C-X-C Motif Chemokine Receptor 3 Splice Variants Differentially Activate Beta-Arrestins to Regulate Downstream Signaling Pathways


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Running title page

CXCR3 Splice Variants

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Nonstandard abbreviations

βarr1, beta-arrestin 1 (also known as arrestin-2); βarr2, beta-arrestin 2 (also known as arrestin-3); BRET, bioluminescence resonance energy transfer; CKR, chemokine receptor; CXCL4, C-X-C motif chemokine ligand 4; CXCL9, C-X-C motif chemokine ligand 9; CXCL10, C-X-C motif chemokine ligand 10; CXCL11 C-X-C motif chemokine ligand 11; CXCR3, C-X-C Motif Chemokine Receptor 3; ERK, extracellular regulated kinase; GFP, green fluorescent protein GPCR, G protein coupled receptor; GRK, G protein receptor kinase; HEK, human embryonic kidney; Rluc, renilla luciferase; siRNA, small inhibitory ribonucleic acid; SRE, serum response element; SRF, serum response element response factor; YFP, yellow fluorescent protein
Abstract

Biased agonism, the ability of different ligands for the same receptor to selectively activate some signaling pathways while blocking others, is now an established paradigm for GPCR signaling. One group of receptors in which endogenous bias is critical is the chemokine system, consisting of over 50 ligands and 20 receptors that bind one another with significant promiscuity. We have previously demonstrated that ligands for the same receptor can cause biased signaling responses. The goal of this study was to identify mechanisms that could underlie biased signaling between different receptor splice variants. The receptor CXCR3 has two splice variants, CXCR3A and CXCR3B, which differ by 51 amino acids at its N-terminus. Consistent with an earlier study, we found that CXCL4, CXCL9, CXCL10, and CXCL11 all activated G\textsubscript{ai} at CXCR3A, while at CXCR3B, these ligands demonstrated no measurable G\textsubscript{ai} or G\textsubscript{as} activity. β-arrestin (βarr) was recruited a reduced level to CXCR3B relative to CXCR3A, which was also associated with differences in βarr2 conformation. βarr2 recruitment to CXCR3A was attenuated by both GRK2/3 and GRK5/6 knockdown, while only GRK2/3 knockdown blunted recruitment to CXCR3B. ERK1/2 phosphorylation downstream of CXCR3A and CXCR3B was increased and decreased, respectively, by βarr1/2 knockout. The splice variants also differentially activated transcriptional reporters. These findings demonstrate that differential splicing of CXCR3 results in biased responses associated with distinct patterns of βarr conformation and recruitment. Differential splicing may serve as a common mechanism for generating biased signaling and provides insights into how chemokine receptor signaling can be modulated post-transcriptionally.
Introduction

Chemokine receptors (CKRs) are a family of G protein-coupled receptors (GPCRs) that bind small cognate peptide ligands, chemokines. Chemokines are so named for their ability to induce chemotaxis and guide leukocyte migration. Chemokines are produced by a variety of cell types at sites of inflammation, and not only mediate the extravasation and chemotaxis of inflammatory mediators, but also are involved in cell activation, differentiation, actin polymerization, and direct immune cell function (Koelink et al., 2012; Thelen, 2001). CKRs often interact with multiple chemokines, and chemokines often bind to multiple chemokine receptors, supporting the prevailing sentiment that the chemokine system is both promiscuous and redundant. However, recent work demonstrates non-overlapping intracellular pathway activation by various chemokines at the same CKR (Drury et al., 2011; Kohout et al., 2004; Rajagopal et al., 2010; Zidar et al., 2009; Zohar et al., 2014), suggesting a unique signaling repertoire may be encoded with each distinct ligand-receptor complex.

GPCRs typically interact with three classes of proteins – heterotrimeric G proteins, β-arrestins (βarrs), and GRKs. Some chemokines selectively activate certain GPCR signaling pathways, such as the G protein pathway, while blocking others, like the βarr pathway (Karin et al., 2016; Kohout et al., 2004; Rajagopal et al., 2013; Rajagopal et al., 2010; Zidar et al., 2009). This signaling paradigm is known as biased agonism or functional selectivity (Rajagopal et al., 2011; Urban et al., 2007). Many GPCRs, including chemokine receptors, show evidence of biased signaling through G-proteins or βarrs (Kohout et al., 2004; Rajagopal et al., 2013; Rajagopal et al., 2010; Zidar et al., 2009). In addition, some chemokine receptors were thought to act as ‘decoys’ for ligands given their inability to active classical G protein pathways. However, we now appreciate some of these receptors, such as CXCR7, are βarr-biased receptors that do not signal through G protein pathways but do couple to βarrs and mediate βarr dependent signaling (Rajagopal et al., 2010). However, the mechanisms underlying biased signaling at chemokine receptors remain only partially characterized (Busillo et al., 2010).
CXCR3 has two seven-transmembrane spanning splice variants, CXCR3A and CXCR3B. CXCR3A and CXCR3B have identical intracellular sequences and only differ by the replacement of the four most distal residues on the N-terminus of CXCR3A with 51 amino acids unique to CXCR3B due to alternative splicing at the 5’ end of the second exon (Lasagni et al., 2003) (Figure 1). These splice variants are known to signal in response to four chemokines: CXCL4 (Platelet Factor-4; PF-4), CXCL9 (Monokine induced by IFN-γ; MIG), CXCL10 (IFN-induced protein 10; IP-10), and CXCL11 (IFN-inducible T-cell α chemoattractant; I-TAC) (Cole et al., 1998; Hermodson et al., 1977; Taub et al., 1993; Tensen et al., 1999). CXCR3 was first discovered through its selective recruitment of effector T-cells (Loetscher et al., 1996), and is now known to be a critical mediator of inflammation, vascular disease, and cancer (Van Raemdonck et al., 2015). Aberrant CXCR3 signaling is implicated in inflammatory diseases, inhibition of blood vessel formation, and both tumor repression and tumorigenesis (Kawada et al., 2004; Koch et al., 2009; Peng et al., 2015; Villarroel et al., 2014). Due to differential regulatory promoter elements, the endogenous ligands of CXCR3 are spatially and temporally separated by expression pattern (Groom and Luster, 2011), suggesting distinct functional properties of CXCL4, CXCL9, CXCL10, and CXCL11. CXCR3A is primarily expressed on activated T-lymphocytes (Qin et al., 1998), but expression is also noted on a variety of other cell types including dendritic cells, natural killer cells, B-cells, and macrophages (Garcia-Lopez et al., 2001). In contrast, CXCR3B is expressed predominantly on microvascular endothelial cells (Lasagni et al., 2003), as well as on T-lymphocytes, although at a lower level than CXCR3A. A recent report demonstrated differential effects of endogenous ligands at CXCR3B compared to CXCR3A (Berchiche and Sakmar, 2016), however, the role of β-arrestin recruitment and how recruitment propagates to downstream signaling between CXCR3A and CXCR3B remains unclear. The goal of this study was to understand how the different N-termini of CXCR3A and CXCR3B influence intracellular signaling through G-proteins, βarrs and GRKs.
Materials and methods

