $G\alpha_i$ signaling. Moreover constitutive CCR1 internalization required β-arrestin. This mechanism shows parallels with that of the "professional" scavenging chemokine receptor, D6, which also utilizes a G protein-independent and β-arrestin-dependent pathway (95). Moreover, in this same publication, it was noted that uncoupling of CCR5 from a G protein-dependent pathway was insufficient to convert this chemotactic receptor into a chemokine scavenger and that other specific structural/signaling features are required. CCR1 constitutive activity and its persistent association with β-arrestin could very well be that missing signaling feature that allows a canonical chemokine receptor to convert to a scavenging modality. However, in contrast to D6, CCR1 possesses the ability to signal through both a canonical G protein pathway as well as through a G protein-independent/β-arrestin-dependent internalization/recycling pathway. Given that this receptor is activated by at

least 10 pro-inflammatory chemokines, consumption of ligand without activation of G protein could represent a means by which CCR1 can remodel the local concentration of the chemoattractant gradient while maintaining receptor responsiveness. However, further experiments are necessary to determine whether CCR1 can mediate leukocyte migration along an increasing gradient of chemokine without desensitization while continuously consuming ligand.

In summary, this report has shown for the first time that CCR1 exhibits constitutive activity sufficient to induce agonist-independent migration of multiple CCR1-expressing cell types. The receptor undergoes continual internalization mediated by constitutive phosphorylation and association with β-arrestin-2. Surprisingly, CCR1 was also shown to be concurrently engaged in a complex with both β-arrestin-2 and $G\alpha_i$, which can possibly be explained by the formation of CCR1 homo-oligomers. While possible that separate sub-populations of CCR1 are selectively engaged with either G protein or β-arrestin, the data provides a model of a CCR1 “signalsome” that facilitates close proximity of the receptor with signaling and regulatory proteins enabling agonist-independent signal transduction from some receptors and continuous down-modulation from others (**Fig. 2.10**). Chemokine scavenging by non-signaling CCR1 may serve as a functional explanation for this behavior. The constitutive activity of CCR1 also suggests a new pharmacological axis for drug development. In principle, the non-canonical behavior of CCR1 could be exploited in the context of inflammatory diseases with drugs that block G

protein activation but are permissive or agonistic for the non-canonical β-arrestin-mediated receptor internalization and chemokine scavenging.

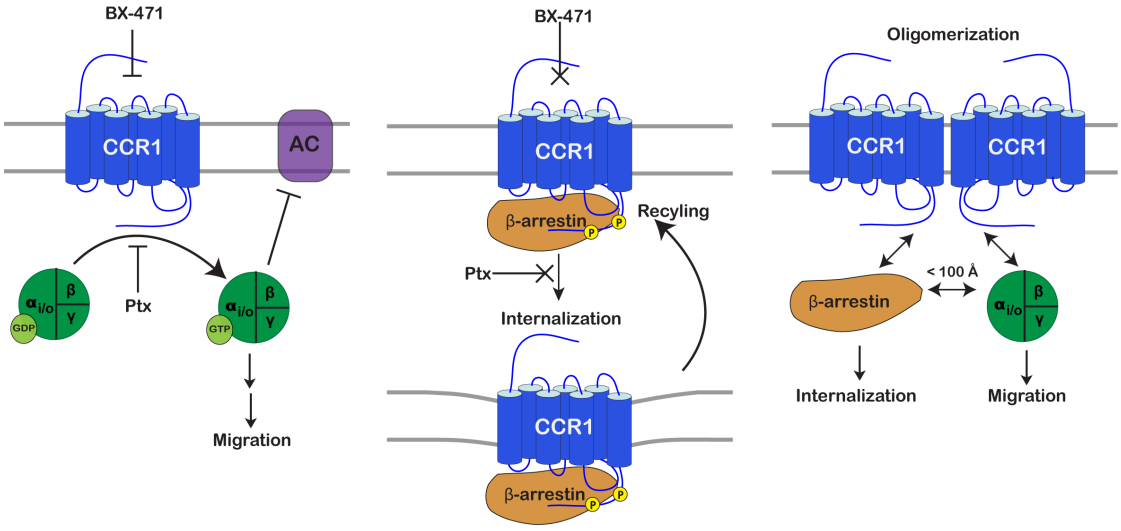


**Figure 2.10. Model of CCR1 constitutive activity**. CCR1 expression is sufficient for inducing basal migration and G protein signaling, which can be blocked with a CCR1-specific inhibitor or pertussis toxin (PT) treatment (*left*). At the same time, CCR1 is also constitutively phosphorylated leading to β-arrestin-2 recruitment, receptor internalization and recycling (*middle*). The fate of the internalized receptor and whether it is sent for degradation or eventually recycled back to the cell surface in the presence of ligand stimulation remains to be determined. CCR1 inhibition with BX-471 was unable to block constitutive internalization or prevent basal association with β-arrestin-2. Additionally, a pre-formed complex that brings CCR1, $G\alpha_i$ and β-arrestin-2 into close proximity may provide precise regulation of signal transduction by a constitutively active or agonist-activated receptor. The observation that CCR1 forms a homo-oligomer may also explain how the receptor is physically able to form concurrent interactions with these intracellular proteins (*right*).

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## 2.7 Acknowledgements

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# CHAPTER 3

## STRUCTURAL ANALYSIS OF THE CONSTUTIVE ACTIVITY OF CCR1 AND ITS BASAL ASSOCIATION WITH β-ARRESTIN-2

### 3.1 Abstract

It has recently been discovered that the chemokine receptor CCR1 possesses significant constitutive activity; however, the structural basis of this behavior is unknown. The purpose of this study was to identify the residues responsible for CCR1 basal phosphorylation and ligand-independent association with β-arrestin-2 and $G\alpha_i$. Using the bioluminescence resonance energy transfer (BRET) assay, mutation or deletion of a Ser/Thr-rich cluster in the receptor distal C-terminal tail was shown to reduce CCR1 basal phosphorylation, ablate constitutive β-arrestin-2 interaction, and inhibit receptor desensitization and internalization. Chimeric CCR1 receptors with ICL3 and C-tail domains from CCR5 demonstrated that the C-tail is necessary, but not sufficient, for basal pre-coupling to β-arrestin-2. Lastly, select point mutations within the TM domains and conserved DRY motif were shown to affect CCR1 constitutive activity. This report provides an initial picture of the structural aspects of CCR1 that are involved in maintaining the receptor in a constitutively active state and modulating its behavior.

### 3.2 Introduction

G protein coupled receptors (GPCRs) are capable of undergoing spontaneous isomerization from an incactive to an active state resulting in elevated basal signaling to effector proteins and consequent cellular responses (1). This ligand-independent behavior, known as constitutive activity, has been demonstrated for a number of both wild-type and mutant receptors and may be an intrinsic property of most (if not all) members of the GPCR family (2). While the amount of high resolution structural data on receptors is rapidly expanding (3), the majority of available structures are snapshots of the inactive/antagonist-bound state leaving relatively little information on the precise molecular mechanisms involved in receptor activation. From studies on constitutively active mutant GPCRs, coupled with patterns of sequence conservation amongst the class A/rhodopsin-like receptors, common structural motifs within the transmembrane helices (TMs) have been identified as potentially contributing to stabilizing the receptor in a basally active state (4).

Our laboratory has recently shown for the first time that CCR1 exhibits constitutive activity sufficient to induce agonist-independent migration of CCR1-expressing leukocytic cell types (see Chapter 2). The receptor undergoes continual internalization mediated by constitutive phosphorylation and ligand-independent association with β-arrestin-2. Surprisingly, CCR1 was also shown to be concurrently engaged in a complex with both β-arrestin-2 and $G\alpha_i$, which can possibly be explained by the formation of CCR1 homo-

oligomers. The data provides a model of a CCR1 “signalsome” that facilitates close proximity of the receptor with signaling and regulatory proteins enabling agonist-independent signal transduction from some receptors and continuous down-modulation from others. Chemokine scavenging by non-signaling CCR1 was proposed and initially verified as a functional explanation for this behavior. Constitutive activity is unique to CCR1 compared to the other receptors tested, suggesting it possesses structural traits that set it apart from chemokine receptors requiring agonist binding to drive activation and internalization.

While many proteins have been shown to directly bind to GPCRs and regulate their function, the arrestin family of proteins modulate receptor signaling over the greatest diversity of GPCRs. Historically, it was thought that arrestins could only be recruited to agonist-occupied and phosphorylated receptors (5); however, recent studies including ours have shown that some GPCRs are able to interact with β-arrestins in a ligand-independent manner (6, 7). The arrestins are typically recruited to a GPCR following phosphorylation of serine or threonine residues primarily in the carboxy-terminal tail region and/or intracellular loop 3 (ICL3) (5). Some data are available regarding the structural motifs within various chemokine receptors that mediate receptor internalization and association with regulatory proteins, including β-arrestins. However, there is little consensus amongst this family of GPCRs as to the importance of different intracellular domains as well as the necessity for Ser/Thr phosphorylation. (8-12). Given this heterogeneity, coupled with the fact that relatively little is known about the mechanisms

surrounding CCR1 desensitization (under either constitutive or agonist-induced conditions), one purpose of this study was to determine which amino acid residues in the receptor are essential for basal phosphorylation and β-arrestin-2 association and to measure their effect on CCR1 signaling and internalization. Additionally, residues in the receptor transmembrane domains and conserved DRY motif were selectively mutated to measure their effect on constitutive activity through basal association with β-arrestin-2 and $G\alpha_i$. These data collectively provide an initial picture of the structural aspects of CCR1 that are involved in maintaining its constitutively active state.

**3.3 Experimental procedures**

*Cell Culture and Transfections* — HEK293t cells, which express the SV40 large T antigen, were maintained in Dulbecco's modified Eagle medium (DMEM) with Glutamax (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% $CO_2$. Transfection of HEK293t cells was carried out in 6-well plates at 50-60% confluency using TransIT-LT1 reagent (Mirus Bio) per the manufacturer's protocol.

*Bioluminescence Resonance Energy Transfer (BRET) Assay* — The BRET assay protocol has been described extensively elsewhere (13-15). In brief, the chemokine receptors CCR1 and CCR5 were genetically fused to *A. victoria* YFP on the receptor C-terminus without its stop codon followed by a short linker in a pcDNA3.1 vector (receptor-pYFP3.1). The *R. reniformis* luciferase (Rluc) genetic sequence was fused to the C-terminus of β-arrestin-2

in the phRluc-N2 vector. YFP and Rluc constructs were generous gifts from M. Bouvier, University of Montréal, Québec, Canada. HEK293t cells were transiently transfected in defined stoichiometric ratios of each vector, usually 1.5-2 µg of a receptor-YFP construct and 0.05-0.1 µg of β-arrestin-2-Rluc. 48 h post-transfection, cells were washed and suspended in phosphate buffered saline (PBS) supplemented with 0.5% (w/v) glucose. 1 x $10^5$ cells were aliquoted in triplicate into each well of a white, clear bottom 96-well plate (BD Biosciences) and incubated for 1h at 37°C prior to BRET measurement. YFP-tagged receptor expression was quantified by measuring fluorescence of the wells at 485nm excitation and 538nm emission wavelengths on a SpectraMax fluorescence spectrometer (Molecular Devices). The luciferase substrate coelenterazine-*h* (Biotium) was added to a final concentration of 50 µM in each well 10 min prior to the beginning of the BRET assay. Luminescence and fluorescence measurements were collected at room temperature with 1 sec exposure times using a VictorX Light multilabel plate reader (Perkin-Elmer) at repeating time intervals. The BRET signal was calculated as the ratio of YFP emission (550±40 nm) to Rluc emission (470±30 nm). The $BRET_{net}$ signal is calculated by subtracting the background BRET ratio of cells expressing only the Rluc fusion from the BRET ratio of cells expressing both the YFP- and Rluc-fused proteins. Ligands were diluted in PBS and added following incubation with coelenterazine-*h* but prior to BRET measurement. Mutations in the CCR1 sequence were introduced using the QuikChange site-directed mutagenesis kit (Stratagene).

*Co-Immunoprecipitation and Western Blotting* — HEK-293t cells were transfected as above in 6-well plates with FLAG-CCR1 and β-arrestin-2-HA constructs in pcDNA3.1 vectors. 48 h after transfection, cells were washed with ice-cold PBS and lysed in radio-immunoprecipitation assay (RIPA) buffer with protease inhibitor mixture and phosphatase inhibitor cocktail II and III (Sigma-Aldrich) for 1 h at 4°C. The supernatant fraction of the cell lysate was collected after 10 min of centrifugation at 20,000xg. Anti-FLAG M2 affinity gel (Sigma-Aldrich) was added to the sample and incubated overnight at 4°C on a rotating platform. The affinity gel was then washed 3-4 times in RIPA buffer, and elution performed with 3X-FLAG peptide. Eluted proteins were mixed with 5X Laemmli buffer and separated via SDS-PAGE in a 10% polyacrylamide gel. The presence of FLAG-CCR1 and β-arrestin-2-HA was measured by Western blotting and ECL Plus chemiluminescent detection (GE Healthcare) using anti-FLAG (Sigma-Aldrich) and anti-mouse-HRP (horseradish peroxidase, Promega) and anti-HA-HRP (Roche) monoclonal antibodies. For ERK1/2 phosphorylation analysis, HEK293t cells were transfected as above with CCR1 or the C-tail Ser/Thr mutants C4 or CtSTA. 24 h post transfection, cells were serum-starved overnight and treated with 10 μM BX-471 for 1 h or 1 μM CCL14 for 6 min the following day. Lysates were prepared as above and 25 μg protein from each sample subjected to Western blotting using anti-phospho or anti-total ERK1/2 antibodies (Upstate).

*CCR1 Phosphorylation* — HEK293t cells were transiently transfected in 6-well plates as described above with vector containing FLAG-CCR1 or FLAG-

CCR1 with various C-terminal tail mutations. 48 h post-transfection, cells were labeled with 250 μCi [$^{32}$P] orthophosphate (Perkin Elmer) in phosphate-free DMEM for 2 h at 37°C. Cells were incubated with or without 1 μM CCL14 for 6 min and then lysed in RIPA buffer supplemented with protease inhibitor mixture (Sigma) and phosphatase inhibitor cocktail II and III (Sigma) for 1 h at 4°C. Cell lysates were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma) and immunoprecipitates were resolved by SDS- PAGE and transferred to PVDF membrane. Membranes were analyzed by autoradiography and then probed with anti-FLAG (Sigma) antibody to detect FLAG-CCR1 expression by Western blotting.

*Calcium Mobilization and Receptor Desensitization* — HEK293t cells were transiently transfected as above with 1.5-2 μg FLAG-receptor vector with TransIT-LT1 (Mirus Bio). 48 h post-transfection, cells were lifted off the plate with 1 mM EDTA and resuspended in assay buffer (1X Hanks balanced salt solution, HBSS, 20 mM HEPES pH 7.4, and 0.1% (w/v) bovine serum albumin, BSA) at a concentration of 1.75 x $10^6$ cells/mL. 100 μL of the cell suspension was aliquoted in triplicate into a black, clear bottom Biocoat assay plate (BD Biosciences). 100 μL of assay dye from the FLIPR calcium 4 explorer format kit (Molecular Devices) was added to each well, and the plate was incubated for 1 h at 37°C. Receptor ligands were diluted in assay buffer in a V-bottom 96-well assay plate. Calcium mobilization at 37°C was measured by fluorescence excitation of the dye following ligand addition at two timepoints (16 sec and 250 sec) using a Flex Station 3 microplate reader (Molecular

Devices). Data was plotted as the percent of the maximal signal obtained after the first ligand injection.

*Flow Cytometry and Receptor Internalization* — HEK293 cells stably expressing FLAG-CCR1, FLAG-CCR1 CtSTA, or FLAG-CCR1 340-43,45-46A (abbreviated C4) were cultured in 6-well plates and stimulated with 1 μM CCL14 for various periods of time. Cells were washed with PBS + 0.5% BSA and cell surface receptor labeled with CCR1 antibody conjugated to phycoerythrin (PE) (R&D Systems). The relative amount of receptor remaining on the surface at each time point was quantified using a Guava flow cytometer (Millipore) and analysed using FlowJo software (Tree Star).

*Computational modeling of CCR1* — An ensemble of 3D models of CCR1 was built using the homology modeling platform of the Internal Coordinate Mechanics (ICM) software (16-18). Multiple structures and chains of CXCR4 (PDB 3odu and 3oe0, (19)) and CCR5 (PDB 4mbs, (20)) were used as homology templates. Initially, well-aligned regions of the target sequence were threaded through the backbone coordinates of the template structure. Loops, insertions and deletions were searched against a large database of PDB fragments for similar sequence and termini topology; well-scoring fragments were incorporated into the nascent model and minimized in its context. For polar backbone atoms and polar side-chain atoms of the conserved residues, template hydrogen bonds were converted into distance restraints; the model side-chains were thoroughly sampled to find the global minimum of the energy function that included soft van der Waals, electrostatic,

hydrogen bonding, torsional strain, and distance restraint terms. To resolve the remaining steric conflicts, each model was subjected to gradient minimization with both side-chain and backbone variables relaxed.

### 3.4 Results

*Ser/Thr residues in the CCR1 C-terminal tail mediate constitutive β-arrestin-2 association* — Binding of arrestin to GPCRs is generally mediated by phosphorylation of threonine and/or serine residues in the exposed intracellular domains of the receptor, primarily intracellular loop 3 (ICL3) and the C-terminal tail (C-tail) (21). Typically, receptor phosphorylation of intracellular domains is carried out by G protein-coupled receptor kinases (GRKs) following receptor activation, while second messenger kinases (PKA, PKC) have been shown to occasionally play a role as well. Some GPCRs have been demonstrated to be constitutively phosphorylated without agonist binding, which was important in mediating their interactions with arrestins (22-24). CCR1 has eight Ser/Thr residues in its C-tail at positions 332, 340-43, 345-36 and 352 (**Fig. 3.1A**), with an additional exposed Ser in ICL3 at position 235. In order to test the importance of residues along the entire length of the CCR1 C-tail, a series of sequential residues were mutated in sets of four to alanine and the impact on the basal $BRET_{net}$ signal between CCR1-YFP and β-arrestin-2-Rluc was measured (**Fig. 3.1B**). Importantly, the constitutive and ligand-independent association between wild-type CCR1 and β-arrestin-2 was confirmed (see Chapter 2, **Fig. 2.5**). Mutation of the amino acid regions

containing S332 (CCR1 332-35A) and S352 (CCR1 352-55A) had a minimal impact on the basal $BRET_{net}$ signal, whereas mutation of the Ser/Thr residues between positions 340-47 (CCR1 340-43A and 344-47A) led to a significant decrease in the basal $BRET_{net}$ value. Truncation of the C-tail at different positions along the CCR1 C-tail confirms that this region of high Ser/Thr residue density is necessary for mediating constitutive β-arrestin-2 association (**Fig. 3.1C**). A receptor with C-tail residues extending to at least position 347 (CCR1d348) was the minimal construct able to yield a high basal $BRET_{net}$ signal. To determine whether Ser/Thr residues at positions SSTS340-43 and ST345-46 are independently or collectively essential for β-arrestin-2 association, each set was mutated and the impact on the basal $BRET_{net}$ signal was measured. CCR1 340-43A (abbreviated C2) and 345-36A (C3) caused a significant reduction in the $BRET_{net}$ signal independently of one another, while mutation of all Ser/Thr residues in the C-tail (CtSTA) caused a slightly greater reduction in the $BRET_{net}$ signal (**Fig. 3.1D**). Additionally, in coimmunoprecipitation experiments between β-arrestin-2 and CCR1 wild-type or C-tail mutants (abbreviated C1-C5 and CtSTA), no basal β-arrestin-2 interaction was observed with the mutants other than C1 and C5, which confirms the observations above of the relative insignificance of Ser332 (C1) and Ser352 (C5) **(Fig. 3.1E)**. Therefore, Ser/Thr residues in both the 340-43 and 345-56 positions are important for constitutive β-arrestin-2 association, suggesting their involvement in receptor basal phosphorylation.