Cell Culture – HEK293 cells stably expressing a modified firefly luciferase enzyme linked to the cyclic AMP (cAMP) sensor Epac (Glosensor, Promega) active in the presence of cAMP and Luciferin (Promega), were used to quantify G protein signaling. HEK293T cells were used for bioluminescence resonance energy transfer (BRET) experiments to assess βarr recruitment. The cells were maintained in Minimum Essential Media (MEM) (Corning) containing 1% penicillin/streptomycin and 10% Fetal Bovine Serum (FBS) (Corning). Cells were grown at 37°C in a humidified 5% CO₂ incubator in poly-d-lysine coated tissue culture plates. Media was changed every 48-72 hours.

Transfections - Luciferase attached to the C-terminus of CXCR3A and CXCR3B was used in donor constructs for BRET assays. Luciferase constructs were encoded either in a Renilla luciferase (Rluc) pN-3 vector (Promega) or Nanoluc Luciferase vector pNL1.1 (Promega). YFP was attached to the C-terminus of βarr1/2; YFP tags for βarrs and GRKs were used as acceptors in BRET assays. Transient transfections were conducted with calcium phosphate for all BRET assays as previously described (Peterson et al., 2015). Briefly, calcium phosphate transfections were conducted with HEPES buffered saline with 4 µg of the CXCR3-RLuc receptor, 10 µg of βarr2-YFP, 10 µg βarr1-YFP, 4 µg of GRK2-YFP, or 4 µg of GRK6-YFP and 0.25M CaCl₂. 50 ng of the Nanoluc βarr2 biosensor was determined to be the optimal concentration for conformational assessment, and transfected with untagged receptor (4 µg CXCR3A, 5 µg CXCR3B) for biosensor studies. Media was changed 30 minutes prior to transfection, and again 4 hours after CaCl₂ transfection. Receptor expression was quantified through CXCR3A-Rluc and CXCR3B-Rluc signal and surface staining. When equal amounts of expression vector were transfected, CXCR3B signal was found to be approximately 80% of CXCR3A (normalized CXCR3A expression 100 ± 2.0, n=56, normalized CXCR3B expression 79 ± 1.6, n=41; p<0.05 by unpaired student’s t-test, consistent with prior findings that surface expression of CXCR3B is lower than CXCR3A (Korniejewska et al., 2011; Mueller et al., 2008). For assays dependent on absolute quantity of receptor expression (i.e., non-BRET assays),
splice variant expression was normalized by increasing the concentration of CXCR3B by 25% relative to CXCR3A. This resulted in equivalent surface expression levels of CXCR3A and CXCR3B (Supplemental Figure 1).

**Bioluminescence Resonance Energy Transfer (BRET)** –24 hours after transfection, cells were plated onto a 96-well plate at 50,000-100,000 cells/well. Approximately 44 hours after transfection, media was changed to MEM (Corning) supplemented with 10mM HEPES, 0.1% bovine serum albumin and 1% penicillin/streptomycin. After approximately three to four hours of serum starvation, cells were washed with room temperature PBS. Next, 80 \( \mu \)L of a coelenterazine-h/HBSS solution (3 \( \mu \)M coelenterazine-h) was added. Ligands were prepared at 5x concentration, and read by a Mithras LB940 instrument (Berthold) with 485nm and 530nm emission filters. The BRET ratio was calculated using equation 1.

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(1) \text{Net BRET ratio} = \left[ \frac{\text{YFP emission at 530 nm}}{\text{Rluc emission at 485 nm}} \right] - cf
\]

In equation 1, \( cf \) represents BRET ratio in the vehicle control group. For the bystander BRET total internalization assay, titration response experiments determined that 400ng of myr-palm-mVenus and 2.5\( \mu \)g of 2xFYVE-mVenus and a constant 4\( \mu \)g of Rluc labeled CXCR3 maximized signal:noise, and these concentrations were used throughout for BRET internalization experiments. For CXCR3-\( \beta \text{arr} \) interaction, the net BRET ratio was quantified five minutes following ligand addition. For bystander BRET-based receptor internalization assays (receptor association with FYVE-mVenus or dissociation from a myristoyl-palmitoylated-mVenus (myr-palm)), cells were not serum starved and instead incubated in assay buffer with ligand for one hour before adding coelenterazine-h as described above. Recombinant human CXCL4, CXCL9, CXCL10, and CXCL11 were obtained from either Peprotech (Rocky Hill, NJ) or BioLegend (San Diego, CA).
**Generation of FLAG-tagged CXCR3 and C-terminal Truncation Mutants**

FLAG-tagged CXCR3 isoform constructs were created by PCR amplification with the signal sequence hemagglutinin (HA) 5’ to the FLAG-epitope sequence as previously described (Guan et al., 1992). The PCR product was gel purified and inserted into pcDNA3.1 using XbaI endonuclease cleavage sequence 5’ to the HA sequence and an EcoRI endonuclease cleavage sequence 3’ to the stop codon. L344X and L391X C-terminal CXCR3 truncation mutants were created by PCR amplification of WT CXCR3A or CXCR3B, introduction of a HindIII endonuclease cleavage sequence 5’ to the start codon on the forward primers (CXCR3A: 5’-CGCGGTAAGCTTATGGTCCTTGAG-3’; CXCR3B: 5’-CGGACCGAAGCTTATGGAGTTGAG-3’) and the introduction of a KpnI endonuclease cleavage sequence 3’ to either the 343rd or 390th amino acid on the reverse primer (5’-ACCCATGGTACCATCCCTCTCTGG-3’). The amplified PCR product was gel purified and inserted in the pRLuc-N3 expression vector. All constructs were verified by Sanger DNA sequencing.

**GRK inhibition on βarr recruitment**

For siRNA GRK knockdown studies, HEK 293T cells were transfected with Lipofectamine 3000 (Thermo Fisher) per manufacturer specifications within a 96 well plate with 5ng of either CXCR3A-Rluc or CXCR3B-Rluc, 12.5ng of βarr2-YFP, and 30ng GRK2/GRK3 siRNA (60ng total), or 30ng GRK5/6 siRNA, or 60ng control siRNA per well. Cells were then stimulated with 500 nM of CXCL11 for 5 minutes, and the BRET signal of βarr recruitment recorded. GRK2, GRK3, GRK5, GRK6, and control siRNA sequences were used as previously validated and described (Kim et al., 2005).

**GloSensor cAMP Assay** –Lipofectamine 2000 (Thermo Fisher) transfected cells were plated on 96-well plates 24 hours after transfection with either CXCR3A or CXCR3B at a density of 10-25,000 cells/well. At approximately 44 hours post transfection, cells were serum starved for two hours. Next, the plate was washed with room temperature PBS, and 60 μL of GloSensor cAMP Reagent (Promega) in HBSS with 20
mM HEPES (Gibco) was then added per well. The plate was incubated for two hours in the dark at room temperature. For G_{ia} pathway assay, the cells were then stimulated with 100 nM isoproterenol, incubated for five minutes, and luminescence over one second was quantified by a Mithras LB940 instrument. Vehicle or CXCR3 ligands were then added, incubated for ten minutes, and the plate read a second time. Data were normalized to vehicle treated wells, and to 1μM CXCL11 for G_{ia} studies. For G_{as} studies, baseline luminescence was determined prior to the addition of CXCR3 ligand, and the plate read a second time after ligand addition.

DiscoverX Active Internalization Assay: This assay was conducted as previously described (Rajagopal et al., 2013) and in accordance with manufacturer protocols. Briefly, an Enzyme Acceptor-tagged βarr and a ProLink tag localized to endosomes are stably expressed in U2OS cells. Untagged CXCR3A or CXCR3B was transiently expressed. βarr-mediated internalization results in the complementation of the two β-galactosidase enzyme fragments that hydrolyze a substrate (DiscoveRx) to produce a chemiluminescent signal.

SRE/SRF pathway assay- 293T cells were transiently transfected with SRE or SRF and either unlabeled CXCR3A (4 μg) or CXCR3B (5 μg). 4 hours after transfection, the cells were plated on a 96 well plate at a concentration of 25,000 cells/well. 24 hours after transfection, cells were serum starved overnight. The next day, cells were incubated with ligand for five hours and subsequently lysed with passive lysis buffer (Promega) for ten minutes as previously described (Evron et al., 2014). Luciferin was added to the lysate and luminescence was quantified using a Mithras LB940 instrument with no wavelength filter between the cells and the photomultiplier as previously described (Peterson et al., 2015). To observe MEK dependent effects of CXCL11, the MEK inhibitor PD98059 (Tocris) was applied to cells at 20μM for 15 minutes prior to ligand stimulation, similar to a previously described protocol (Gesty-Palmer et al., 2005).
Immunoblot—HEK 293 cells were transiently transfected with 4 μg of CXCR3A or 5 μg of CXCR3B using Lipofectamine 2000. After 48 hours, cells were starved in serum-free DMEM >4 hours and treated with the indicated ligand for the indicated duration. Cells were lysed in ice-cold RIPA buffer containing phosphatase and protease inhibitors (Phos-STOP (Roche), cOmplete EDTA free (Sigma)) for 15 minutes, sonicated, and cleared of insoluble debris by centrifugation at 12,000 x g (4 °C, 15 min) after which the supernatant was collected. Protein was resolved on SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted at with the indicated primary antibody overnight (4 °C). Phospho-ERK (Cell Signaling Technology, #9106) and total ERK (Millipore #06-182) were used to assess ERK activation. GRK knockdown was assessed by immunoblot of GRK2 (Santa Cruz #sc-13143), GRK3 (Santa Cruz #sc-365197), GRK5 (Santa Cruz #sc-11396), GRK6 (Santa Cruz #sc-377494) and protein loading assessed with alpha-tubulin (Sigma #T6074). β-arrestin knockout was assessed using anti-βarr1 (A1-CT) and anti-βarr2 (A2-CT) antibodies kindly provided by Dr. Robert J. Lefkowitz (Duke University) and previously validated (Attramadal et al., 1992). Horseradish peroxidase-conjugated polyclonal mouse anti-rabbit-IgG or anti-mouse-IgG were used as secondary antibodies. Immune complexes on nitrocellulose membrane were imaged by SuperSignal enhanced chemiluminescent substrate (Thermo Fisher). For quantification, phospho-ERK 1/2 relative intensity was normalized to total ERK 1/2 relative intensity using ImageLab (Bio-Rad).