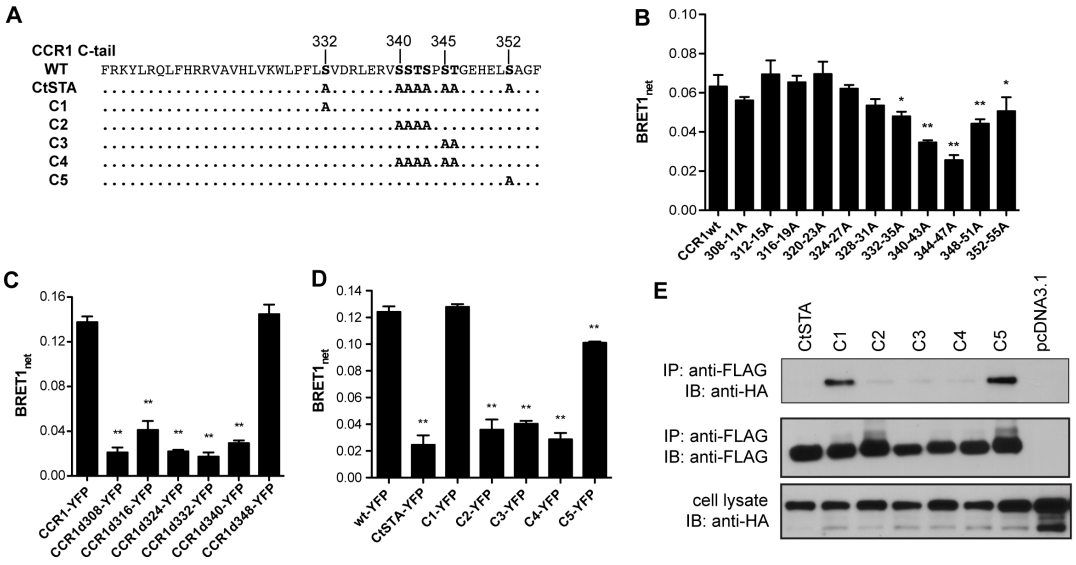


**Figure 3.1. Ser/Thr-rich regions within the CCR1 C-terminus are responsible for constitutive association with β-arrestin-2. A.** Amino acid sequence of the C-terminal tail residues of CCR1 with the Ser/Thr residues identified. The series of receptor C-terminal tail mutants with individual or collective Ser/Thr mutations are shown with their corresponding abbreviations. **B.** The C-terminal tail sequence of CCR1 was sequentially mutated to Ala in series of four amino acids and the effect on the basal $BRET1_{net}$ value between these mutant CCR1-YFP constructs and β-arrestin-2-Rluc was measured. **C.** Alternatively, the C-terminal tail sequence of CCR1 was serially truncated at positions 8 residues apart and the effect on β-arrestin-2 association with CCR1 measured via BRET. **D.** Effect of selective mutation of individual or sets of Ser/Thr residues in the CCR1 C-terminus and the impact on basal $BRET1_{net}$ signal with β-arrestin-2-Rluc. Statistical significance compared to wild-type was calculated using a one-way ANOVA with Dunnetts multiple comparisons post-test, *$p$<0.05, **$p$<0.01. **E.** Co-immunoprecipitation of β-arrestin-2-HA with FLAG-tagged CCR1wt or C-tail mutant receptors in transiently transfected and non-stimulated HEK293t cells.

*CCR1 is basally phosphorylated at a Ser/Thr cluster in the C-terminal tail* — The findings above suggest that the constitutive association between CCR1 and β-arrestin-2 is dependent upon a subset of Ser/Thr residues in the receptor's C-terminal tail. It was previously demonstrated that CCR1 is basally phosphorylated in the absence of agonist (Chapter 2, **Fig. 2.4**); however, it remained to be determined which residues within the intracellular domains of the receptor were involved. Therefore, HEK293t cells expressing wild-type or C-tail Ser/Thr mutant CCR1 were labeled with $^{32}$P orthophosphate and either left untreated or stimulated with CCL14. Wild-type CCR1 was extensively labeled prior to agonist treatment with only a minor increase in phosphorylation following agonist addition, in agreement with earlier observations (**Fig. 3.2)**. The CtSTA mutant exhibited a significant reduction in basal phosphorylation, suggesting that the C-terminal tail contains the entirety of basally phosphorylated Ser/Thr residues. Intriguingly, the majority of receptor phosphorylation was limited to positions 340-43 and 345-46, as evidenced by the lack of significant difference in $^{32}$P labeling between CtSTA and C4. However, when the two clusters of potentially phosphorylated residues were mutated to Ala independently of one another (C2 and C3) some receptor phosphorylation was re-established when only residues 345-46 (C3) were mutated. This suggests that 340-43 may play a more prominent role in receptor basal phosphorylation; however, it is clear from the basal BRET association data that both clusters are important for β-arrestin-2 binding. Also in agreement with previous BRET data was the relative insignificance of

Ser332 or Ser352 in mediating receptor constitutive phosphorylaiton or β-arrestin-2 association. In summary, CCR1 is constitutively phosphorylated at specific residues in the C-terminal tail crucial for mediating β-arrestin-2 association.

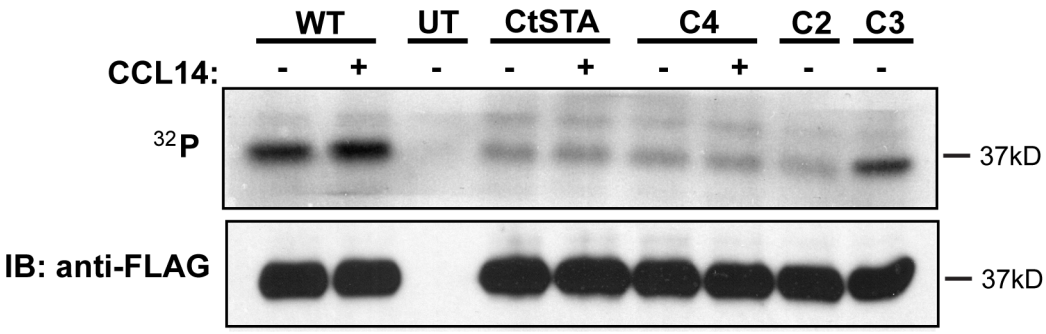


**Figure 3.2. CCR1 is basally phosphorylated in the C-terminal Ser/Thr-rich regions.** HEK293t cells expressing each wild-type or C-tail Ser/Thr mutant construct (see Fig. 1A for legend) were orthotopically labeled with the $^{32}P$ radioisotope and either left unstimulated (-) or stimulated (+) with 1 μM CCL14 for 6 min. Similar levels of CCR1wt and mutant expression were demonstrated by Western blotting against the anti-FLAG-HRP antibody.

*The CCR1 C-tail is not sufficient to induce constitutive association with β-arrestin-2* — Given that residues within the C-terminal tail are crucial for CCR1 basal phosphorylation and interaction with β-arrestin-2, it remained unknown whether this structural domain was sufficient to induce this behavior in a highly related chemokine receptor. To determine this, the entire C-terminal

tail sequence of CCR1 immediately following the conserved NPXXY motif was replaced with that of CCR5 in a YFP fusion construct, and *vice versa.* Additionally, the residues constituting the third intracellular loop of each receptor were swapped and the resulting chimeras assessed for basal $BRET1_{net}$ signal with β-arrestin-2-Rluc (**Fig. 3.3A**). It has previously been demonstrated that recruitment of β-arrestins to CCR5 is ligand-dependent (Chapter 2, **Fig. 2.4B**) while others have shown no evidence of basal receptor phosphorylation (9, 25). Replacement of the CCR1 C-tail with that of CCR5 led to a significant reduction, albeit not a complete ablation, in the basal $BRET1_{net}$ signal between the chimeric CCR1-$R5_{Ctail}$ receptor and β-arrestin-2 (**Fig. 3.3B**). The alternative conformation in which the CCR5 C-tail was replaced with that of CCR1 did not lead to any significant increase in β-arrestin-2 association (**Fig. 3.3C**). The third intracellular loop (ICL3) of either receptor did not exhibit any role in affecting the $BRET1_{net}$ signal, either independently or in combination with C-tail chimeras (**Fig. 3.3B-C**). Taken together, these data suggest that the CCR1 C-tail in and of itself is not sufficient to induce ligand-independent association of β-arrestin-2 with CCR5 and implicates additional structural motifs in driving a receptor conformation conducive to basal phosphorylation.

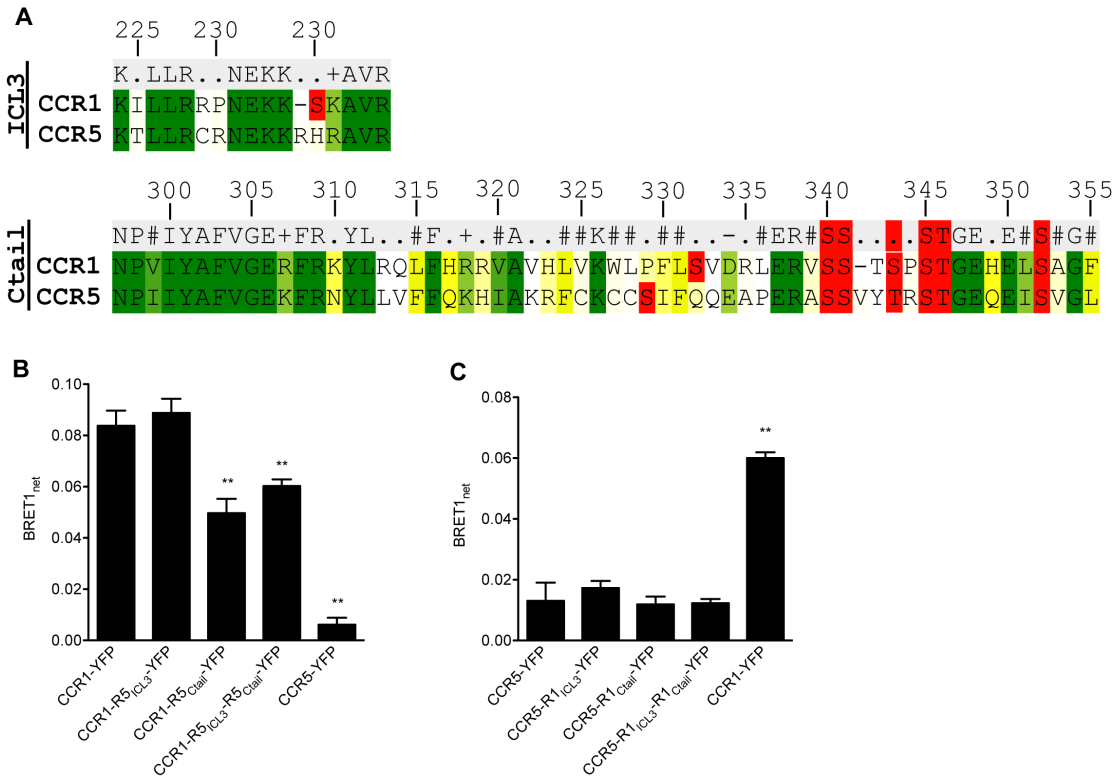


**Figure 3.3. The CCR1 C-tail is necessary but not sufficient to mediate basal association with β-arrestin-2. A.** Sequence alignment of intracellular domain 3 (ICL3, top) and the carboxy-terminal tail (C-tail, bottom) of CCR1 and CCR5. Ser/Thr residues are highlighted in red. **B.** Effect of swapping the third intracellular loop (ICL3) and/or C-terminal tail (Ctail) residues of CCR1 with those of CCR5, and *vice versa* (**C.**), on the basal association with β-arrestin-2 measured via BRET. Statistical significance compared to each wild-type receptor was calculated using a one-way ANOVA with Dunnetts multiple comparisons post-test, **p*<0.05, ***p*<0.01.

*CCR1 C-tail mutations inhibit receptor desensitization and internalization* — One of the primary functions of arrestins is to initiate desensitization of receptor signaling by binding to phosphorylated receptors, theoretically preventing further G protein coupling through steric occlusion. To

assess the impact of the Ser/Thr mutations on CCR1 desensitization, the ability of HEK293 cells expressing wild-type or mutant receptor to induce calcium mobilization upon agonist stimulation was measured. Specifically, cells were treated with an initial saturating injection of CCL14 followed by a second injection of the chemokine at the same final concentration approximately four minutes later. This assay has been used previously to monitor the initial signal decay kinetics as well as the relative ability of receptors on the cell surface to initiate a second wave of calcium mobilization, the latter serving as an indirect measure of receptor desensitization (26, 27). When the relative signals of CCR1wt and the C4 and CtSTA mutants were compared, cells expressing C4 and CtSTA displayed longer signal decay kinetics following an initial injection of CCL14 (**Fig. 3.4A**). These C-tail mutant receptors were also less efficiently desensitized, as evidenced by their ability to induce greater calcium mobilization following the second injection of CCL14. Taken together, decreased basal association with β-arrestin-2 by these mutant receptors leads to prolonged signaling and a weakened ability to be down-regulated after initial ligand stimulation.

In an alternate measurement of receptor signaling activity, the ability of CCL14 to induce ERK1/2 kinase phosphorylation in HEK293t cells expressing CCR1wt, CtSTA or C4 was measured. First, it was observed that CCR1wt-mediated ERK1/2 phosphorylation is dependent upon ligand activation of the receptor, as evidenced by the lack of detectable phospho-ERK1/2 signal in cells left untreated and the significant up-regulation of ERK1/2 phosphorylation

in chemokine-treated cells (**Figure 3.4B, left**). Therefore, constitutive β-arrestin-2 association is not sufficient to stimulate this particular signaling cascade, suggesting that CCR1 utilizes a G protein-dependent pathway. Indeed, previous work done in our laboratory has shown CCR1-mediated ERK1/2 phosphorylation to be fully sensitive to pertussis toxin (PT) treatment, indicating a Gi/o-coupled signaling dependence (data not shown). When CCR1wt was compared to mutants deficient in basal β-arrestin-2 association, C4 and CtSTA were able to stimulate more than 2.5-fold higher levels of ERK1/2 phosphorylation following CCL14 stimulation (**Figure 3.4B,** quantification on right). These data suggest that CCR1 receptors exhibiting decreased association with β-arrestin-2 consequently experience an inhibition of receptor desensitization following activation and prolonged signaling.

Receptor internalization often immediately follows receptor desensitization and can be mediated by the ability of β-arrestins to scaffold proteins involved in endocytosis, such as adaptin and clathrin, to the receptor. To test the effect of CCR1 C-tail mutations on receptor internalization, the cell surface levels of CCR1 in HEK293 cells expressing CCR1wt, CtSTA or C4 following chemokine stimulation were measured via flow cytometry. The percent of receptor remaining on the cell surface was calculated by dividing the median fluorescence intensity of cells treated with agonist to cells left untreated. It was observed that significant levels of CCR1wt were removed from the cell surface following ligand addition, whereas neither CtSTA nor C4 were efficiently internalized (**Fig. 3.4C,** quantification on right). Thus, mutation

of C-tail Ser/Thr residues significantly impaired the removal of activated receptor from the cell surface, which can most likely be attributed to the decreased ability of these mutant receptors to bind to β-arrestin-2 and be scaffolded to intacellular internalization machinery.