Intact Cell Phosphorylation—These assays were performed as described previously (Freedman et al., 2002; Wu et al., 2005). HEK293 cells were transfected with pcDNA3.1 plasmids encoding N-terminal FLAG-tagged constructs of CXCR3A, CXCR3B, or no protein. Confluent cells in 6-well dishes were aliquoted to metabolic labeling or to cell surface immunofluorescence and flow cytometry. For metabolic labeling, cells were serum-starved overnight, rinsed and then labeled with $^{32}$P$_i$ (100 μCi/ml, 37 °C, 1 hr) in phosphate-free Dulbecco's modified Eagle's medium, 20 mM HEPES, pH 7.4, with 0.1% (w/v) fatty acid-free bovine serum albumin (Sigma), 100 μg/ml streptomycin and 100 units/ml penicillin. Cells were
then exposed to the same medium lacking or containing 100nM CXCL11 for 5 min (37 °C), and transferred to ice. After 2 washes with Dulbecco’s PBS, cells were solubilized in “M2 buffer”: 1% (w/v) Triton X-100™, 0.05% SDS, 5 mM EDTA, 50 mM Tris-Cl, pH 8.0 (25 °C), 200 mM NaCl, 50 mM NaF, 10 mM disodium pyrophosphate, and protease inhibitors (1 mM benzamidine, 5 ug/ml aprotinin, 10 μg/ml leupeptin). After solubilization (4 °C, 1 hr), cell lysates were cleared of insoluble debris by two sequential centrifugations at 10,000 × g (4 °C, 30 min). Supernatant aliquots were subjected to immunoprecipitation and modified Lowry protein assay (DC Protein Assay Kit, Bio-Rad). The FLAG-CXCR3A and –CXCR3B were immunoprecipitated from 500 μl of cell lysate by inversion mixing for 60 min (4 °C) with 10 μl of agarose beads conjugated to M2 monoclonal anti-FLAG IgG 1 (Sigma-Aldrich) (these beads were suspended in M2 buffer supplemented with 3% (w/v) bovine serum albumin). Beads were then washed thrice with M2 buffer and finally incubated in 1× Laemmli buffer at 37 °C for 2 h to dissociate immune complexes, which were resolved on SDS-10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, as described (Freedman et al., 2002), and subsequently processed for autoradiography with an intensifying screen at -80 °C for 2-4 days. After autoradiography, each nitrocellulose membrane was immunoblotted for CXCR3 with mouse IgG (R &D systems clone #49801), as described (Wu et al., 2012); chemiluminescence was imaged and quantitated with a Bio-Rad ChemiDoc™ XRS+, which was also used to photograph and quantitate autoradiography films. 32P signals in each CXCR3 band were normalized to cognate receptor immunoblot signals, after subtracting nonspecific signals obtained from lanes loaded with immunoprecipitations of mock-transfected-cell lysates.

**Generation of β-arrestin1/2-double knockout (ΔARRB1/2) HEK293 cells.** ΔARRB1/2 HEK293 cells were generated by simultaneously targeting the ARRB1 and the ARRB2 genes with a CRISPR/Cas9 genome editing technology using a similar strategy that was employed for Gq/11-double knockout cells and as previously described. (Alvarez-Curto et al., 2016; Schrage et al., 2015). Cells were transfected with 0.8μg
of CXCR3A or 1 μg of CXCR3B with Fugene 6 (Promega) per manufacturer specifications. For β-arrestin rescue studies, either 1 μg of βarr1 and 1 μg βarr2 or 2 μg empty vector (pcDNA3.1) was also transfected. Lysates were collected and immunoblotted as described above.

Flow Cytometry- HEK 293 cells were transiently transfected with either 4 μg CXCR3A or 5 μg CXCR3B as described above. After 48 hours, transfected cells and untransfected controls were harvested with trypsin and washed twice with ice cold PBS supplemented with 1% bovine serum albumin (w/v). Cells were then resuspended in flow cytometry buffer (PBS supplemented with 3% FBS and 10mM EDTA). One million cells were transferred to each flow cytometry tube, spun down, and blocked with flow cytometry buffer supplemented with 5% normal human serum and 5% normal rat serum for 15 minutes at 4°C. Anti-human CXCR3 phycoerythrin (PE) conjugated antibody (R&D systems clone #49801) was applied to cells at a 1:150 dilution (100 mcL final volume). Cells were incubated at 37°C for 30 minutes and then washed with 2mL of FACS buffer, fixed for 10 minutes with 0.4% paraformaldehyde, and resuspended in FACS buffer. Cells were immediately analyzed with a Guava EasyCyte HT cytometer (Millipore). To enlarge axis titles of representative signal, a high resolution image of the unaltered histogram was placed on identical axis titles and value that were created in adobe illustrator.

Statistical Analyses – Dose-response curves were fitted to a log agonist vs stimulus with three parameters (Span, Baseline, and EC50) with the minimum baseline-corrected to zero using Prism 7.0 (GraphPad, San Diego, CA). For comparing ligands in concentration-response assays, a two-way ANOVA of ligand and concentration with all four ligands was first conducted. If a significant ligand by concentration interaction was observed (p<0.05), then comparative two way ANOVAs between individual ligands were conducted and corrected for multiple comparisons (e.g., for studies with four ligands, p < 0.05/6 was used as the cutoff for statistical significance), with comparisons finding a significant effect of ligand noted with the figures. Further details of statistical analysis and replicates are included in figure captions. In figure 9H,
one value of eight initial replicates in the CXCL11 treated CXCR3A ARRB 1/2 over expression rescue group was excluded by the Grubbs outlier test (excluded value 164%; prior to exclusion mean value ± SEM was 54% ± 18%, after exclusion 39%± 9% ). Error bars shown in dose response analysis signify the standard error of the mean unless otherwise noted. * indicates $p < 0.05$ throughout the paper to indicate statistical significance from pertinent comparisons detailed in the figure legends, unless otherwise noted.
Results

Confirmation that CXCR3B is βarr-biased compared to CXCR3A

After optimizing transfection conditions for equivalent CXCR3 splice variant surface expression (Supplemental Figure 1), we first examined G protein signaling at CXCR3A and CXCR3B. Prior studies identified selective affinity of CXCL4, CXCL9, CXCL10, and CXCL11 for CXCR3A and CXCR3B through radioligand binding (Lasagni et al., 2003; Mueller et al., 2008). We tested CXCR3A and CXCR3B signaling via G\(_{\text{ai}}\) by assessing inhibition of cyclic AMP (cAMP) production through a modified cAMP dependent firefly luciferase (schematic in Supplemental Figure 2A) with the four known endogenous ligands. In agreement with previous results at CXCR3A (Rajagopal et al., 2013), CXCL10 and CXCL11 were found to be full agonists, while CXCL9 was found to be a partial agonist, in their ability to suppress cAMP production in a highly amplified assay (Figure 2A, Table 1). Slight inhibition of cAMP signal was observed with CXCL4 at high concentrations. In contrast, none of these four ligands were observed to inhibit cAMP at CXCR3B (Figure 2B, Table 1). As CXCR3B has previously been reported to couple to G\(_{\text{as}}\) (Lasagni et al., 2003), we also tested these four ligands’ ability to stimulate cAMP. No signal was observed with CXCL4, CXCL9, CXCL10, or CXCL11 while a positive control of 100nM isoproterenol demonstrated a ~15 fold increase in signal from baseline (Supplemental Figure 2B). We then used BRET to test βarr1 and βarr2 recruitment to CXCR3A and CXCR3B following CXCL4, CXCL9, CXCL10, and CXCL11 stimulation (Figure 2C-F). At CXCR3A, we found that all the ligands recruited βarr2 with rank order of efficacy of CXCL11 > CXCL10 > CXCL9 > CXCL4 (Figure 2C). A bias plot of relative intrinsic activity (Onaran et al., 2017) displayed divergent efficacy of CXCL11 towards the βarr2 pathway relative to the other three endogenous CXCR3 ligands (Supplemental Figure 3). At CXCR3B, only CXCL11 recruited βarr2, with no significant recruitment noted for the other endogenous ligands (Figure 2D). The association of βarr2 with CXCR3A was of longer duration than with CXCR3B (Supplemental Figure 4). CXCL4 displayed inverse agonist characteristics for βarr2 recruitment to CXCR3B, with significantly less signal than CXCL9, which displayed no significant
activity. CXCL11 recruited βarr1 to a significantly greater efficacy to CXCR3A than other ligands (Figure 2E). No statistically significant interaction of ligand and concentration was observed for βarr1 recruitment to CXCR3B (Figure 2F). These findings are consistent with those of Berchiche et al. who found that CXCR3B acted as a βarr-biased receptor relative to CXCR3A (Berchiche and Sakmar, 2016).

*Both CXCR3A and CXCR3B undergo agonist-dependent phosphorylation*

We next focused on assessing whether the mechanisms underlying βarr recruitment to CXCR3A and CXCR3B differed from each other. Phosphorylation of intracellular residues is necessary to recruit βarrs. Given the differential isoform trafficking, we examined agonist-induced phosphorylation of both CXCR3A and CXCR3B by 32P metabolic labeling. CXCL11 treatment resulted in phosphorylation of both receptor isoforms to comparable levels (Figure 3), consistent with the observation that βarr2 is recruited to both receptors. Based on the CXCR3 immunoblot, much of the expressed receptor is not glycosylated; the phosphorylated receptor corresponds to the glycosylated form of the receptor that would be expressed on the plasma membrane (Kobilka, 1990). While a slight difference in mobility of CXCR3B is present in the unglycosylated form, which is likely related to the small predicted shift in molecular weight, there was no significant difference in the mobility of the glycosylated splice variants.