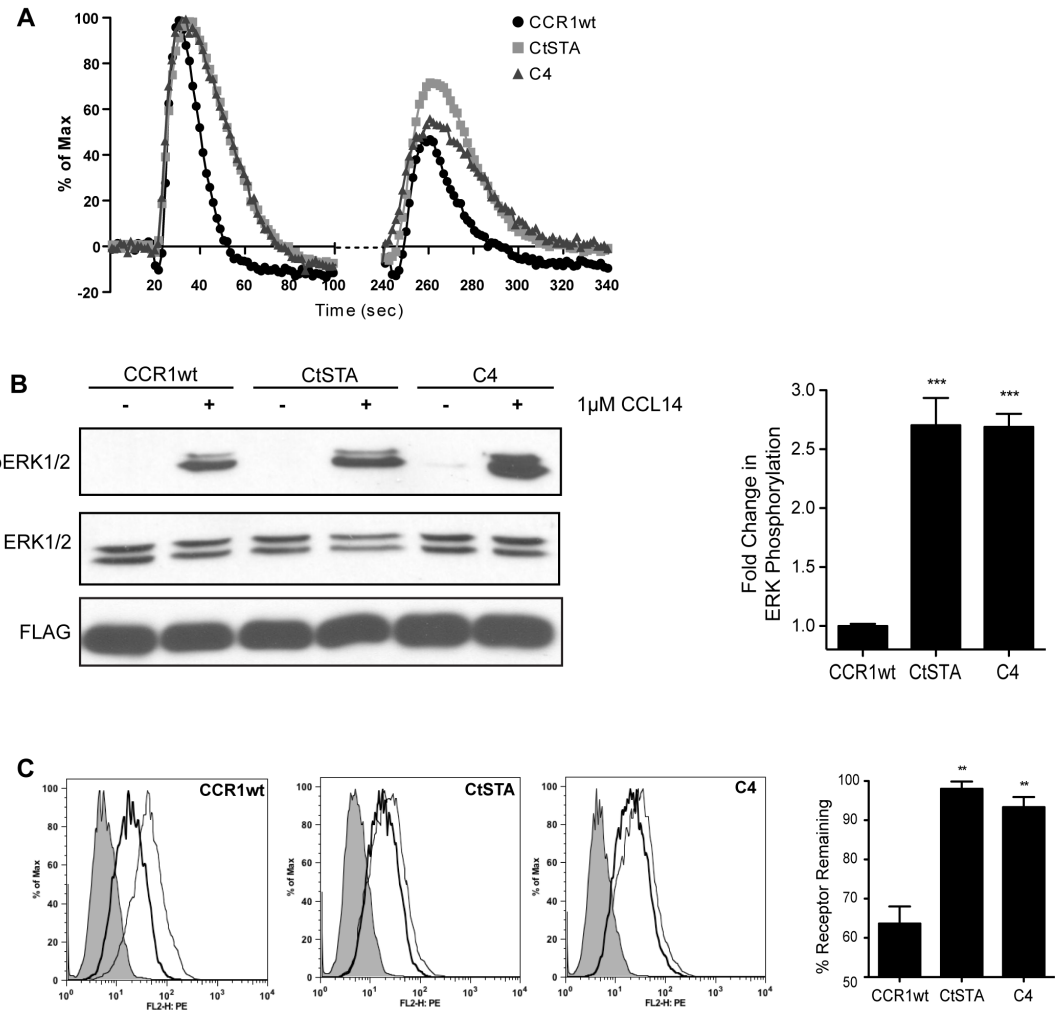


**Figure 3.4. Mutation of Ser/Thr residues in the C-tail of CCR1 reduces receptor desensitization and internalization. A.** Calcium mobilization in HEK293t cells transiently transfected with the C4 (dark gray ▲), CtSTA (light gray ■) or CCR1wt (black ●) constructs. The initial flux was initiated by stimulation of the cells with a final concentration of 1 μM CCL14, with a repeated injection yielding the same concentration of CCL14 at 240 seconds. Data is plotted as the percent of the maximal signal obtained by each CCR1 construct following the first ligand addition. **B.** The phosphorylation of cellular ERK1/2 in HEK293t cells transfected with CCR1wt, CtSTA or C4 and stimulated with 1 μM CCL14 was detected using Western blotting. Quantification of ERK1/2 phosphorylation is shown on the right. Data is plotted as the fold change in phospho-ERK1/2 levels relative to CCR1wt. Expression of each construct was shown to be similar. **C.** Internalization of CCR1wt, CtSTA or C4 as measured by flow cytommetry following surface labeling of transiently transfected HEK293t cells with a PE-conjugated CCR1 antibody (R&D Systems). The distribution curves of unstained cells (gray filled line) and cells stained with CCR1-PE following treatment with either 1 μM CCL14 (black unfilled line) or PBS control (gray unfilled line) for 10 minutes at 37°C are shown. Quantification of the percent of receptor remaining on the cell surface for each of the constructs following CCL14 treatment is shown to the right. Statistical significance was calculated using a one-way ANOVA with Dunnetts multiple comparisons post-test, *$p$<0.05, **$p$<0.01.

*Additional residues within CCR1 transmembrane domains are involved in constitutive association with β-arrestin-2 and G protein* — The identified cluster of Ser/Thr residues in the distal C-terminal tail of CCR1 is necessary, but not sufficient, for the ligand-independent association of CCR1 with β-arrestin-2. The location of these amino acids is important to understanding the structural mechanisms of how a constitutively active CCR1 protein is functionally regulated to prevent excessive signaling; however, they do not shed light on what is driving the constitutive activity of CCR1 in the first place. The residues stabilizing the receptor in an intermediate active state are likely to be found in the transmembrane helices. To investigate this, a molecular model of CCR1 was created using the newly released crystal structure of CCR5, which has 55% sequence identity with CCR1 but no evidence of constitutive activity, as a reference (**Fig. 3.5A**) (20). Amino acids that differed between the two receptors at positions thought to be important for receptor activation were mutated in CCR1 to the corresponding residue in CCR5 or to other residues that may be illustrative as to the potential role of that amino acid in promoting a constitutively active state in CCR1. Over two dozen candidate positions for mutation were identified at the interface of the third, sixth and seventh TM helices (TM3, TM6 and TM7). Previous analyses of GPCR crystal structures in various active and inactive states have identified this region as one that undergoes significant repacking and reorientation upon receptor activation (28). Additionally, mutations in the highly conserved DRY motif at the base of TM3 were tested as these residues are crucial for G

protein activation by class A GPCRs, while some evidence exists for a role in β-arrestin binding (9, 29, 30). The impact of each mutation was then screened by measuring the basal association of the mutant and YFP-tagged receptor with β-arrestin-2-Rluc. The assumption was that if a single point mutation, or some combination of them, was essential for receptor constitutive activity it should prevent basal phosphorylation of the receptor and ablate basal β-arrestin-2 association as measured by a reduction in the $BRET1_{net}$ signal.

The results, plotted as fold change of the basal $BRET1_{net}$ signal for the mutant CCR1-YFP construct compared to wild type CCR1, indicate that the majority of residues tested had little to no effect on basal association with β-arrestin-2 (**Fig. 3.5B**). None of the mutations were able to have the same inhibitory effect as the CtSTA mutant which displays a near total loss of affinity for β-arrestin-2 in the absence of agonist. The single point mutations at the TM3/TM6/TM7 interface that caused a greater than 20% reduction in basal β-arrestin-2 association were E120A and T253L, whereas the L117F mutation actually increased association by roughly 20% (**Fig. 3.5B, left**). The T115I/E120A dual mutation in TM3 and quadruple V288T/I289L/A290G/Y291M (abbreviated TM7-4X) mutation reduced the $BRET1_{net}$ signal by nearly 40% and 20%, respectively (**Fig. 3.5B, left**). Surprisingly, alterations introduced into the DRY motif brought about the most marked changes in either direction in association between a mutant CCR1 and β-arrestin-2 (**Fig. 3.5B, right**). Disruption of the negatively charged Asp at position 130 with either Ala or Asn reduced the basal $BRET1_{net}$ signal by 38%

and 50%, respectively. Combination of the D130A mutation with another TM3 mutation E120A, which showed a modest reduction as a single mutation, displayed no combinatory effect. Conservation of the positive charge at position 131 by substitution of Arg to Lys actually inhibited β-arrestin-2 association to the same extent as the D130 mutations, whereas neutralization of the charge by mutation to other residues appeared to increase the basal $BRET1_{net}$ signal (**Fig. 3.5B, right**).

Previous work by our laboratory has shown that CCR1 also exhibits basal association with G protein, specifically $G\alpha_i$, in addition to β-arrestin-2 and that these signaling and regulatory molecules are able to interact simultaneously with the receptor (Chapter 2, **Fig. 2.7**). Therefore, the mutational screen was extended to include ligand-independent association of CCR1-YFP with $G\alpha_i$-Rluc in combination with the muscarinic acetylcholine receptor 3 (M3) and $G\alpha_{12/13}$-Rluc as negative controls. Immediately, some parallels between residues important for β-arrestin-2 and $G\alpha_i$ association became apparent (**Fig. 3.5C**). While G protein association is more sensitive to mutation at position 120, the E120A mutant is clearly important for receptor association with both proteins (**Fig. 3.5C, left**). Additionally, the T253L and TM7-4X mutations also exhibited similar reductions in the basal $BRET1_{net}$ signal, whereas L117F appears to increase the signal in both assays. However, none of the mutations introduced into the TM3/TM6/TM7 interface were able to completely ablate constitutive association with $G\alpha_i$ to the level of random interactions exhibited by M3, which does not signal through the Gi/o

heterotrimeric G protein complex. As would be expected, mutation of D130 brought about a drastic decrease in the basal $BRET1_{net}$ signal, demonstrating this residue to be important for both $G\alpha_i$ and β-arrestin-2 association (**Fig. 3.5C, right**). However, quite unexpectedly and in contrast to results with β-arrestin-2, no mutation at R131 resulted in a greater than 20% change in the signal in either direction. Collectively, these data provide an initial assessment of residues important for stabilizing the ligand-independent complex formation with β-arrestin-2 and $G\alpha_i$ and, by extension, the constitutive activity of CCR1.

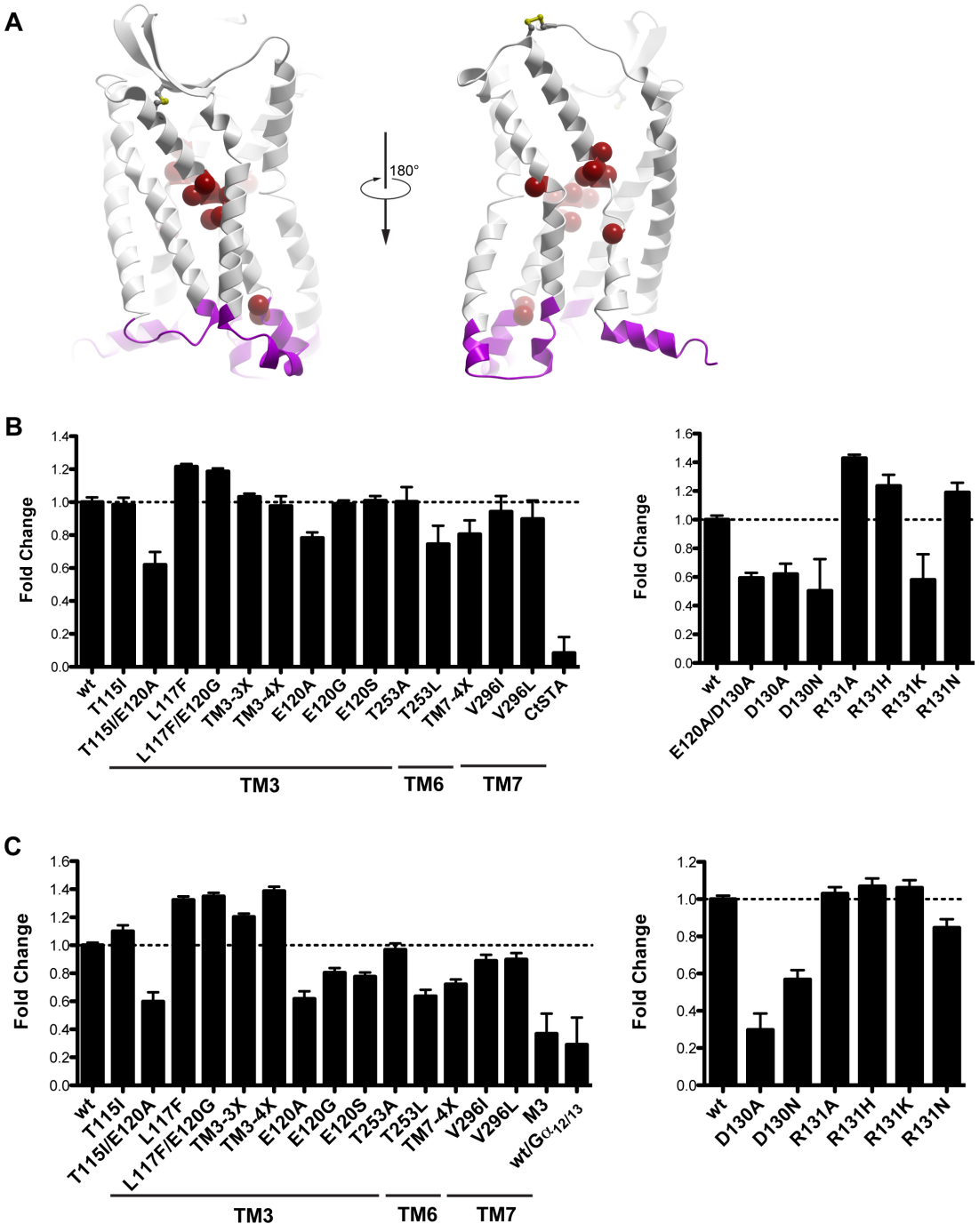


**Figure 3.5. Mutagenesis of the CCR1 transmembrane interface domain and DRY motif reveals residues mediating constitutive association with β-arrestin-2 and Gα$_i$. A.** Molecular model of the three-dimensional structure of CCR1 using the CCR5 structure (PDB: 4MBS) as a reference. Amino acids selected for mutational analysis are represented as red spheres, while the intracellular domains are colored purple. **B-C.** Effect of single point or multiple mutations in TM3, TM6 and TM7 (**left**) or the DRY motif (**right**) on the basal association with β-arrestin-2-Rluc (**B**) or Gα$_i$-Rluc (**C**) measured via BRET.

### 3.5 Discussion

Constitutive activity is the ability of a GPCR to adopt an active conformation (or set thereof) independently of agonist binding. The result is signaling through G proteins and association with allosteric regulators of receptor activation, including β-arrestins. Classically, recruitment of β-arrestin to a GPCR is initiated by phosphorylation of serine or threonine residues in exposed intracellular domains by G protein-coupled receptor kinases (GRKs) or second messenger kinases (such as PKA or PKC). Once associated with a receptor, β-arrestins play an essential role in the regulation of GPCR pharmacology and trafficking, subcellular localization of the receptor, signaling pathway selection, and molecular scaffolding of downstream effectors for activation (31, 32).

Our laboratory has previously demonstrated that CCR1 exhibits a rather unique pattern of association with β-arrestin-2 that is independent of ligand stimulation and attributable to CCR1's behavior as a constitutively active receptor (see Chapter 2). Through the extensive use of the bioluminescence resonance energy transfer (BRET) assay, as well as co-immunoprecipitation and confocal microscopy, it was demonstrated that CCR1 is basally associated with β-arrestin-2. This behavior has not been observed for any other G protein-coupled chemokine receptor, with the exception of the recently described CXCR4-CXCR7 heterodimer that is able to constitutively recruit β-arrestin (33). Ligand-independent association with β-arrestin has been reported for a limited number of other GPCRs including the D6 chemokine

scavenging receptor (11), a constitutively active mutant of the melanocortin 1 receptor (34), a naturally-occuring and constitutively active splice variant of the 5HT2c serotonin receptor (35), and mutants of the vasopressin-2 receptor involved in nephrogenic diabetes insipidus (36-38). A common trait amongst the majority of the receptors that exhibit high basal levels of association with arrestins is high basal levels of receptor activity, as was observed with CCR1. Additionally, many of these receptors are also basally phosphorylated at Ser/Thr residues in their intracellular domains, most likely contributing to constitutive arrestin recruitment. A mutant of CXCR4 that exhibits constitutive activity was shown to be phosphorylated in the absence of CXCL12 treatment (39), as was the virally-encoded chemokine receptor US28 (40) and the previously mentioned mutant V2R (38). CCR1 remains unique in that the wild-type sequence was sufficient for constitutive phosphorylation and β-arrestin-2 association. This non-canonical interaction is specific for CCR1 compared to the other chemokine receptors tested in this study and others, suggesting that CCR1 possesses structural traits that set it apart from the majority of GPCRs requiring agonist binding to drive β-arrestin recruitment. Despite the clinical importance of understanding the molecular mechanisms that regulate CCR1 signaling, no studies have previously investigated the impact of receptor structural domains on the direct interaction between CCR1 and β-arrestin. Therefore, the purpose of this study was to identify those structural traits involved in agonist-independent phosphorylation and β-arrestin-2 association.

The identification of the specific set of Ser/Thr residues in the C-terminal tail at positions 340-43 and 345-46 as key sites for receptor phosphorylation and β-arrestin-2 association confirms the results of two previous studies that partially analyzed CCR1 phosphorylation. Oppermann *et al*. (41) showed that CCR1wt phosphorylation in transfected CHO cells is induced by stimulation with CCL3, CCL5, or PMA, with the latter suggesting a role of PKC in heterologous desensitization of the receptor. In a more detailed analysis Richardson *et al.* (42) found that mutation of all serine and threonine residues in the C-tail to alanine nearly completely blocked phosphorylation of CCR1 in CCL3, CCL5, CCL8, or PMA treated rat basophilic leukemia (RBL-2H3) cells. Mutation of Ser332 or Ser352 appeared to have no effect on the extent of CCR1 phosphorylation. Additionally, the general PKC inhibitor staurosporine was shown to partially decrease CCR1 phosphorylation, providing support to the idea that CCR1 is phosphorylated in both a GRK- and PKC-dependent manner. Studies of other chemokine receptors have observed similar structural requirements for phosphorylation and arrestin binding, albeit only following ligand stimulation. Through a familiar approach of progressive C-tail truncations and serine to alanine mutations, CCR5 phosphorylation was localized to its C-terminal tail following activation with CCL5, and intact C-tail serine phosphorylation sites were required for high-affinity association with β-arrestin (8). In a subsequent publication on CCR5, the four Ser residues in the C-terminal tail cluster were individually, and in various combinations, mutated to Ala and assessed for their effect on association with β-arrestin and

internalization (9). A minimum of two out of the four potential Ser phosphorylation sites were found to be necessary for β-arrestin association at levels similar to wild-type receptor; however, it did not appear to matter which two that specifically remained intact. More extensive mutagenesis of the two C-tail Ser/Thr clusters in CCR1 is needed to determine whether a similar minimum of phosphorylated residues is necessary. A study of CXCR4 also demonstrated the importance of the C-terminal tail as truncation abolished the desensitization effect of β-arrestin on CXCR4 signaling (10). Using mass spectroscopy and phosphosite-specific antibodies, a later study identified the specific Ser residues of CXCR4 that were phosphorylated following CXCL12 treatment and demonstrated that β-arrestin recruitment is dependent upon phosphorylation of these distal C-terminal tail Ser residues (12). Our study represents the first analysis of the sites of CCR1 phosphorylation and their relationship to β-arrestin association in human cells, which may explain why the previous studies did not observe extensive basal phosphorylation of the receptor. The constitutive activity of the receptor may be dependent upon the unique cellular environment and regulatory machinery in place to modulate basal CCR1 signaling.

The use of intracellular domain swapping between the constitutively active CCR1 and non-constitutively active CCR5 was conducted in order to determine whether the C-terminal tail (with or without ICL3) was sufficient in and of itself for constitutive phosphorylation and β-arrestin-2 association. The usefulness of this approach was demonstrated to the extreme with the

gonadotropin-releasing hormone receptor (GnRHR) and thyrotropin-releasing hormone receptor (TRHR). Although both belong to the rhodsopin-like/class A family of GCPRs, GnRHR is unique in that it lacks a C-terminal tail leading to slow internalization kinetics and an inability to undergo acute desensitization (43, 44). While TRHR undergoes agonist-induced phosphorylation and β-arrestin-dependent internalization, GnRHR is not phosphorylated and is internalized independently of β-arrestin (43, 45). The addition of the C-terminal tail of TRHR to the carboxy-terminal end of GnRHR results in a chimeric receptor that is phosphorylated and internalized in a β-arrestin-dependent manner (45, 46). While CCR1 and CCR5 do not exhibit such striking structural differences, the effect of switching the ICL3 and C-terminal tails between the two receptors was tested. The results demonstrate that ICL3 does not play a major contributing role to basal association with β-arrestin-2, whereas the C-terminal tail is necessary but not sufficient for it. Switching the CCR5 C-tail with that of CCR1 did not presumably confer a sufficient structural alteration that would lead to basal phosphorylation and β-arrestin-2 recruitment. Likewise, the CCR1 chimera with the CCR5 C-tail did not completely lose its basal affinity to β-arrestin-2, suggesting that the global conformation of CCR1 is sufficient to induce ligand-independent phosphorylation of non-native C-tail residues. These findings implicate other structural motifs within the transmembrane helices that stabilize a constitutively active conformation of CCR1, which will be addressed in a later figure.

The C-tail residues essential for constitutive β-arrestin-2 association and phosphorylation were also shown to play a key role in receptor desensitization and internalization. The CCR1 C4 and CtSTA constructs displayed enhanced signaling, resistance to desensitization, and slower internalization kinetics compared to CCR1wt. Prevention of CCR5 phosphorylation through truncation or Ser/Ala mutation also reduced agonist-induced receptor desensitization and internalization (8). This behavior is most likely attributable to decreased affinity for β-arrestin, as demonstrated for the CCR1 mutants via BRET and immunoprecipitation, leading to diminished ability of the receptor to be down-regulated and of β-arrestin to sterically inhibit continued G protein-coupling and signaling. The lack of significant differences between the responses of C4 and CtSTA in the calcium mobilization, ERK1/2 phosphorylation, and receptor internalization assays once again demonstrates that residues at positions 340-43 and 345-46 are primarily responsible for mediating CCR1's association with β-arrestin. Somewhat related was the observation that CCR1 expression and constitutive β-arrestin-2 recruitment did not induce basal phosphorylation of ERK1/2; rather, it was entirely an agonist-dependent signaling event. This is similar to a constitutively active melanocortin 1 receptor that showed high basal recruitment of β-arrestin but did not lead to ERK activation or increased internalization (34). Therefore, the "active" structure, or set of structures, adopted by CCR1 when it is basally pre-coupled to β-arrestin-2 is not sufficient to initiate certain signaling cascades. These data support the emerging notion that the conformational state of a

receptor associated with constitutive activity may be different and distinct from that of the conformational state stabilized by an agonist (34).

As mentioned previously, the CCR1 C-terminal tail is not sufficient for conferring constitutively active behavior onto a basally quiescent CCR5. The constitutive activity of a receptor would suggest the absence or weakening of the intramolecular interactions that would normally restrain the receptor in the inactive state (47). To determine what additional residues beyond the cytoplasmic domains contribute to the ligand-independent signaling of CCR1, a three-dimensional structural model of the receptor was created using computational modeling based upon the recently released crystal structure of CCR5 (20). This model, in combination with previous knowledge of the conformational changes that occur upon GPCR activation (28), enabled the identification of residues potentially involved in stabilizing an active state (or, conversely, destabilizing the inactive state) in the absence of agonist binding and that differed from the corresponding amino acid in CCR5. The selected positions are primarily found in the interface between TM3, TM6 and TM7 and were initially mutated to their CCR5 equivalent or to alternative amino acids for select residues. The BRET screen for basal association with β-arrestin-2 has thus far identified the E120A (individually or in conjunction with T115I) in TM3 and T253L in TM6 mutations as contributing to a greater than or equal to 20% reduction in the constitutive activity of CCR1; however, none of the mutations were capable of ablating basal association to the extent of the CCR1 CtSTA mutant. Similar positions were identified when the mutations were assessed

for effect on basal $G\alpha_i$ association. This suggests that the intramolecular interactions involved in maintaining CCR1 constitutive activity are sufficiently robust such that single or multiple point mutations within an individual transmembrane domain are insufficient to destabilize them.

Alternatively, these data may indicate that the residues responsible for constitutive activity are located in other structural regions of the receptor. The E/DRY motif at the boundary between the base of TM3 and the beginning of ICL2 is highly conserved amongst the rhodopsin-like family of GPCRs and plays an essential role in G protein activation (48). The crystal structure of inactive rhodopsin demonstrates that the Arg is engaged in a salt bridge with the adjacent Glu/Asp and with a Glu at the base of TM6, with the latter electrostatic interaction referred to as the “ionic lock” (49). These salt bridges are thought to constrain GPCRs in the inactive conformation, and disruption through mutation can induce constitutive activity (50); however, the necessary acidic residue in TM6 is not found in approximately 75% of GPCRs, including chemokine receptors (28, 51). In CCR1, the residue at this position is Arg; therefore, the receptor is not capable of forming the “ionic lock.” Mutation of Arg in the DRY motif has been shown to induce constitutive activity in multiple GPCRs including the vasopressin 2 receptor (V2R), $\alpha_{1B}$ and $\alpha_{1A}$ adrenergic receptors ($\alpha_{1B}$AR and $\alpha_{1A}$AR), angiotensin II type 1A receptor ($AT_{1A}R$), and others (37, 38, 48, 50, 52). In V2R a mutation of this conserved Arg to His, Cys or Leu leads to basal association with β-arrestin and subsequent constitutive internalization (37). An Arg to His mutation in $\alpha_{1B}$AR leads to

similar ligand-independent interaction with β-arrestin (50). Mutation of the Glu/Asp in the E/DRY motif of multiple GPCRs is thought to reposition Arg from its polar pocket and act as a constitutively activating mutation (48). However, the complete opposite has been observed for other receptors in which mutation of this residue leads to an impairment of agonist-induced responses and no observable constitutive activity. For example, an intact DRY motif was shown to be necessary for β-arrestin recruitment to activated *N*-formyl peptide receptors (FPR) and mutation ablated their association (29). To determine the role of this motif in the constitutive activity and basal associations of CCR1 with β-arrestin-2 or $G\alpha_i$, mutations at D130 and R131 were introduced. For both β-arrestin and G protein, mutation of D130 to either Ala or Asn resulted in a significant reduction in basal association, suggesting this residue plays an important role in maintaining receptor constitutive activity. Mutation of R131 resulted in somewhat mixed results; replacement with Ala, His, Lys, or Asn appeared to have no effect on $G\alpha_i$ association, whereas R131A, R131H, and R131N mutations all increased the basal β-arrestin association with CCR1. The contribution of R131H to constitutive activity was described above, while the R131A mutation has been shown to induce ligand-independent signaling in the oxytocin receptor (53). Therefore, the positive effect of these mutations on β-arrestin are in agreement with published data of other GPCRs. However, the negative effect of R131K on β-arrestin-2 association is more surprising as this mutation in the $\alpha_{1B}$AR increases constitutive activity and initiates some additional β-arrestin recruitment (52).

These results demonstrate that the role of the E/DRY motif in stabilizing ligand-free or ligand-bound conformations is quite complex and remains not fully elucidated.

In summary, the structural domains within the C-terminal tail of CCR1 responsible for basal phosphorylation and ligand-independent association with β-arrestin-2 are identified. Initial data is presented on the residues involved in the transmembrane intramolecular network supporting receptor constitutive activity, although more thorough mutational and signaling analyses are needed. Determination of the structural underpinnings of CCR1 constitutive activity will enable the development of biased ligands capable of selectively inhibiting basal signaling and possibly avoiding harmful side effects.

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### 3.7 Acknowledgments

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# CHAPTER 4

## DIFFERENTIAL EFFECTS OF CHEMOKINES AND SMALL MOLECULE AGONISTS ON THE REGULATION OF CCR1

### 4.1 Abstract

G protein-coupled receptors (GPCRs) are able to detect and respond to an extraordinarily broad array of extracellular stimuli and display a remarkable diversity in their function. Diversity of ligand binding is observed at the level of individual receptors as well, with ligand promiscuity amongst chemokine receptors serving as a prime example. CCR1 itself is recognized and activated by at least ten endogenous chemokines. Whether this promiscuity in ligand:receptor relationships leads to redundant physiological effects or functionally selective signaling remains a matter of open debate. In this report we examine the effect of CCL3, CCL5, CCL7 and CCL14 on β-arrestin-2 association with CCR1 and receptor internalization. While all chemokines were able to act as agonists in both assays, CCL3 and CCL14 exhibited full agonist behavior with higher potency and efficacy compared to CCL5 and CCL7. Activation of CCR1 was shown to lead to a conformational rearrangement in a pre-formed complex between CCR1 and β-arrestin-2. Additionally, small metal ion chelator complexes were demonstrated, for the first time, to be capable of inducing receptor down-modulation. Together, these data provide a basis for further investigating the functional selectivity of CCR1 chemokine ligands.

## 4.2 Introduction

The chemokine family has approximately 50 members that are known to bind to at least 23 different receptors. This family displays a wide range of promiscuity in which single chemokines can bind to multiple receptors while a single receptor can respond to multiple chemokines. Whether multiplicity in ligand binding is indicative of functional redundancy or selectivity remains a matter of open debate. Knockout of inflammatory chemokine receptors, particularly of the CC group, that share common ligands with other receptors most often is not phenotypically lethal and only shows an effect following immune system challenge, suggesting overlapping effects of chemokines (1). Additionally, drug development efforts targeting an individual chemokine receptor have largely been halted due to lack of efficacy; “promiscuous” inhibitors that act upon multiple receptors are now being suggested (2). Some have argued that redundancy is an essential component of a “robustness” that is needed by the immune system to properly function over evolutionary time (3). However, other studies have demonstrated that activation of a single receptor by multiple chemokines can lead to differential signaling events, suggesting specificity and non-overlapping roles within the system (4). This has been exhibited most clearly by the two endogenous ligands of CCR7: CCL19 and CCL21. Whereas both chemokines are equipotent in terms of G protein activation and calcium mobilization, CCL19 induces significant CCR7 desensitization, phosphorylation, and ERK1/2 phosphorylation compared to CCL21 (5). Even though multiple chemokine ligands can activate the same

receptor and potentially yield a similar intracellular signal, chemokines themselves are spatiotemporally regulated by cell-type specific expression, proteolytic processing, hetero-oligomerization, and the availability of and binding affinity to specific cell surface glycosaminoglycan structures (6).

The C-C chemokine receptor type 1 (CCR1) was originally cloned in 1993 and classified as a receptor for CCL3 and CCL5 (7, 8). Since then, 8 additional chemokines have been demonstrated to activate CCR1: CCL3L1, CCL7-8, CCL13-16, CCL23 (9). CCR3 is the only other non-atypical (*i.e.* G protein-coupled) chemokine receptor that displays such ligand promiscuity. Few studies have set out to measure whether each chemokine yields a similar or distinct cellular response upon activation of CCR1, whereas none have specifically assessed ligand-induced effects on recruitment of β-arrestins**.** The available studies have shown differential activation of non-Gi/o heterotrimeric G proteins (10), selective activation of p38 mitogen activated protein kinase and calcium flux (11), contrasting abilities to mediate leukocyte chemotaxis (12), different binding sites within the receptor ligand binding pocket (13), and differing rates of receptor internalization and recycling (14). Additionally, small molecule metal ion chelators have been shown to act as efficacious CCR1 agonists and allosteric enhancers, adding an additional layer of complexity to distinctive modulation of CCR1 activation and signaling (15). Given the extensive structural and functional analysis of the constitutive activity of CCR1 presented in the previous Chapters, this Chapter focuses on the potentially unique effects of different chemokines and metal ion chelators on the

overlooked association between CCR1 and β-arrestin and subsequent receptor internalization.

**4.3 Experimental procedures**

*Cell Culture and Transfections* — HEK293t cells, which express the SV40 large T antigen, and HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) with Glutamax (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% $CO_2$. Transfection of HEK293t cells was carried out in 6-well plates at 50-60% confluency using TransIT-LT1 reagent (Mirus Bio) per the manufacturer's protocol.

*Bioluminescence Resonance Energy Transfer (BRET) Assay* — The BRET assay protocol has been described extensively elsewhere (16-18). In brief, the chemokine receptors CCR1 and CCR5 were genetically fused to *A. victoria* YFP on the receptor C-terminus without its stop codon followed by a short linker in a pcDNA3.1 vector (receptor-pYFP3.1). The *R. reniformis* luciferase (Rluc) genetic sequence was fused to the C-terminus of β-arrestin-2 or β-arrestin-1 in the phRluc-N2 vector. YFP and Rluc constructs were generous gifts from M. Bouvier, University of Montréal, Québec, Canada. HEK293t cells were transiently transfected in defined stoichiometric ratios of each vector, usually 1.5-2 μg of a receptor-YFP construct and 0.05-0.1 μg of β-arrestin-2-Rluc or β-arrestin-1-Rluc. Fourty-eight h post-transfection, cells were washed and suspended in phosphate buffered saline (PBS) supplemented with 0.5% (w/v) glucose. 1 x $10^5$ cells were aliquoted in

triplicate into each well of a white, clear bottom 96-well plate (BD Biosciences) and incubated for 1h at 37°C prior to BRET measurement. YFP-tagged receptor expression was quantified by measuring fluorescence of the wells at 485nm excitation and 538nm emission wavelengths on a SpectraMax fluorescence spectrometer (Molecular Devices). The luciferase substrate coelenterazine-*h* (Biotium) was added to a final concentration of 50 μM in each well 10 min prior to the beginning of the BRET assay. Luminescence and fluorescence measurements were collected at room temperature with 1 sec exposure times using a VictorX Light multilabel plate reader (Perkin-Elmer) at repeating time intervals. The BRET signal was calculated as the ratio of YFP emission (550±40 nm) to Rluc emission (470±30 nm). The $BRET_{net}$ signal is calculated by subtracting the background BRET ratio of cells expressing only the Rluc fusion from the BRET ratio of cells expressing both the YFP- and Rluc-fused proteins. Chemokine ligands were prepared as described previously (19), diluted in PBS, and added following incubation with coelenterazine-*h* but prior to BRET measurement. Preparation of the 1,10-phenanthroline and 4,7-dimethyl-1,10-phenanthroline (Sigma) complexes with zinc were carried out as described previously (15). Mutations in the CCR1 sequence were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). BRET saturation curve experiments were carried out in which the levels of $\beta arrestin_2$-Rluc were kept constant while increasing amounts of CCR1-YFP were co-transfected. The BRET signal was then plotted against the energy acceptor/donor ratio; a hyperbolic curve is indicative of a specific

interaction as opposed to random collisions within the cell that would yield a quasi-linear relationship (16). The $BRET_{max}$ (the maximal BRET signal obtained at saturating conditions) and the $BRET_{50}$ (the acceptor/donor ratio at which the half-maximal BRET signal is obtained) values in the presence or absence of 100 nM CCL14 were calculated. The extended BRET (eBRET) assay was carried out using a chemically modified coelenterazine-*h* substrate EnduRen (Promega) that exhibits slow decay kinetics and permits BRET measurement over a period of many hours at 37°C (20).

*Confocal Fluorescence Microscopy* — HeLa cells were cultured on glass coverslips coated with 10 µg/mL human plasma fibronectin (Millipore) and transiently transfected with CCR1-mCherry and/or $\beta arrestin_2$-GFP using Lipofectamine®2000 (Life Technologies). Twenty-four h post-transfection the media was replaced with serum-free DMEM with or without 100 nM CCL14 for specified periods of time. The cells were then washed 3 times with cold serum-free DMEM and then fixed with 4% paraformaldehyde (PFA) for 5 min at room temperature. The coverslips with fixed cells were mounted onto microscope slides with FluoroSave (Calbiochem). Images were collected using an Olympus DSU spinning disk confocal microscope.

*Flow Cytometry and Receptor Internalization* — HEK293 cells stably expressing HA-CCR1 were cultured in 10 cm plates and induced with 5 mM sodium butyrate 18 h prior to beginning the assay. Cells were then lifted from the plate with PBS + 1 mM EDTA and washed with cold assay buffer (DMEM + Glutamax, 0.5% BSA, 10 mM HEPES pH 7.4) to yield $1.5x10^6$ cells/mL.

Aliquots of 200 µL of the cell suspension were distributed into 2 mL eppie tubes followed by addition of pre-warmed for various periods of time. Cells were washed with cold PBS + 0.5% BSA and cell surface receptor labeled with CCR1 antibody or IgG isotype control conjugated to phycoerythrin (PE) (R&D Systems). The relative amount of receptor remaining on the surface at each time point was quantified using a a Guava® EasyCyte™ Flow Cytometer (Millipore) and analyzed using FlowJo software (Tree Star).

*Calcium Mobilization* — HEK293 cells stably transfected with CCR1 were resuspended in assay buffer (1X Hanks balanced salt solution, HBSS, 20 mM HEPES pH 7.4, and 0.1% (w/v) bovine serum albumin, BSA) at a concentration of 1.75 x $10^6$ cells/mL. 100 µL of the cell suspension was aliquoted in triplicate into a black, clear bottom Biocoat assay plate (BD Biosciences). 100 µL of assay dye from the FLIPR calcium 4 explorer format kit (Molecular Devices) was added to each well, and the plate was incubated for 1 h at 37°C. Receptor ligands were diluted in assay buffer in a V-bottom 96-well assay plate. Calcium mobilization at 37°C was measured by fluorescence excitation of the dye following ligand addition using a Flex Station 3 microplate reader (Molecular Devices).

**4.4 Results**

*CCR1 is constitutively associated with β-arrestin-2* — CCR1 has previously been demonstrated to behave in a constitutively active manner, which leads to basal phosphorylation and agonist-independent association

with β-arrestin-2 (see Chapters 2 and 3). To further confirm the specificity of this interaction, a BRET saturation assay was conducted in which the expression of the energy donor (β-arrestin-2-Rluc) is kept constant while expression of the energy acceptor (CCR1-YFP) is continually increased. When the BRET signal is plotted against the energy acceptor:donor ratio, a hyperbolic and saturable curve is indicative of a specific interaction between the two proteins being studied (16). A quasi-linear and un-saturable curve suggests the two proteins are undergoing random interactions. Using this assay under basal conditions, CCR1 and β-arrestin-2 clearly exhibited a specific interaction (**Fig. 4.1A**). To further address any concerns over potential random interactions giving rise to the signal, the BRET saturation assay was repeated with the dominant-negative V54D mutant of β-arrestin-2 that has weakened affinity to phosphorylated residues of a receptor and inhibits receptor internalization (21). The saturation curve between CCR1 and β-arrestin-2-V54D was quasi-linear and demonstrated that the basal association between CCR1 and β-arrestin-2 was indeed specific and not the chance result of random collisions within the cell (**Fig. 4.1A**).