*Activation of CXCR3A and B generate distinct patterns of β-arrestin recruitment and conformation*

We then examined CXCL11-induced βarr-GFP trafficking in cells transfected with CXCR3A or CXCR3B by confocal microscopy (Figure 4). At CXCR3A, βarr2-GFP was strongly recruited to the plasma membrane immediately following treatment with 100nM CXCL11, with βarr2-GFP complexes forming in the cytosol in a ‘class B’ recruitment pattern 30-40 minutes post ligand treatment. In contrast, CXCL11 treatment of CXCR3B-transfected cells only induced weak βarr2-GFP recruitment to the plasma membrane in a ‘class A’ pattern (Oakley et al., 2000), with maximal βarr2-GFP complex formation at the
cell membrane observed 20-30 minutes post stimulation. No appreciable βarr2-GFP complexes in CXCR3B-transfected cells were observed in the cytosol at 40 minutes or longer (data not shown).

To assess whether CXCR3A and CXCR3B generate distinct βarr conformations, we designed a βarr2 biosensor consisting of a nanoluc (Promega) donor linked to the N-terminus and a YFP acceptor on the C-terminus of βarr2 (Figure 5A), which produces a higher intensity signal than a previously-designed βarr biosensor (Charest et al., 2005). A significant change in the magnitude of the BRET ratio could be due to a change in distance and/or dipole orientation between the donor and acceptor or a change in avidity in the β-arrestin-receptor interaction. We performed a dose-receptor titration of the biosensor to find the optimal biosensor concentration that attempts to limit avidity effects in our assay by utilizing the lowest concentration of biosensor that produced a reliable signal and reducing excess biosensor remaining in the cellular pool (Supplemental Figure 5). Therefore, the changes seen in the net BRET ratio primarily imply a conformational change of βarr2. We transiently transfected cells with this biosensor as well as untagged CXCR3A or CXCR3B and subsequently stimulated with CXCL4, CXCL9, CXCL10, or CXCL11. We detected a significant change in BRET signal in the βarr2 biosensor after stimulation of CXCR3A with CXCL11 (Figure 5B), but not after stimulation of CXCR3B (Figure 5C). This suggests that stimulation of CXCR3A with CXCL11 leads to a distinct change in βarr conformation.

Differences in internalization and β–arrestin activity between CXCR3 splice variants

Because recruitment does not necessarily correlate with function, we next investigated if CXCL11 stimulation resulted in a functional difference compared to the other three endogenous ligands. The earlier study by Berchiche et al. demonstrated that both CXCR3A and B undergo similar agonist-induced internalization, although the kinetics for internalization depended on the specific agonist. However, there are distinct mechanisms for GPCR internalization, which can be dependent or independent of βarrs, which can act as clathrin adapters (Shenoy and Lefkowitz, 2011). Therefore, we quantified receptor internalization in three separate assays: 1) a bystander BRET-based membrane
dissociation assay, in which receptor-Rluc internalization results in a decrease in the net BRET ratio from myr-palm labeled mVenus (Figure 6A, middle panel); 2) a bystander BRET-based early endosome association assay, in which receptor-Rluc association with 2xFYVE labeled mVenus localized to causes an increase in the net BRET ratio (Figure 6A, right panel); and 3) a βarr-complex mediated internalization using an assay in U2OS cells permanently expressing split β-galactosidase fragments on βarr2 and endosomes (DiscoveRx). In this last assay, when βarr2 and endosomes are in close proximity for a sustained duration, complementation of β-galactosidase fragments in the presence of a substrate produces a chemiluminescent signal. Signal is produced in ‘Class B’ stable endosome βarr2 interactions, however, limited or no signal is produced in ‘Class A’ transient βarr2-endosome interactions. At CXCR3A, total internalization, early endosome association, and βarr associated internalization at CXCR3A mirrored rank order of efficacy of βarr2 recruitment, with the order of CXCL11 > CXCL10 > CXCL9 > CXCL4. CXCL4 stimulation resulted in negligible internalization and displaying weak inverse agonist activity in the early endosome association assay (Figure 6B, D, F). At CXCR3B, only CXCL11 induced statistically significant total internalization and early endosome association (Figure 6C, E). No CXCR3B-mediated chemiluminescent signal in the βarr-mediated internalization assay was observed (Figure 6G), consistent with the ‘Class A’ pattern of βarr recruitment noted on confocal imaging (Figure 4). Thus, although both receptors undergo agonist-induced internalization, they appear to do so using different underlying mechanisms.

*C-terminus truncation, GRK knockdown, and pharmacologic GRK2 inhibition reduce βarr2 recruitment*

Given our findings in the GRK BRET experiments, we hypothesized that inhibition of GRKs would attenuate βarr2 recruitment at CXCR3A. We first generated C-terminal truncation mutants of CXCR3A and CXCR3B that lack putative C-terminal phosphorylation sites (Figure 7A). The mutants’ truncation location is at the identical C-terminal residue given the extended N-terminus of CXCR3B (344th residue on CXCR3A, 391st residue on CXCR3B). Truncation resulted in ~50% reduction, but not elimination, of
βarr2 recruitment efficacy to both CXCR3A and CXCR3B as quantified by BRET (Figure 7B), similar to other GPCRs such as the Angiotensin Type 1A receptor (Wei et al., 2004), but unlike other GPCRs, such as CCR5 and the Apelin receptor, where βarr2 recruitment is not observed after removal of the C-terminus (Chen et al., 2014; Huttenrauch et al., 2002). Given this data, CXCR3 C-terminal phosphorylation-dependent βarr2 recruitment would not be expected to completely eliminate βarr2 recruitment to the receptor. Indeed, siRNA knockdown of either GRK2/3 or GRK5/6 decreased, but did not eliminate, CXCR3A – βarr2 association following CXCL11 stimulation as measured by BRET (Figure 7C). Interestingly, GRK2/3, but not GRK5/6, siRNA knockdown prominently attenuated CXCR3B-βarr2 association (Figure 7D). This suggests, although both receptor splice variants undergo agonist-dependent phosphorylation, that different GRKs are playing distinctive roles at each receptor.