*CCR1 chemokines induce an increase the BRET signal between CCR1 and β-arrestin-2, but not β-arrestin-1* — Given the ligand-independent interaction between CCR1 and β-arrestin-2, the question remained as to what effect receptor activation may have on this interaction. Four CCR1 chemokine agonists (CCL3, CCL5, CCL7 and CCL14) were tested in the BRET assay for their dose-dependent effect on the interaction between CCR1-YFP and β-

arrestin-2-Rluc (**Fig. 4.1B**). Each chemokine was able to induce a significant increase in the BRET signal from its basal level; however, differences were apparent in the potency and efficacy. CCL3 and CCL14 were the most potent chemokine ligands with $EC_{50}$ values in this β-arrestin-2 association assay of 4.6 nM and 8.7 nM, respectively. CCL5 and CCL7 were roughly two to three times less potent in stimulating an increase in the BRET signal between CCR1 and β-arrestin-2 with $EC_{50}$ values of 15.4 nM and 14.9 nM, respectively. These values are comparable to the estimates of potency derived from other CCR1 functional assays (22, 23). CCL3 and CCL14 were also the most efficacious agonists as they yielded a higher maximal BRET value compared to CCL5 and CCL7 over the range of concentrations tested. In an $^{125}$I-CCL3 displacement assay on CCR1 expressing membranes from transfected Ba/F3 cells the chemokine ligands displayed a rank order binding affinity of CCL3>CCL7>CCL14 with $K_i$ values ranging from 0.056-40 nM (CCL5 binding was not determined) (23). In the same study, the rank order of potency of G protein activation was CCL3>CCL5>CCL7>CCL14. Unfortunately, binding data is somewhat variable depending on the approach and cell type used; therefore, it is difficult to determine whether the observed differences in β-arrestin-2 association are simply due to differences in affinity or a consequence of distinct CCR1 conformation(s) stabilized by each chemokine.

Seeing as each chemokine was able to dose-dependently increase the BRET signal between CCR1 and β-arrestin-2, it was tested whether this effect was specific to this isoform of β-arrestin. Previously, it has been demonstrated

that CCR1 exhibits a strong preference for β-arrestin-2 over β-arrestin-1 when assessed for association under basal conditions (Chapter 2, **Fig. 2.5C**); however, this selective coupling has not been tested following chemokine stimulation. When the dose-response effect of CCR1-YFP association with β-arrestin-1-Rluc was tested with each of the chemokines, there was no significant agonist-promoted change in the $BRET1_{net}$ signal for any ligand (**Fig. 4.1C**). This suggests that CCR1 exhibits significant selectivity and only associates with β-arrestin-2 under both basal and ligand-stimulated conditions independent of the identity of the activating chemokine.

The specificity of the ligand-induced effect on the BRET signal was demonstrated by the ability of the CCR1 antagonist, BX 471, to completely block the increase in the maximal $BRET1_{net}$ signal following CCL14 addition (**Fig. 4.1D**). This effect was repeated with CCL3, CCL5 and CCL7 in which BX 471 prevented CCR1 activation and any subsequent increase in the BRET signal with β-arrestin-2 (data not shown). Interestingly, BX-471 did not appear to stabilize a receptor conformation that was antagonistic to constitutive association with β-arrestin-2, as evidenced by the lack of a significant reduction on the basal BRET signal between CCR1-YFP and β-arrestin-2-Rluc prior to ligand stimulation. Furthermore, incubation of the cells with BX 471 for up to 4 hours prior to the assay did not disrupt this constitutive association (data not shown).

In order to determine the length of the interaction between ligand-activated CCR1 and β-arrestin-2, the extended BRET (eBRET) assay was

used. Inherent instability of the coeleneterazine-*h* substrate limits reliable measurement of the BRET signal to approximately 45 minutes; however, the eBRET assay substitutes coelenterazine-*h* for a chemically protected coelenterazine (EnduRen) that requires the action of cellular esterases to convert it into a suitable substrate for luciferase (20). Using this assay it was demonstrated that the agonist-promoted increase in the BRET signal reaches a maximum after approximately 30 minutes following ligand addition and remains near this value for the lifetime of the assay (approximately 3 additional hours) suggesting the formation of a persistent and stable complex (**Fig. 4.1E**). CCL14 was used in this and subsequent experiments as it is one of the most potent chemokines we have tested in the β-arrestin-2 BRET assays. One caveat to this experiment, however, is that the eBRET signal in this assay is an average of a population of cells and does not necessarily illuminate the behavior of individual complexes of CCR1 and β-arrestin-2. This leaves open the possibility of rapid association followed by dissociation that, on average, favors the associated state.

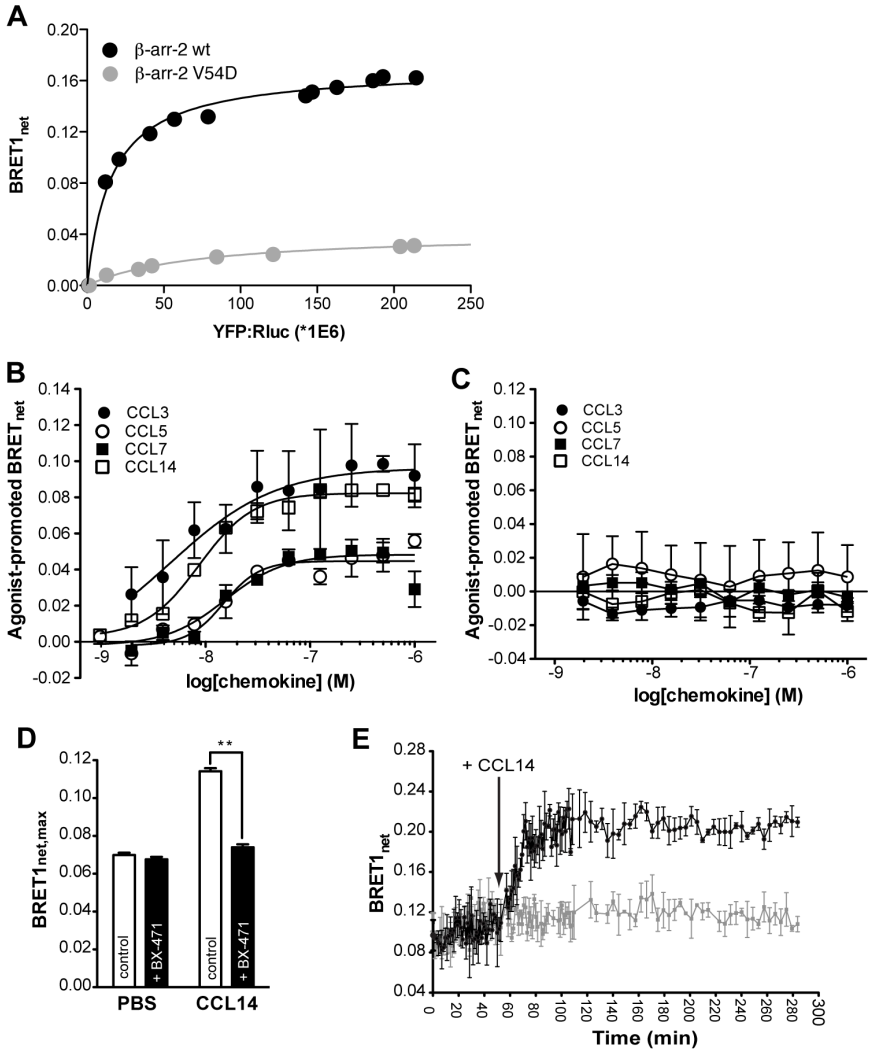


**Figure 4.1. Chemokine agonists induce an increase in the BRET signal between CCR1 and β-arrestin-2 above basal levels. A.** BRET1 saturation assay between CCR1-YFP and β-arrestin-2-Rluc (black) or the dominant-negative β-arrestin-2-V54D-Rluc mutant (gray) under basal conditions. **B** and **C.** Dose-reponse effect of the chemokines CCL3, CCL5, CCL7, and CCL14 on the interaction between CCR1-YFP and β-arrestin-2-Rluc (**B) or** β-arrestin-1-Rluc (**C**). Data is plotted as the mean ± S.D. (*N* = 3) of the agonist-promoted $BRET1_{net}$ signal from cells transiently co-expressing CCR1-YFP and β-arrestin-Rluc following stimulation with 100 nM chemokine for 25 min at room temperature. Each curve in **B** was fitted using non-linear regression analysis (Graph Pad Prism) and the $EC_{50}$ values calculated: CCL3 = 4.6 nM; CCL5 = 15.4 nM; CCL7 = 14.9 nM; CCL14 = 8.7 nM. **D.** Effect of the antagonist BX 471 on the ability of CCL14 to induce an increase in the $BRET1_{net}$ signal between CCR1-YFP and β-arrestin-2-Rluc. Cells were treated with 1 μM BX 471 (black bar) or left untreated (white bar) for 10 min prior to stimulation with 100 nM CCL14. Data is plotted as the mean ± S.D. of the maximal $BRET1_{net}$ signal 30 min post chemokine stimulation. Statistical significance was calculated using an unpaired t test (**$p < 0.01$). **E.** The extended BRET (eBRET) assay was used to measure the long-term interaction of CCR1-YFP and β-arrestin-2-Rluc for approximately 3 hours at 37°C following addition of 100 nM CCL14 (black line) or PBS (gray line).

*Activation of CCR1 induces a conformational change in the pre-formed complex with β-arrestin-2* — At least two possible explanations can account for the positive impact of chemokines on the $BRET_{net}$ signal: either CCR1 activation induces increased β-arrestin-2 affinity and recruitment to receptors that may or may not be currently bound by β-arrestin-2, or chemokines induce a conformational rearrangement within pre-formed CCR1 and β-arrestin-2 complexes that brings Rluc and YFP into closer proximity or into a more favorable orientation for energy transfer. The BRET saturation assay has been shown by others to be a useful method for distinguishing between these mechanisms (16, 24). In this assay, the expression of β-arrestin-2-Rluc was held constant while the expression of CCR1 was continually increased. Under basal conditions (PBS control), we observed a hyperbolic saturation curve indicative of a specific interaction between CCR1 and β-arrestin-2 (**Fig. 4.2A**), as opposed to a non-saturable and quasi-linear curve that indicates random and non-specific collisions between the two proteins. This confirms data in **Fig. 4.1A** conducted in the absence of agonist. Data collected following CCL14 addition resulted in a hyperbolic curve that was significantly shifted upwards with comparably little change in the half-maximal BRET signal ($BRET_{50}$). These results are most consistent with a ligand-induced intramolecular conformational change between a pre-formed complex of CCR1 and β-arrestin-2, as opposed to an increase in the affinity of association which would have shifted the $BRET_{50}$ to the left while reaching a similar $BRET_{max}$. This interpretation is in agreement with **Fig. 2.5F** which shows no

significant increase in the amount of β-arrestin-2 that co-immunoprecipitates with CCR1 following CCL14 treatment compared to untreated cells.

Validation of this interpretation was obtained using a “double-brilliance” β-arrestin-2 reporter with Rluc fused to the N-terminus and YFP fused to the C-terminus of β-arrestin-2. This construct was designed on the basis of the general model of βarrestin activation in which the N- and C-lobes move into closer proximity following binding to phosphorylated residues on the receptor C-tail (25). Along these lines, CCL14 induced a significant increase in the BRET signal in HEK293 cells expressing CCR1 and the double brilliance reporter, indicative of an intramolecular conformational change of β-arrestin-2 (**Fig. 4.2B**). Coupled with the BRET saturation and co-immunopreciptiation data, the double-brilliance experiment provides a model in which chemokine binding to CCR1 primarily stimulates a repositioning of CCR1 and β-arrestin-2 relative to one another rather than a dramatic increase in the number of β-arrestin-2 molecules associated with the receptor as would classically be expected. However, it remains likely that chemokine stimulation increases recruitment of β-arrestin-2 to a subpopulation of CCR1 is not basally pre-associated.

To investigate the effect of receptor activation on subcellular distribution of β-arrestin-2, HeLa cells were transiently co-transfected with CCR1-mCherry and β-arrestin-2-GFP. As shown previously, the vast majority of the intracellular pool of β-arrestin-2-GFP was co-localized with the receptor and in discrete puncta prior to stimulation (**Fig. 4.2C**). This is in agreement with the

finding in Chapter 2 of β-arrestin-2-mediated constitutive internalization of CCR1. However, a small pool of β-arrestin-2-GFP appears to be homogeneously distributed within the cell under basal conditions. After treatment with 100 nM CCL14 for 60 min, nearly all of the homogenously distributed β-arrestin-2-GFP translocated and co-localized with CCR1-mCherry. Therefore, receptor activation causes a slight (albeit minor) increase in the recruitment of β-arrestin-2 to CCR1, which is consistent with the slight reduction of the $BRET_{50}$ in **Fig. 4.2A**. This result suggests conformational heterogeneity in which a small proportion of the CCR1 population adopts an inactive conformation that does not exhibit basal β-arrestin-2 association while the major population exists in a conformational state that does. Agonist binding to the receptor apparently shifts this structural equilibrium towards the β-arrestin binding-competent state(s).

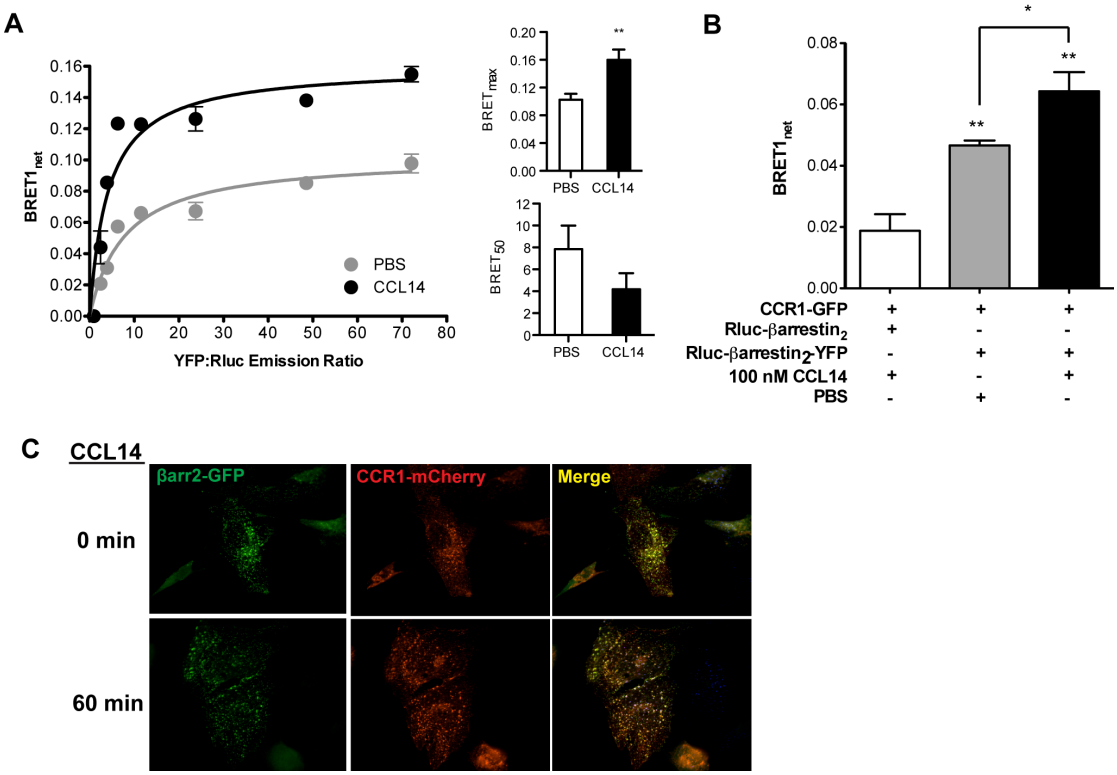


**Figure 4.2. Chemokine-mediated activation of CCR1 induces a conformational change between the receptor and β-arrestin-2. A.** BRET saturation assay in which the levels of β-arrestin-2-Rluc were held constant with increasing expression of CCR1-YFP. The data are plotted against the ratio of YFP:Rluc emission, and the effect of 100 nM CCL14 (black line) was compared to PBS (gray line). Individual data points are shown as the mean ± S.D. of a representative experiment. The bar graphs to the right show the significant effect of CCL14 on the $BRET_{max}$ but not on the $BRET_{50}$ (the ratio of energy acceptor emission to energy donor emission that corresponds to the half maximal point of the curve). Statistical significance was calculated using an unpaired t test. **B.** The $BRET1_{net}$ signal resulting from expression of the double-brilliance β-arrestin-2 reporter (Rluc-β-arrestin-2-YFP) with co-expression of CCR1-GFP and with (black bar) or without (gray bar) the addition of 100 nM CCL14. Statistical significance was calculated using a one-way ANOVA with Tukey's post test. **C.** Immunofluorescence microscopy of HeLa cells cultured on glass coverslips and transiently transfected with β-arrestin-2-GFP (green) and CCR1-mCherry (red). The top panel corresponds to cells left untreated, the bottom panel are images collected from cells following treatment with 100 nM CCL14 for 60 min at 37°C. Co-localization of β-arrestin-2-GFP and CCR1-mCherry is shown in yellow in the merged image.

*CCR1 chemokines induce receptor internalization* — Recruitment and association of β-arrestins to agonist-occupied GPCRs typically results in down-modulation of the receptor through clathrin-mediated endocytosis (26). Flow cytometry was used to measure the amount of CCR1 remaining on the cell surface at any given time point following stimulation with 100 nM CCL3, CCL5, CCL7 and CCL14 (**Fig. 4.3**). Similar to the results from the β-arrestin-2 BRET association assays, CCL3 and CCL14 were the most efficacious in stimulating receptor removal from the cell surface resulting in 70-80% internalization of CCR1. CCL7 was less efficacious with only 55% internalization of the receptor at the later time points. Interestingly, CCL5 exhibited significant variability over the time course of the assay and was the weakest in its ability to down-modulate CCR1.