**CXCL11 activates ERK 1/2 at both CXCR3A and CXCR3B**

G proteins and βarrs can activate similar intracellular pathways, but with different spatial and temporal patterns that result in distinct cellular responses (Shenoy and Lefkowitz, 2011; Smith and Rajagopal, 2016). One well-characterized example is activation and phosphorylation (pERK) of the ERK 1/2 MAP kinases (MAPKs) (Wei et al., 2003). For many receptors, such as the Angiotensin Type IA and Parathyroid hormone receptors, G proteins, βarr1, and/or βarr2 significantly contribute to early phase pERK (Gesty-Palmer et al., 2006; Kim et al., 2009; Lee et al., 2008), while at others, G protein-mediated pERK includes both a ‘fast’ and ‘slow’ phase (Luo et al., 2008). Thus, kinetics alone cannot distinguish between G protein- and βarr-dependent pERK, and the contributions of these different pathways to the pERK response requires more detailed characterization. The earlier study by Berchiche et al. demonstrated that agonist-induced activation of CXCR3A and CXCR3B results in different intensities of activation with similar kinetics at early time points. We observe a similar response at early time points, as the pattern of pERK activation at 5 minutes by the four endogenous ligands was not different between HEK293 cells transiently transfected with CXCR3A or CXCR3B (Figure 8A, C). Given that differential
βarr signaling often occurs at time points beyond 15 minutes, we evaluated signaling up to one hour. Interestingly, a differential pattern of signaling emerged between splice variants at the one hour time point, as CXCR3A transfected cells showed greater ERK activation at 60 minutes following stimulation with CXCL11, while CXCR3B transfected cells did not (Figure 8B, D).

Given the differential pattern of observed ERK activity, we tested the effects of βarr knockout on ERK activation by CXCR3 splice variants. To perform this, we used a different HEK 293 cell line in which both βarr1 and βarr2 (Arrb 1/2 knockout (KO)) were removed by CRISPR/Cas9 genome editing. βarr1 and βarr2 knockout was confirmed by immunoblot with polyclonal anti-βarrestin antibodies recognizing either the C-terminus of βarr1 (A1-CT) or the C-terminus of βarr2 (A2-CT) (Supplemental Figure 6). Phospho-ERK signal in these Arrb 1/2 KO cells was compared to WT cells following 100nM CXCL11 stimulation. Both WT and Arrb 1/2 KO cells from this HEK 293 line differed in morphology and growth rate compared to HEK 293 cells used in other experiments. The basal pERK activity of the Arrb 1/2 KO vehicle control group of both CXCR3A and CXCR3B transfected cells was greater than in identically treated WT cells (Figure 9A, B). In both CXCR3A transfected WT and Arrb 1/2 KO cells, CXCL11 at 5 minutes caused a significant increase in pERK signal (Figure 9A, C). In contrast, at CXCR3B, WT cells displayed a significant increase in pERK signal compared to vehicle while Arrb 1/2 KO cells did not (Figure 9B, D). The residual ERK phosphorylation by CXCR3B in Arrb 1/2 KO cells may represent very low residual Gαi signaling not detected in our second messenger assays or coupling to alternative G proteins. To further probe the differential pERK signal observed between WT and Arrb 1/2 KO cells at 5 minutes expressing either CXCR3A or CXCR3B, we rescued βarr1 and βarr2 through transient overexpression in the Arrb 1/2 KO cells and compared pERK signal at 5 minutes to empty vector transfection controls. Similar to the trend observed between WT and Arrb 1/2 KO cells, 5 minute pERK signal in CXCR3A transfected cells was significantly decreased by βarr overexpression rescue relative to vehicle (Figure 10A, D). Conversely, the pERK signal was significantly potentiated in CXCR3B
transfected cells relative to vehicle (Figure 10B, D), with a significant difference also noted between isoforms (Figure 10D).

*Differential transcriptional regulation by CXCR3 splice variants*

When assessing bias, testing activity distal to direct receptor transducers is helpful in modeling physiological relevance. For example, if biased signaling is observed at proximal effectors such as G proteins and β-arrestins, but not distal transducers such as regulators of transcription, the significance of the observed bias is less clear. We further probed distal signaling by evaluating transcriptional activation through serum response reporter assays. Serum response element SRE is known to respond to the ternary complex (TCF) dependent ERK/MAPK activity, while serum response factor-response element (SRF) is a mutant form of SRE lacking the TCF binding domain that also responds to SRF dependent and TCF independent signals, such as RhoA (Cheng et al., 2010; Hill et al., 1995). Either SRE or SRF reporters were transiently cotransfected with either CXCR3A or CXCR3B. Transcriptional activity was assessed following incubation with the four CXCR3 endogenous ligands. Stimulation with CXCL11 resulted in robust signal from both SRE and SRF-RE reporters co-transfected with CXCR3A (Figure 11A, C), but not when co-transfected with CXCR3B (Figure 11B, D). Both SRE and SRF signals were attenuated, but not eliminated, by mitogen activated protein kinase kinase (MEK) inhibition prior to CXCL11 stimulation (Figure 11E, F). This is consistent with ERK activation at later time points resulting in some transcriptional regulation of SRE and SRF.
Discussion