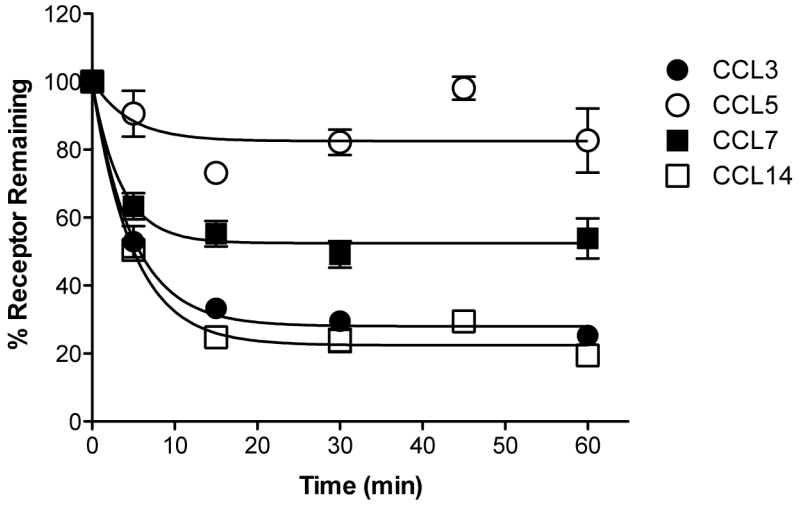


**Figure 4.3. Internalization of CCR1 induced by different CCR1 chemokines.** HEK293 cells stably expressing HA-CCR1 were incubated with 100 nM CCL3, CCL5, CCL7, or CCL14 at 37°C and aliquots removed at the indicated time points. Cells were washed and the amount of cell surface receptor remaining at each time point was measured by flow cytometry with a PE-conjugated anti-CCR1 antibody. Data are plotted as the percent ratio of antibody labeling relative to the amount of cell surface receptor prior to ligand stimulation and fitted using a one-phase exponential decay (Graph Pad Prism).

*The activity of a metal ion chelator complex on CCR1* — Metal ion chelators, specifically Zn(II) or Cu(II) in complex with 2,2'-bipyridine or 1,10-phenanthroline (Phen) scaffolds, have been shown to display agonistic properties on CCR1 (13, 15). However, the function of these chelators has only been tested in inositol phosphate (IP) turnover assays using a chimeric and promiscuous $G\alpha_{\Delta 6qi4myr}$ that couples normally $G\alpha_i$-associated receptors to the $G\alpha_q$ pathway leading to phospholipase C activation. No published reports have assessed whether these ligands can also induce desensitization and internalization of the receptor. 4,7-dimethyl-1,10-phenanthroline was chosen for analysis because it has one of the highest affinities for Zn(II) and potencies for CCR1 activation (15). In a standard assay of chemokine receptor function, the Zn(II):4,7-dimethyl-1,10-phenanthroline (abbreviated 4,7/1,10-ZnPhen) complex was able to specifically induce calcium mobilization in CCR1-expressing HEK293 cells (**Fig. 4.4A**). Independently, 4,7-dimethyl-1,10-phenanthroline and Zn(II) were unable to induce activation of CCR1, which demonstrates the necessity for complex formation for agonist function. The chelator complex was also able to induce an increase in the BRET signal between CCR1 and β-arrestin-2 over time (**Fig. 4.4B**), and cause receptor internalization and removal from the cell surface (**Fig. 4.4C**) to an extent similar to CCR1 chemokines. However, the potency of the metal ion chelators as a whole is significantly lower than that of the chemokine ligands, demonstrated in each of these assays by the significantly higher concentration (approx. 100 fold) of 4,7/1,10-ZnPhen needed to activate CCR1. To the best of

our knowledge, this is the first demonstration of the ability of a small molecule agonist on CCR1 to trigger association with β-arrestin-2 and receptor internalization.

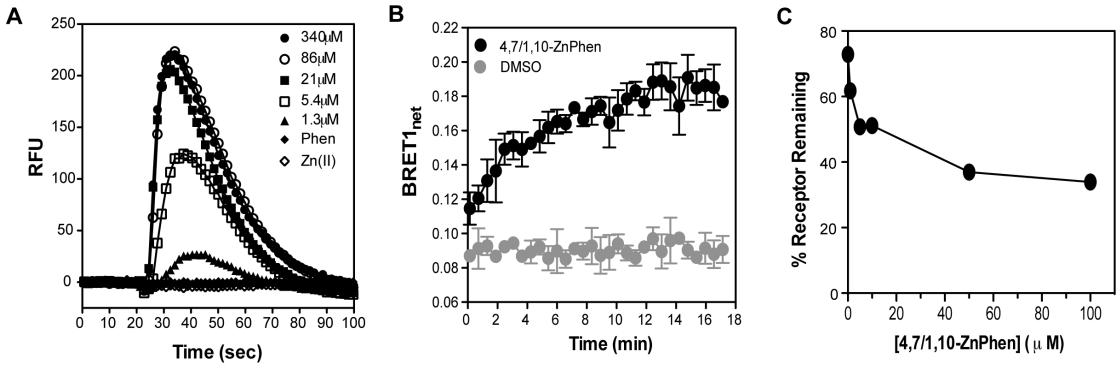


**Figure 4.4**. **Functional effects of a metal ion chelator molecule on CCR1 activity. A.** Calcium flux of HEK293 cells stably expressing CCR1 following stimulation with various concentrations of Zn(II):4,7-dimethyl-1,10-phenanthroline. **B.** Ability of 10 μM Zn(II):4,7-dimethyl-1,10-phenanthroline (abbreviated 4,7/1,10-ZnPhen) to induce an increase in the $BRET1_{net}$ signal over time between CCR1-YFP and β-arrestin-2-Rluc in co-transfected HEK293t cells. **C.** Dose-dependent effect of Zn(II):4,7-dimethyl-1,10-phenanthroline on the internalization of HA-CCR1 in stably-expressing HEK293 cells. The amount of cell surface receptor remaining at 30 minutes post-stimulation was measured using flow-cytometry and compared to the amount of cell surface receptor prior to ligand stimulation.

A previous study carried out a thorough analysis of the effect of different chemical group substitutions to the 1,10-phenanthroline scaffold on activation of CCR1, as well as CCR5 and CCR8 (15). The unsubstituted 1,10-phenanthroline, when complexed with Zn(II), is approximately 10-fold less potent than 4,7-dimethyl-1,10-phenanthroline in stimulating IP turnover through CCR1. However, both chelator compounds were able to displace radiolabeled CCL3 from CCR1 with similar potencies, suggesting they bind

with similar affinity to the receptor. To determine whether this difference in activation also translates to association with β-arrestin-2, the ability of each Zn(II) chelator complex to increase the BRET signal over time between CCR1 and β-arrestin-2 was tested. For each concentration, the 4,7-dimethyl compound yielded a significantly higher response than the unsubstituted 1,10-phenanthroline (**Fig 4.5**). At the lowest dose of 3.125 μM, only 4,7-dimethyl was able to increase the BRET signal at all. These results provide the potential for developing functionally selective small molecule agonists of CCR1 that are β-arrestin "biased."

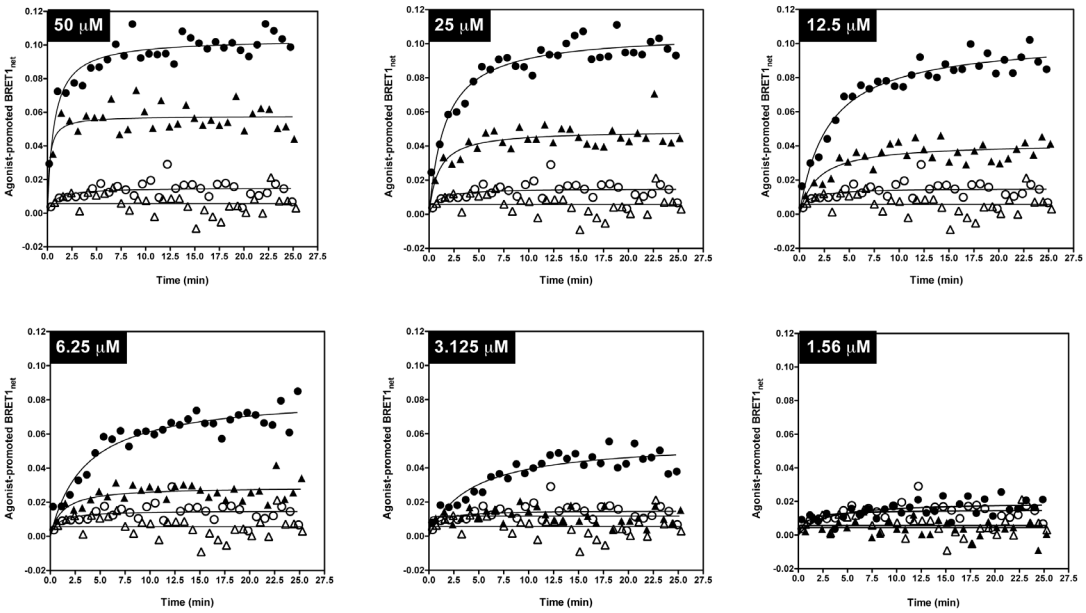


**Figure 4.5**. **Structure-function relationship between metal ion chelator agonists of CCR1.** HEK293t cells transiently co-transfected with CCR1-YFP and β-arrestin-2-Rluc were stimulated with the given concentrations of either Zn(II):1,10-phenanthroline (▲), Zn(II):4,7-dimethyl-1,10-phenanthroline (●), 1,10-phenanthroline (△), or 4,7-dimethyl-1,10-phenanthroline (○). The $BRET1_{net}$ signal was measured over time and the curves fitted using a non-linear regression analysis (GraphPad Prism).

## 4.5 Discussion

As a family, G protein-coupled receptors (GPCRs) are able to detect and respond to an extraordinarily broad array of extracellular stimuli and display a remarkable diversity in their function. Diversity of ligand binding is observed at the level of individual receptors as well, with the chemokine receptors serving as role models amongst the rhodopsin-like subfamily of GPCRs. Multiple members (CCR1, CCR2, CCR3, CCR5, CXCR2 and CXCR3) are activated by five or more chemokines while CCR1 and CCR3 bind to ten chemokines each (9). Whether this promiscuity in ligand:receptor relationships leads to redundant physiological effects or functionally selective signaling remains a matter of open debate.

Receptor ligands have historically been classified into the following categories based upon their affinity (the interaction between the ligand and receptor) and efficacy (the response of the receptor to the ligand): full agonists, partial agonists, neutral antagonists, or inverse agonists. Full agonists maximally stimulate all cellular responses associated with a particular receptor, while partial agonists are only capable of stimulating sub-maximal responses. On the other hand, inverse agonists reduce constitutive (ligand-independent) receptor signaling, and neutral antagonists merely occupy the receptor and block the effects of other ligands without affecting basal activity. It was assumed that all ligands would exhibit the same signaling behavior regardless of the system in which the receptor:ligand pair were being tested. In other words, the intrinsic efficacy of a ligand was thought to be system-

independent and differences in signaling were limited to a “strength-of-scale” response along a uniform pathway (27, 28). As understood in terms of classical pharmacology, all agonists of a particular GPCR would activate the same G protein-signaling pathway and vary from one another only in terms of their affinity for the receptor and whether their induced responses are maximal or sub-maximal. Data collected within the past decade and a half have cast doubt on the validity of this concept; multiple studies have demonstrated that certain ligands have quite diverse functional consequences that are mediated through a single receptor (29). The simple two-state model in which all agonists of a given receptor stabilize a similar active conformation has given way to a multiple-state model wherein any given ligand stabilizes a unique receptor conformation that is then capable of directing coupling towards specific intracellular signaling pathways (30). The concept in which two or more ligands activate the same receptor while leading to differential G protein-coupling or β-arrestin recruitment, for example, is known as “ligand bias” or “functional selectivity,” among other terminologies (27).

Functional selectivity has been observed widely in the GPCR family, with the beta-2 adrenergic receptor ($\beta_2$AR) being the most well-studied (31-34). The histamine H2 and H4 receptors (35, 36), serotonin receptors (37), and type 1 parathyroid hormone receptor (38) have all displayed ligand-selective behavior, just to reference a few of the most recent reports. Amongst the chemokine receptor family, the most prominent example is of the two endogenous agonists of CCR7: CCL19 and CCL21. These two chemokines

bind to the receptor with similar affinity, have equal efficacy for G protein activation, calcium flux, and chemotaxis, and share 32% sequence identity (39). However, multiple reports have shown differential regulation of CCR7 activity by CCL19 and CCL21. Early on, CCL19 was shown to induce rapid CCR7 desensitization and internalization followed by recycling back to the cell surface, whereas CCL21 stimulation did not result in receptor down-modulation (40). Differences in ligand ability to induce rapid dendrite extension (41), C-terminal tail phosphorylation and β-arrestin recruitment (5, 42), and selective engagement of particular GPCR kinase (GRK) isoforms (43) have also been uncovered. Beyond CCR7, a study of five different chemokines acting upon CCR2 found qualitative and quantitative differences in G protein signaling, β-arrestin recruitment, receptor internalization, and in stabilizing different conformations of the CCR2 homodimer (44). For CCR1 itself, differences in the binding affinity and potency of eight different CCR1 chemokines were observed in radiolabeled ligand displacement, [$^{35}$S]-GTPγS exchange, calcium flux and chemotaxis assays, with the most striking difference of CCL4 acting as an antagonist of CCR1 function (23). While CCL3, CCL7, CCL5 and CCL15 were all able to activate $G\alpha_i$-mediated pathways through CCR1, only CCL15 was unable to signal via $G\alpha_{14/16}$ and promote intracellular kinase phosphorylation (10). Additionally, CCL15, but not CCL3, CCL5, or CCL16, was found to activate the human leucine zipper protein (LZIP) transcription factor (45). CCL26 was shown to stimulate chemotaxis in a p38-depdendent manner and failed to bring about calcium

flux, in direct contrast to CCL3, CCL5 and CCL15 (11). Lastly, one study has shown CCL5 to induce a more prolonged internalization of the receptor compared to CCL3 (46). However, no studies thus far have sought to measure any functional differences in chemokine activity in terms of association with β-arrestins. This, coupled with the previous observation that CCR1 forms a ligand-independent association with β-arrestin-2, motivated the research described in this Chapter.

When CCL3, CCL5, CCL7 and CCL14 were each tested in the BRET-based β-arrestin association assay, it was immediately observed that all chemokines were able to induce a dose-dependent increase in the BRET signal between CCR1 and β-arrestin-2. CCL3 and CCL14 displayed greater potency and efficacy compared to CCL5 and CCL7. The effect of CCL3 was expected as it was shown to be the most potent of the CCR1 chemokines in terms of G protein activation; however, the response to CCL14 was surprising as it has one of the lower affinities for CCR1 binding and potencies for [$^{35}$S]-GTPγS exchange (23). CCL5 and CCL7 act as partial agonists, while CCL3 and CCL17 behave as full agonists. The potency rankings were mirrored in the receptor internalization assays where CCL3 and CCL14 stimulated the greatest amount of receptor internalization, followed by CCL7. The behavior of CCL5 in this assay was contradictory to a previous report showing significant internalization of CCR1 in primary eosinophils by CCL5 at the same concentration (100 nM) (46). CCL5 is known to undergo significant oligomerization, which is crucial for its binding to cell surface

glycosaminoglycans and its proper function (47). Perhaps, under the conditions of our study and given the different cell types used, CCL5 was in a different oligomeric state that rendered it less functional.

While differences in the behavior of the chemokines in these assays are evident, they were relatively minor and made it challenging to claim a level of functional selectivity in relation to β-arrestin-2 association or receptor internalization. Perhaps more interestingly, none of the chemokines were able to increase the BRET signal between CCR1 and β-arrestin-1 to any measurable extent. The strong preference of CCR1 for association with β-arrestin-2 over β-arrestin-1 in the absence of agonist (see Chapter 2, **Fig. 2.5C**) was replicated in the presence of agonist as well. GPCRs can be loosely divided into two classes based upon their preferential coupling to a particular β-arrestin isoform and the length of the association following ligand stimulation (26, 48). Class A receptors exhibit preferential binding to β-arrestin-2 and form transient interactions with the receptor (characterized by a lack of co-localization in endocytic vesicles) while Class B receptors bind to β-arrestin-1 and β-arrestin-2 with equal affinity and form more stable interactions with the receptor (characterized by joint trafficking to endocytic vesicles). CCR1 is unique in that it displays qualities of both classes: it couples selectively to β-arrestin-2, yet the association remains stable for an extended period of time, suggesting that CCR1 is a dual Class A/B receptor.

The ability of each chemokine to cause an increase in the BRET signal between CCR1 and β-arrestin-2, given that the pair already exhibit a high

basal association, raised the question as to whether agonists were inducing even more recruitment of β-arrestin molecules to the receptor or a conformational change in the pre-formed receptor:β-arrestin complex. Multiple experiments demonstrated the latter to be the most probable explanation, but left room for increased recruitment to previously unoccupied receptors as well. The intermolecular conformational change between CCR1 and β-arrestin-2 most likely positions the β-arrestin molecule into a more permissive arrangement for scaffolding proteins involved in receptor endocytosis or in β-arrestin-mediated signaling. The structural plasticity of β-arrestin is an emerging concept with previous reports demonstrating that β-arrestin can adopt multiple “active” conformations dependent upon the activation state of the receptor and that the functionally-selective action of biased agonists can be translated downstream through β-arrestin (49, 50). Additionally, the agonist-induced structural re-arrangement between CCR1 and β-arrestin-2 was shown to be stable over an extended period of time. Such a stable association with β-arrestin-2 suggests that CCR1 is sequestered into endocytic vesicles and either targeted for degradation or slowly recycled back to the cell membrane similar to CCR2, CCR5 and CXCR4 (51-53).

With the effect of endogenous chemokine agonists on the association with β-arrestin-2 now elucidated, the ability of small molecule agonists to modulate CCR1 function was tested. Another group has conducted an extensive structural analysis of Zn(II) and Cu(II) metal ion chelators and their unique ability to activate a subset of chemokine receptors, including CCR1

(13, 15, 54). Given the relatively large size of chemokines as GPCR ligands (compared to most other GPCR ligands like biogenic amines, neurotransmitters, and ions) and the extensive contacts they make with multiple structural domains within chemokine receptors, it is quite fascinating that small chemical compounds can stabilize active conformational states within a wild-type chemokine receptor yielding similar signaling efficacies. One of the most potent metal ion chelator complexes identified was Zn(II):1,10-phenanthroline, whose $EC_{50}$ values in terms of IP turnover could be decreased from approximately 6.3 μM to 0.8 μM with the addition of methyl groups at the $p^2$ position (15). This Zn(II):4,7-dimethyl-1,10-phenanthroline complex was used in our experiments to measure calcium mobilization, β-arrestin-2 recruitment, and receptor internalization. In all previous studies of these metal ion chelators, activity at CCR1 was measured only through IP turnover using transfection of an artificial G protein into a non-human cell line. It was demonstrated here that Zn(II):4,7-dimethyl-1,10-phenanthroline is capable of activating CCR1 coupled to endogenous heterotrimeric G protein to initiate calcium flux in HEK293 cells. Additionally, for the first time it was shown that a metal ion chelator induces increased association of CCR1 with β-arrestin-2 and receptor internalization. Although the potency of Zn(II):4,7-dimethyl-1,10-phenanthroline to stimulate these responses was many fold lower than native chemokines, the chelator compound displayed similar efficacy in terms of the maximal BRET signal attained and percent of receptor internalized. The importance of the substituent methyl groups was clearly demonstrated by the

significantly lower BRET signal induced by 1,10-phenanthroline. This provides a glimpse into the structural features necessary for activation of CCR1 beyond its basal state and could provide an initial scaffold in the design of small molecule agonists that exhibit functional selectivity. While not well suited for pharmaceutical development, the ease and low cost of preparation of the Zn(II) chelators provide a unique opportunity to probe the residues involved in the activation mechanisms of CCR1, as has been demonstrated for CXCR3 (55).

Overall, the four endogenous chemokines as well as the Zn(II) chelator examined here were each able to increase the association of CCR1 with β-arrestin-2 and induce receptor internalization. Chemokine agonists were shown to activate a conformational change in a pre-formed complex between CCR1 and β-arrestin-2 as well as increase recruitment to previously unoccupied receptors. Knowledge of the intrinsically different effects of multiple chemokines acting upon a single receptor should aid in the design and development of more effective therapeutics that selectively activate signaling or regulatory pathways through CCR1.

## 4.6 References


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# CHAPTER 5

## DISCUSSION & FUTURE DIRECTIONS

### 5.1 Future of CCR1 drug development

Chemokine receptors are considered one of the most druggable targets in the immune system, and nearly half have been targeted in clinical trials (1). Multiple strategies have been employed to affect the role of chemokine receptors and their ligands in human disease including small molecule inhibitors of receptors, neutralizing antibodies to receptors and ligands, chemically modified chemokines, and inhibitors of chemokine presentation or oligomerization (2). Despite the massive academic and pharmaceutical investment placed into chemokine receptor drug discovery, the development of safe and effective therapeutics has proven to be exceedingly challenging with few successes. To date, only two drugs that directly target chemokine receptors have received FDA approval: the CCR5 antagonist Selzentry (maraviroc) and the CXCR4 antagonist Mozobil (plerixafor/AMD3100). Even though most CCR1 small molecule antagonists have displayed quite favorable safety profiles and were well tolerated in Phase I studies, the Phase II reports are littered with failures to reach clinical endpoints. Of the published reports of CCR1 inhibitors that have reached this stage of development, only one out of seven (CCX354 for treatment of RA) has any promise of moving forward (3).

The question remains as to why compounds that exhibited such promise *in vitro* and in animal models have largely floundered once entering into humans? Multiple potential explanations have been advanced in the literature, ranging from issues in clinical trial design to the heterogeneity of autoimmune diseases (1, 4-6). The significant differences in the expression and function of CCR1 between humans and animal species commonly used as disease models has added to the difficulty in testing new drug candidates. For example, CCR1 is a potent chemoattractant receptor for neutrophils but not monocytes in mice, while just the opposite is true in humans (7). On the flip side, many of the original antagonists developed for CCR1 suffered from a lack of species cross-reactivity and were weak in their affinity to rodent forms of the receptor (1). This significantly limited the options for testing pre-clinical efficacy of CCR1 inhibitors. One solution created by a team at Pfizer involved replacing the murine CCR1 gene with that of the human receptor in order to create a “humanized” mouse model to further test their antagonist (8). One of the most commonly described hurdles to drug development is the potential redundancy of chemokine receptors arising from the promiscuity of chemokine ligands, especially for CC-type chemokines. If a particular inflammatory disease causes up-regulation of chemokines capable of binding to and activating more than one receptor, then a drug development strategy targeting just one chemokine receptor may allow disease progression through another companion receptor. CCR1 ligands CCL3 and CCL5 also bind to CCR5, while CCL7 and CCL8 are similarly recognized by CCR2. Both CCR5 and CCR2

have been implicated in the pathogenesis of RA and MS (9-13); therefore, blockade of a single receptor may be insufficient for attaining efficacy as other receptors can compensate for the original loss of function. This explanation is controversial, however, as it implies that multiple chemokines or chemokine receptors can carry out the same biological functions *in vivo*. It is quite possible that some level of redundancy in the chemokine system developed during evolutionary history in order to impart robustness and ensure continued function of the immune system in case of an impairment of one branch of the system, but the widespread application of this concept to all chemokine receptors involved in inflammatory and autoimmune diseases may not be warranted (6).

Another hurdle to successful drug development is inherent within how the antagonists were developed: the design of competitive inhibitors requires a consistently high concentration in the patient in order to reach sufficient receptor occupancy levels to compete with the up-regulated chemokine ligand levels in inflammatory environments. To borrow an analogy from Thomas Schall, chemokine receptor antagonists are like a sea wall designed to hold back a flood of immune cells into inflamed tissue (6). In order for a sea wall to be effective in this analogy, it must constantly be sound enough to hold back all of the water at all times; if the integrity of the wall is anywhere below a near 100% threshold the resultant inundation from the breach will be sufficient to cause destruction. Additionally, the sea wall must not only remain fully intact but also be of sufficient height; otherwise, it will be overtopped. Chemokine

receptor drugs must be both “breach-resistant” and dosed sufficiently high enough to prevent leukocyte infiltration into inflamed tissue. One study suggested that the anti-inflammatory effects of CCR1 inhibition require antagonist occupancy rates over 90% for a 24-hour period (14). For this reason, the development of non-competitive antagonists that do not require such high dosage levels to prevent chemokine binding to the receptor could be advantageous.

Perhaps, part of the failure in targeting CCR1 could lie within the behavior of the receptor itself. Despite two decades of research since the cloning of CCR1, our laboratory was recently the first to describe the ability of the receptor to signal in the absence of agonist and potentially function as a chemokine scavenger. This means that a fundamental aspect of CCR1 biology has eluded scientists, until now. It seems plausible that the constitutive activity of CCR1 could play a role in disease physiology where receptor expression is significantly up-regulated. Expression of a constitutively active CCR1 is sufficient to induce basal chemotaxis of leukocytic cells and could provide the initial stimulus for immune cell migration in inflammatory disease states. Infiltration of a relatively small number of cells into a particular tissue could lead to the secretion of pro-inflammatory chemokines and other cytokines and recruitment of additional cells to establish a positive feedback loop and enhance tissue damage. Therefore, drug development efforts should consider developing inverse agonists that not only prevent receptor activation by chemokines but also reduce basal signaling. This would open up a new

avenue for targeting CCR1 in the prevention of inflammation and autoimmunity.

### 5.2 Mapping the structural basis of CCR1 constitutive activity

Constitutive activity of wild-type GPCRs has been observed in more than 60 receptors from mammalian, avian and amphibian origin, thereby implicating constitutive activity as an important biological phenomenon in the animal kingdom (15). High basal signaling of GPCRs is also likely to play an important role in pharmacological manipulation of the receptors for the amelioration of disease; many of the clinically used antagonists of GPCR function have been re-classified as inverse agonists that reduce constitutive activity. The functional significance of this re-classification to GPCR drug design remains to be determined; however, examples of naturally-occurring constitutive activity mutations (CAMs) in GPCRs that lead to human disease suggest a pathophysiological role for constitutive activity (16). Understanding the structural basis for this ligand-independent behavior will aid in the development of therapeutics that selectively modulate constitutive activity and could prove useful in the treatment of diseases such as hypertension, heart failure, depression, and schizophrenia (15, 16).

The majority of disease-associated CAMs occur in transmembrane (TM) domains 3, 6, and 7, which are the helices that undergo the most significant conformational change upon GPCR activation (15). Despite the vast chemical diversity amongst endogenous ligands for class A (rhodopsin-like)

GPCRs, it is believed the receptors share a common “global toggle switch” activation mechanism (17). Recent crystal structures of a $\beta_2$AR stabilized in its fully active state confirmed that the most pronounced structural changes occur at the cytosolic face of the receptor and involve an outward movement of TM5 and TM6 and inward motion of TM3 and TM7 (18, 19). These motions are governed by a series of TM domain “micro-switches” that collectively alter the global conformation of the receptor and allow it to populate various active and inactive states (17).

We have begun efforts to map the structural basis of CCR1 constitutive activity by identifying residues in the receptor that differ in strategic positions from analogous residues in CCR5, a non-constitutively active receptor, and measuring the effect of mutation on ligand-independent receptor behavior (see Chapter 3). This effort was aided by molecular modeling of the three-dimensional structure of CCR1 using the newly solved crystal structure of CCR5 (20). Residues at positions within the TM3/TM6/TM7 interface and conserved DRY motif have, thus far, exhibited variable effects on CCR1 basal association with β-arrestin-2 or $G\alpha_i$. We will continue to assess the effects of site-directed mutagenesis on CCR1 intermolecular BRET and advance selected mutants into other assays of constitutive activity including receptor internalization, cAMP inhibition, and basal migration. We will continue to guide our efforts with data on the residues essential for constitutive activity in other chemokine receptors and, more generally, other GPCRs. A summary of all published CAMs of CC- and CXC-type chemokine receptors is shown in **Table**

**5.1**. For example, a Gly→Phe mutation at position $286^{7.42}$ (superscript refers to Ballesteros-Weinstein numbering (21)) in CCR5 was recently shown to induce constitutive activity as measured by elevated basal $G\alpha_i$-mediated signaling, but resulted in a complete loss of β-arrestin recruitment to the receptor (22). This and other mutations in TM6 and TM7 biased CCR5 towards G protein activation over β-arrestin, thereby implicating these domains as indirect but important mediators of β-arrestin recruitment.

**Table 5.1. Summary of constitutive activity mutations (CAMs) of chemokine receptors.** The position of each mutation is provided as the unique residue number within each receptor as well as the systematic Ballesteros-Weinstein number (21). Abbreviations: IP, inositol phosphate; GTPγS, guanosine 5'-O-[gamma-thio]triphosphate. References: (22-29)

| Chemokine Receptor | Mutation | Position | Cell Type | Assay | Reference |
|---|---|---|---|---|---|
| **CCR2** | T84K | 2.56 | CHO-K1 | [$^{35}$S]GTPγS binding | 23 |
| **CCR5** | G286F | 7.42 | COS-7 | IP accumulation, cAMP reduction | 22 |
| | R126N | 3.50 | HEK293 | receptor phosphorylation, internalization | 24 |
| | T82P/K | 2.56 | CHO-K1 | [$^{35}$S]GTPγS binding | 23 |
| | T82P/R225Q | 2.56/6.32 | HEK293 | IP accumulation | 25 |
| | T82K/R225Q | | | | |
| **CCR8** | F84A | 2.53 | COS-7 | IP accumulation | 26 |
| | Y172A | 4.64 | | | |
| | W251A/Q | 6.48 | | | |
| | F290A | 7.43 | | | |
| **CXCR1** | V247A | 6.40 | COS-7 | IP accumulation | 27 |
| | V247N | | | IP accumulation, cAMP reduction | |
| **CXCR2** | D138V | 3.49 | 3T3 | cellular transformation, IP accumulation | 28 |
| **CXCR4** | N119S | 3.35 | CHO-K1 | [$^{35}$S]GTPγS binding, receptor phosphorylation, internalization | 29 |
| | N119A | | | [$^{35}$S]GTPγS binding | |

### 5.3 Functional selectivity of CCR1 ligands: Signaling and regulation

Functional selectivity in the chemokine system can be described by the ability of multiple chemokine ligands to bind to the same receptor and elicit different biological responses. While extensive reports exist within the literature for this phenomenon within other GPCR families, much less data is available for chemokine receptors. Even less well known is the biological significance of functional selectivity at particular receptors. The purpose of studying the unique signaling and regulatory behavior of each chemokine ligand is to (hopefully) enable the development of biased ligands for therapeutic use that selectively activate or inactivate CCR1 signaling pathways beneficial to desired clinical outcomes while avoiding pathways contributing to decreased efficacy or deleterious side effects. This approach has been suggested to be feasible for CCR5 and CXCR4 (30-32), while it has been put into clinical practice for other receptors (33). Indeed, an entire biopharmaceutical company (Trevena, www.trevenainc.com) was co-founded by 2012 Nobel Laureate Robert Lefkowitz and dedicated to the discovery and development of GPCR biased ligands. Trevena has progressed a functionally selective ligand for the angiotensin II type 1 receptor to Phase II clinical trials for treatment of acute heart failure (34).

The classical example of chemokines acting at the same receptor and inducing distinct behaviors is of the two endogenous ligands for CCR7 (CCL19 and CCL21) where only CCL19 is capable of inducing receptor internalization (35-37). While CC-type chemokines CCL3, CCL3L1, CCL4 and CCL5 were all

equally able to stimulate a G protein signaling pathway through CCR5, CCL3 was significantly less efficacious in inducing receptor internalization (38). Additionally, CXCL12 and the HIV-1 glycoprotein gp120 exhibit different levels of ERK1/2 phosphorylation dependent on neuronal cell type being tested (39, 40). At the level of chemokines, CXCL8 exhibits bias in its down-regulation of CXCR1 and CXCR2 by inducing rapid internalization of CXCR2 but not CXCR1 (41). CCL5 also exhibits bias in its ability to differentially regulate the recycling behavior of the three classical receptors it is recognized by – CCR1, CCR3 and CCR5 (42-44). CCR1 is a prime candidate for studying the potential for functional selectivity in the chemokine system given its extensive diversity in ligand binding and activation by ten endogenous chemokines (45). However, few studies have set out to primarily test this hypothesis and measure whether each chemokine yields a similar or distinct cellular response upon activation of CCR1. The published reports have shown differential activation of non-Gi/o heterotrimeric G proteins (46), selective activation of p38 mitogen activated protein kinase and calcium flux (47), contrasting abilities to mediate leukocyte chemotaxis (48), different binding sites within the receptor ligand binding pocket (49), and differing rates of receptor internalization and recycling (50). However, none of these reports were systematic in comparing the effects of all CCR1 ligands and did not necessarily differentiate biological response with chemokine affinity. Prior to our study, no information was available on any ligand-induced effects on the recruitment of β-arrestins to CCR1. As evidenced above, the majority of studies demonstrating chemokine

receptor functional selectivity outside of CCR1 have done so at the level of receptor desensitization and down-regulation rather than G protein activation. Preliminary results in our laboratory have shown little differences between a subset of CCR1 chemokines in assays of calcium flux and ERK1/2 phosphorylation. Therefore, our efforts in the future will focus on differential ligand effects on non-G protein-mediated signaling and regulatory pathways.

We have shown different quantitative and qualitative effects of four CCR1 chemokines – CCL3, CCL5, CCL7 and CCL14 – on β-arrestin-2 association and receptor internalization. However, all ligands tested were able to induce a positive response in these assays and none could bring about association between CCR1 and β-arrestin-1; therefore, the differences were not as stark as what was observed for CCR7 making the classification of these chemokines as functionally selective difficult. The next step in this work will involve expanding the repertoire of chemokines to include the other CCR1 ligands – CCL3L1, CCL8, CCL13, CCL15, CCL16, and CCL23. The latter three chemokines have recently been cloned into our laboratory's unique expression vector system and are currently undergoing optimization of expression and purification (51). CCL4 will also be included in future studies since the literature is contradictory as to whether it is a partial agonist or antagonist on CCR1 (52, 53). The potential exists for CCL4 to inhibit CCR1 under certain physiological settings or through select pathways and activate the receptor in others, thereby demonstrating functionally selective behavior. All chemokines will be tested in the β-arrestin-1/2 association assay as well as

for agonist-induced receptor phosphorylation and internalization. A recent study of multiple CCR2 chemokine agonists demonstrated quantitative differences in β-arrestin recruitment; however, similar to our study with CCR1 all ligands exhibited an increase in association with differences limited to the overall extent and stability of the interaction (54). However, phosphorylation of receptor intracellular domains has already been shown in some GPCRs to be a consequence of differential activation by distinct ligands and may illuminate unique mechanisms of ligand-induced regulation of CCR1 (36, 55-58). This is complicated by our observation that CCR1 is basally phosphorylated and exhibits minimal, if any, increase in phosphorylation following stimulation with a single concentration of CCL14. Two studies of CCR1 expressed in a rat basophilic leukemia cell line did observe an up-regulation of receptor phosphorylation in following stimulation with CCL3, CCL5 and CCL8 (59, 60). The first step moving forward will be to determine whether any other chemokines significantly increase CCR1 phosphorylation in human cells and subsequently identify the residues (most likely on the receptor C-terminal tail) to be differentially involved in constitutive versus agonist-induced phosphorylation. The region of high Ser/Thr density in the CCR1 C-tail responsible for basal phosphorylation has already been identified; however, the mutational analysis should be extended to determine which specific residues are modified and essential for basal association with β-arrestin-2. Lastly, we have shown that CCR1 undergoes ligand-independent internalization and recycling while ligand-dependent internalization seems to

prolong receptor internalization, at least within the hour-long time frame of the assay. A previous study has shown extended CCR1 internalization in primary human eosinophils following stimulation with CCL5, but extensive recycling following CCL3 treatment (50). We must first confirm whether all CCR1 chemokine agonists are capable of inducing receptor internalization and then determine the post-internalization fate of the receptor, as will be discussed in a later section.

### 5.4 Functional selectivity of CCR1 ligands: Receptor structure

Evidence from cell-based and biophysical studies demonstrates that structurally different agonists of a given GPCR stabilize distinct conformational states within the receptor, rather than simply altering the equilibrium between an inactive and active state (61-63). Characterizing how ligand binding is correlated with unique conformational changes in GPCRs and leads to activation of select downstream signaling and/or regulatory pathways is critical for understanding how these receptors function in complex signaling networks. Connecting the receptor structural data with the *in vitro* or *in vivo* actions of each ligand would also provide a deeper understanding of the molecular mechanisms behind ligand functional selectivity and reveal important principles highly relevant to GPCR drug discovery and development. The prior section is focused on continuing our work on studying the intermolecular associations and downstream effects of CCR1 activation by different ligands, while this section focuses on taking the next step of elucidating the

intramolecular interactions within the receptor that initiate those intracellular events. Such experiments shall ultimately answer questions over what the conformational changes that occur in CCR1 upon ligand binding are and whether different chemokines induce distinguishable active states in CCR1. Additionally, identification of the distinct networks of interactions leading to the fully active state in complex with current data on the mutational analysis of CCR1 will shed light on the structural basis of constitutive activity. Such data can then be compared with the results of similar experiments with other GPCRs to develop family-wide patterns of conformational states/activation mechanisms and to identify those that are unique to specific classes of receptors or individual receptor/ligand pairs. It will be interesting to determine whether or not the activation mechanisms of chemokine receptors can be distinguished from GPCRs that are responsive to drastically smaller ligands with fewer contacts with the receptor. From these studies we will gain a better understanding of how structural changes couple ligand binding on the outside of the cell to activation of signaling molecules inside the cell.

Multiple strategies have been employed to analyze distinct conformational changes within GPCRs stabilized upon ligand binding including NMR spectroscopy (64), site-specific labeling with environmentally sensitive fluorophores for fluorescence spectroscopy (65-69), site-directed spin labeling with nitroxide spin labels for electron paramagnetic resonance measurement (70-72), and *in vivo* intramolecular FRET using genetically encoded fluorescent proteins or motifs for in-cell fluorophore labeling (73-75). The first

three techniques provide the most conformationally sensitive measurements; however, they require the isolation of highly purified receptor and reconstitution into artificial lipid environments, which poses significant technical challenges. Our laboratory has been successful recently in developing novel strategies for recombinant chemokine receptor expression and purification to sufficient purity and quantity for high-resolution structural analysis. We are now capable of generating CCR1 to the levels necessary for fluorescence spectroscopy and EPR analyses with introduced Cys residues for labeling with maleimide-linked fluorophores or nitroxide spin labels, respectively. CCR1 has seven cysteines within its amino acid sequence with four residues engaged in disulphide bonding and another two located in TM7 and not solvent exposed. The only endogenous cysteine that exhibits background labeling is at the base of TM5 and has been mutated to Ser with no effect on ligand binding or receptor function (data not shown). In collaboration with the Ruben Abagyan laboratory at UCSD, we have used computational modeling to identify positions at the interface of the CCR1 TM and intracellular domains that are likely to be sensitive to ligand-induced conformational changes within the receptor. For example, Ser235 of CCR1 is analogous to the highly studied Cys265 of $\beta_2$AR and is well positioned to detect motion of ICL3 that is involved in G-protein coupling (66, 67). We have begun mutating these residues singularly or in pairs to cysteines and will eventually express the constructs in our insect cell or mammalian systems once we have confirmed that mutation has little to no impact on chemokine

binding affinity and receptor signaling. We have the capacity in the laboratory to carry out labeling with environmentally sensitive fluorophores that have been used successfully by other groups to study GPCR conformational change (ex. methylbromobimane, tetramethylrhodamine, and fluoroscein). Measurement of fluorescence intensity or lifetime of the probes will be conducted in collaboration with Judy Kim at UCSD. Fluorescence intensity is used to determine the average of the anticipated multiple discrete conformational states that are induced by ligand binding, while lifetime measurements can detect the discrete conformational changes within the population. Nitroxide spin labeling and subsequent EPR measurement will be accomplished in collaboration with Wayne Hubbell at UCLA whose lab has pioneered the use of this technique to study the structural plasticity of GPCRs, arrestins, and G proteins. Lastly, we have taken the initial steps to create an *in vivo* and intramolecular FRET-based system to measure CCR1 conformational changes in living cells. This involves the fusion of cyan fluorescent protein (CFP, energy donor) to the C-terminal tail of CCR1 and introduction of a tetra-cysteine (CCPGCC) motif into ICL3 that has high affinity for labeling by FlAsH (fluorescein arsenical helix binder, energy acceptor). FlAsH is a small fluorescent probe that remains nonfluorescent until bound specifically to the teta-cysteine motif (76). Additionally, FlAsH labeling has been shown not to affect GPCR function when incorporated into positions in the ICL3 or the C-tail (77-79). Ligand-induced distance/orientation changes measured as the FRET signal between fluorophores in ICL3 and the C-terminal tail of a receptor have

been used for multiple GPCRs as a reporter of receptor activation in an intact cellular environment (74, 75, 80). The CCR1-CFP fusion with the CCPGCC insertion into ICL3 has already been cloned and confirmed as functional in a calcium mobilization assay. HEK293 cells stably expressing the construct under a tetracycline-inducible promoter have been generated and initial attempts at FlAsH labeling have been carried out; however, significant optimization is needed to minimize background cellular labeling and maximize receptor labeling before we can begin testing ligand-induced patterns in the FRET signal between CFP and FlAsH. In summary, the preliminary steps have been taken that lay the foundation for probing the conformational complexity of CCR1 and measuring the structural states stabilized by multiple chemokine ligands. Correlating the distinct signaling and regulatory pathways activated by each chemokine (discussed in the previous section) with the conformational heterogeneity of CCR1 following differential ligand activation (discussed in this section) will provide us with a deeper understanding of the molecular mechanisms that form the basis of functional selectivity.

**5.5 Phosphorylation, internalization, and post-endocytic fate of CCR1**

GPCR signaling is regulated by multiple processes including desensitization, internalization, recycling and degradation. Desensitization is the process by which receptors no longer respond to continued stimulation primarily due to physical uncoupling from G proteins by recruitment of arrestins and typically occurs within a short timeframe of seconds to minutes

following initial agonist exposure. Internalization is the process whereby receptors are sequestered from the cell surface to intracellular compartments most commonly by clathrin coated pit or calveolae-mediated endocytosis and temporally follows desensitization. Receptor recycling and degradation result in the reduction of total cellular receptor numbers typically over a period of several hours and is driven by localization of the receptor into specific endosomal compartments. Relatively little is known of the desensitization and internalization mechanisms that regulate the function and localization of CCR1 and the fate of the receptor following removal from the cell surface. Our work presented in the previous Chapters has demonstrated that CCR1 is constitutively phosphorylated, associated with β-arrestin-2, internalized, and recycled in an agonist-independent manner. Four CCR1 chemokines were able to dose-dependently increase the association between CCR1 and β-arrestin-2, as well as induce receptor internalization, with some observed differences in potency and efficacy. However, many questions still remain regarding the specific proteins that are engaged throughout the CCR1 regulatory process and how those differ between constitutively active and chemokine activated receptor.

One of the first questions our future studies hope to answer is what kinases are involved in the basal phosphorylation of CCR1. A previous report has shown that CCR1 phosphorylation following treatment with either CCL5 or CCL8 was partially inhibited by pretreatment with staurosporine, suggesting that PKC is involved in CCR1 phosphorylation (60). Additionally, treatment of

CCR1 expressing cells with phorbol 12-myristate 13-acetate (PMA, an activator of PKC) partially increased CCR1 phosphorylation while activation of PKA with the cAMP analogue cpt-cAMP had no effect, indicating further that PKC but not PKA plays a role in heterologous receptor phosphorylation. However, PKC was reported to only be partially responsible for CCR1 phosphorylation, thereby implicating G protein-coupled receptor kinases (GRKs) as well. Desensitization of agonist-occupied GPCRs is most commonly initiated through the recruitment of GRKs, which phosphorylate key Ser/Thr residues in receptor intracellular domains (81). Seven mammalian GRKs have been identified (GRK1-7), two of which (GRK1 and GRK7) are limited in their expression to the visual system (82). The role of individual GRKs in mediating receptor phosphorylation has been studied for many chemokine receptors other than CCR1 including CCR2, CCR5, CCR7, CXCR1, CXCR2, and CXCR4 (37, 83-86). Individual over-expression of the non-visual GRKs in cells stably expressing CCR5 each led to increases in CCL5-induced phosphorylation of CCR5 (58). Subsequently, neutralizing antibodies against GRK2 and GRK3, but not GRK4-6, blocked CCR5 phosphorylation in receptor-expressing rat basophilic leukemia (RBL) cell line (87). Differential phosphorylation of specific C-terminal tail Ser residues by GRKs and PKC was also observed for CCR5 indicating differential homologous and heterologous desensitization mechanisms for this receptor (88). Perhaps more relevant to CCR1, the constitutive phosphorylation of the viral chemokine receptor US28 and its association with β-arrestin is up-

regulated by overexpression of GRK2 and GRK5 (89). Most recently, RNA interference (RNAi) was used to determine that GRK2 and GRK6 are responsible for CXCR1 and CXCR2 phosphorylation, desensitization and internalization (83). We plan to utilize a similar knockdown approach and first screen the effect of specific GRK shRNA constructs on constitutive CCR1 association with β-arrestin-2 using the BRET assay in HEK293 cells. Mutation of specific Ser/Thr residues in the C-terminal tail to Ala was sufficient to block basal phosphorylation and β-arrestin-2 interaction; therefore, down-regulation of one of the kinases responsible for CCR1 phosphorylation event(s) should translate into a decrease in the basal BRET signal between CCR1 and β-arrestin-2. Using this assay we can also screen specific PKC and PKA inhibitors to monitor whether CCR1 also undergoes heterologous phosphorylation by second messenger kinases in human cells; the previously mentioned evidence for PKC phosphorylation of CCR1 is limited to a rat cellular expression system (60). The effects of selective GRK knockdown and second messenger kinase inhibition can then be extended to phosphorylation and internalization assays similar to those conducted in Chapter 2 and Chapter 3. The future development of phosphosite-specific antibodies for CCR1 would greatly enhance efforts to understand the functional consequences of selective phosphorylation of C-terminal residues, as has been demonstrated with CXCR4 (85).

GPCR internalization typically follows receptor phosphorylation and β-arrestin-2 recruitment and most commonly occurs through clathrin-coated pits

(CCP) or caveolae. In the first process, GPCRs are scaffolded to clathrin through the binding of β-arrestin to the β2-adaptin subunit of the heterotetrameric AP-2 adaptor complex (90). Receptor-containing CCPs then bud off from the plasma membrane through the action of the GTPase dynamin. The vesicles are then sorted through various endosomal compartments for rapid or slow recycling to back to the cellular surface or eventual degradation (91). The second pathway of internalization is dependent on cholesterol-rich and highly organized membrane structures that are shaped and organized by caveolins, which are a small class of proteins that form large oligomeric complexes and coat the cytoplasmic surface of membrane invaginations (92). The internalization pathways of multiple chemokine receptors have been studied and no family-wide patterns have emerged. Clathrin-mediated endocytosis has been observed for CXCR1, CXCR2, CXCR4, and CCR7, while atypical receptors DARC and CCX-CKR have exhibited calveolae-dependent internalization (93). Some chemokine receptors, including CCR2, CCR4 and CCR5 take advantage of both of these pathways for down-modulation from the cell surface. The specific mechanism utilized by CCR1 remains unknown; however, our observation of the necessity of β-arrestin-2 for CCR1 constitutive internalization in mouse embryonic fibroblasts suggests it is dependent upon association with CCPs. The atypical chemokine scavenging receptor D6, which is similar to CCR1 in that it is constitutively associated with β-arrestin and undergoes continual internalization and recycling, utilizes a dynamin- and arrestin-dependent

mechanism for endocytosis (94, 95). The conventional chemokine receptor CXCR4 has been shown to undergo CCP-dependent constitutive endocytosis as well (96). Whether CCR1 utilizes clathrin-coated pits for constitutive internalization can be tested using a battery of CCP inhibitors (chloropromazine, high sucrose concentrations, dominant negative dynamin K44A mutant) in the loss-of-surface-receptor assay used in Chapter 2. Inhibitors of calveolae formation and function (nystatin and filipin) can be examined in parallel to determine which pathway is primarily utilized by CCR1 under non-stimulatory conditions. These assays of constitutive internalization should also be conducted with chemokine stimulation to determine whether the selection of a particular endocytosis mechanism is ligand-dependent.

Once the receptor is internalized into an membrane vesicle, it must then decide whether to be sorted into late endosomes and lysosomes for degradation or into recycling endosomes for delivery back to the plasma membrane (97). The prior results in long-term attenuation of receptor signaling, whereas the latter enables functional resensitization. Vesicles originating from clathrin-dependent and -independent endocytosis are initially transported to the early endosome where the future of their cargo is determined by the action of different Rab GTPases (98). Rab proteins are low molecular mass GTP-binding proteins that cycle between an inactive GDP-bound an active GTP-bound state and interact with and activate several cellular effector proteins to control intracellular vesicular transport. Rab family members selectively associate with particular endocytic compartments: Rab5

is an important mediator of early endocytic sorting; Rab4 and Rab11a are associated with the slow and fast recycling compartments, respectively; Rab7 plays a role in lysosomal sorting (99, 100). For example, CXCR2 has been shown to localize in Rab5-positive endosomes soon after ligand stimulation and utilize a Rab11a-dependent process for recycling back to the cell surface, but is also sorted into Rab7-positive endosomes for lysosomal degradation under periods of prolonged agonist treatment (101). No information is currently available regarding the role of specific Rab proteins in the post-endocytic fate of CCR1, or many other chemokine receptors, and may be an avenue of future study in our laboratory. A recent study monitored the constitutive internalization of the Wnt pathway-associated GPCR LGR5 and determined that it utilizes a clathrin-dependent pathway, rapidly internalizes into Rab5-positive endosomes, and transits through Rab7- and Rab9-positive vesicles to reach a steady-state distribution in the trans-Golgi network (102). LGR5 trafficking was partially measured using fluorescence microscopic co-localization of multiple endosomal markers as well as GFP-tagged Rab proteins with pre-labeled LGR5. This strategy could be employed with simple modifications to the constitutive receptor internalization protocol in HeLa and COS-7 cells demonstrated in Chapter 2. We have observed a consistent level of CCR1 cell surface expression under basal conditions despite constitutive receptor internalization, suggesting that CCR1 is continually recycled back to the plasma membrane for no net change in surface density. This may implicate the association of the receptor with Rab5- and then either Rab4 or

Rab11a-positive vesicles. Chemokine stimulation of CCR1, however, does lead to a significant down-modulation of total cell-surface receptor levels. It will be interesting to determine whether constitutive versus agonist-induced internalization leads to separate post-endocytic sorting pathways, and whether CCR1 chemokines display any functional selectivity in regulating differential receptor sorting. The limited data available on chemokine-induced internalization of CCR1 claims that stimulation with CCL5 or modified forms of CCL14 induces endocytosis without recycling, whereas CCL3-mediated activation of CCR1 leads to rapid repopulation of receptor levels at the plasma membrane (50, 103, 104). Understanding the behavior of CCR1 once it is internalized may aid in our understanding of its function as a chemokine scavenger as it would need to deliver its chemokine cargo for degradation or continual internalization while also sorting itself for eventual recycling back to the membrane.

### 5.6 CCR1 as a chemokine scavenging receptor

Our work has described a dual role for CCR1 of classical G protein signaling to mediate leukocyte migration and of chemokine scavenging. The removal of soluble chemokines from tissue environments independently of conventional signaling is a hallmark function of the atypical chemokine receptors (also known as “decoy” or “scavenging” receptors) including Duffy antigen for chemokine receptor (DARC), D6, CC chemokine receptor-like 1 and 2 (CCRL1, CCRL2), and CXCR7. These receptors are mainly expressed

by non-leukocytic cells and play an essential and non-redundant role in leukocyte migration by shaping chemokine gradients to ensure directional migration under both homeostatic and inflammatory conditions (105). The loss of D6, perhaps the most thoroughly studied of the scavenger receptors, is associated with increased levels of inflammatory CC-type chemokines in the circulation leading to exacerbated inflammatory responses (106-108). Interestingly, gene knockout studies have correlated a similar increase in chemokine ligand concentration in peripheral blood and certain tissues with the loss of non-atypical chemokine receptor expression (109). Mice lacking CX3CR1, CXCR2, CXCR3, or CCR2 exhibited greater than 10-fold increases in the levels of their cognate chemokines that was not related to transcript expression. Reconstitution of *Cx3cr1*$^{-/-}$ or *Cxcr2*$^{-/-}$ mice with bone marrow from wild-type littermates returned the levels of CX3CL1 or CXCL2 to normal levels. Another study found that IL-10-mediated inhibition of the down-regulation of CCR1, CCR2, and CCR5 in dendritic cells and monocytes led to “frozen” receptors that were functionally uncoupled from G protein signaling but were still able to internalize their chemokine ligands (110). This report suggested that the generation of functional decoy receptors in an inflammatory setting could be a strategy to block excessive leukocyte recruitment and activation. Lastly, CCR2-expressing primary human monocytes were shown to continually scavenge and degrade fluorescently-labeled chemokine in a CCR2-dependent manner in the presence of pertussis toxin, which completely blocked induction of cellular migration (111). Chemokine scavenging by

CCR2 was elegantly observed through the use of time-lapse video microscopy of monocytes under a layer of matrigel with constant perfusion of mCherry-labeled chemokine resulting in a continual chemokine gradient (111). This study will provide a model for the experimental techniques our laboratory is currently pursuing in order to further confirm the scavenging behavior of CCR1.

The role for signaling chemokine receptors in ligand homeostasis through clearance of chemokines from the circulation and tissues is an emerging concept; accordingly, the molecular mechanism enabling these G protein-signaling chemokine receptors to adopt decoy receptor behavior remains undetermined. A recent study illuminated the pathway engaged by the atypical chemokine receptor D6 for its scavenging activity (112). Ligand binding to D6 activated a β-arrestin-1-dependent, G protein-independent signaling pathway that resulted in phosphorylation of the actin-binding protein cofilin and re-organization of the actin cytoskeleton through the Rac1—p21-activated kinase 1 (PAK1)—LIM kinase 1 (LIMK1) cascade. CCR5 activation was also shown to induce cofilin phosphorylation through the Rac1—PAK1—LIMK1 pathway; however, the pertussis toxin-sensitivity of the signal meant it was $G\alpha_i$-dependent and not arrestin-mediated. Additionally, blockade of $G\alpha_i$ signaling did not impart chemokine scavenging behavior upon CCR5 indicating that other signaling features are required to convert a conventional chemokine receptor into a scavenger. Our experiments demonstrated a similar inability of CCR5 to engage in chemokine scavenging. CCR1, however, was

capable of internalizing fluorescently-labeled CCL7 in the presence of pertussis toxin, suggesting it possesses the necessary signaling features for scavenging behavior. Similar to D6, CCR1 is basally phosphorylated, associated with β-arrestin, and undergoes constitutive internalization and recycling. D6 is known to internalize in Rab5-positive vesicles in a CCP- and dynamin-dependent process, and then recycle back to the membrane through Rab11-positive endosomes (95, 113-116). As outlined in the section above, future work on CCR1 will involve deciphering the post-endocytic fate of the receptor and determine whether it engages a similar pathway as D6. We have demonstrated that the constitutive activity of CCR1 is sufficient to induce cellular migration and increased levels of basal F-actin content in L1.2 cells, suggesting that CCR1 is stimulating continual actin remodeling. This could implicate cofilin activity in basal CCR1 internalization and chemokine scavenging since it plays a critical role in cytoskeletal rearrangement through depolymerizing and severing actin filaments and will be examined in future studies (117).

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