In this study, we found that CXCR3 receptor splice variants displayed biased signaling that was associated with distinct β-arrestin conformations and signaling to downstream pathways, including ERK and SRE/SRF. Unlike CXCR3A, which demonstrated robust signaling through G\textsubscript{ia}, CXCR3B did not display any appreciable G\textsubscript{ia} protein signaling in second messenger assays. In rank order of efficacy, CXCL11, CXCL10, and CXCL9, but not CXCL4, stimulation caused internalization of CXCR3A in the same order previously described in human T-lymphocytes and HEK cells (Berchiche and Sakmar, 2016; Korniejewska et al., 2011). This internalization rank order was identical to the rank order of efficacy in β-arrestin recruitment to CXCR3A as measured by BRET. Interestingly, only CXCL11, and not the other CXCR3 ligands recruited β-arrestin2 to CXCR3B and resulted in measureable receptor internalization. The CXCR3B-β-arrestin interaction was confined to the plasma membrane in a ‘class A’ pattern compared to the CXCR3A-β-arrestin interaction displaying an endocytic ‘Class B’ pattern (Tohgo et al., 2003). Notably, a biosensor demonstrated distinct β-arrestin conformations associated with these two patterns of recruitment, with distinct β-arrestin conformations recently to correlate with distinct β-arrestin functions at the β2AR (Cahill et al., 2017). Functional activity of CXCR3B was only observed when stimulated by a ligand that caused β-arrestin2 recruitment, CXCL11. In further support of CXCR3B acting as a β-arrestin-biased receptor, β-arrestin1/2 overexpression in Arrb 1/2 KO cells increased signaling by CXCR3B while decreasing signaling by CXCR3A. Taken in its entirety, these findings suggest that CXCR3B acts as a β-arrestin-biased receptor relative to CXCR3A, albeit with a less stable interaction with β-arrestin compared to CXCR3A (Figure 12, summary). This bias may be encoded at least in part by differential GRK recruitment, as siRNA knockdown of GRK5/6 attenuated β-arrestin recruitment to CXCR3A, but not to CXCR3B.

Phosphorylation of MAP kinases is a well-studied signaling response regulated by G proteins and β-arrestins downstream of GPCRs (Shenoy and Lefkowitz, 2011; Smith and Rajagopal, 2016). Although
pERK signal was diminished in CXCR3B-transfected Arrb 1/2 KO cells, the signal 5 minutes after stimulation was still higher than vehicle. This suggests that while CXCR3B is biased towards β-arrestin signaling compared to CXCR3A, CXCR3B signaling does not absolutely require β-arrestins. CXCR3B may couple to a different G protein, an unidentified signaling effector, or both. Further investigation will be needed to understand how N-terminal splice variants alter intracellular effector coupling. CXCL11 stimulation resulted in phosphorylation of both receptor isoforms to comparable levels, suggesting that the differential signaling by CXCR3A and B is due to the phosphorylation of different residues in the C-terminus and/or different conformational changes induced in the receptors.

In our studies, we focused on receptor coupling to two GRK receptor families, GRK2/3 and GRK5/6, because GRK2- and GRK6-deficient lymphocytes display defective trafficking, and because the expression of both CXCR3A and CXCR3B is high in lymphocytes (Arnon et al., 2011; Fong et al., 2002; Lasagni et al., 2003). Phosphorylation by different kinases of serines and/or threonines in GPCR intracellular loops and/or the C-terminus is often necessary for recruitment of βarrs to the receptor (Ahn et al., 2002; Freedman et al., 1995). Our siRNA knockdown studies demonstrate a role for both GRK2/3 and GRK5/6 in CXCL11 mediated βarr2 recruitment to CXCR3A, but a role only for GRK2/3 in βarr2 recruitment to CXCR3B. At other GPCRs, such as the angiotensin AT1R (Kim et al., 2005), the β2 adrenergic receptor (Nobles et al., 2011), and CCR7 (Zidar et al., 2009), different GRKs have been demonstrated to perform signaling or desensitization functions. The activity of these different kinases is thought to result in a phosphorylation ‘bar code’ of the third intracellular loop and C-terminal tail of other GPCRs that regulates βarr activity (Nobles et al., 2011; Reiter et al., 2012). Indeed, the C-terminus and third intracellular loop of CXCR3 is known to be necessary for chemotaxis, calcium flux, and internalization of CXCR3A, processes that are known to be mediated by βarrs (Colvin et al., 2004). Once activated, βarr is thought to form a core scaffolding complex, which activates ERK1/2 by phosphorylation (Eichel et al., 2016). The findings here support a model for a CXCR3 signaling ‘barcode’, in which post-
translational modifications in the receptor C-terminus regulate the affinity of βarr recruitment and the pattern of GPCR intracellular trafficking (Oakley et al., 2001; Oakley et al., 2000). However, future studies that clearly link the phosphorylation of specific sites in the receptor by distinct kinases will be needed to test this hypothesis at CXCR3.

It is somewhat surprising that an N-terminal CKR modification has such an impact on receptor coupling to G proteins, GRKs and βarrs. This is likely due to the mode of chemokines binding to their CKRs, which is primarily mediated by distinct chemokine recognition sites (CRS) (Qin et al., 2015). Our current understanding of chemokine:CKR binding, which is informed both by earlier biophysical studies (Booth et al., 2002) and now crystal structures (Qin et al., 2015; Tan et al., 2013; Wu et al., 2010), is that the N-terminus of the chemokine inserts itself in the transmembrane regions of the CKR and interacts through an interface termed CRS2. The C-terminal domain of the chemokine interacts with the extracellular N-terminus of the receptor through the CRS1 site. Recent crystal structures have demonstrated that the extracellular loops of the CKR interact with the chemokine through a CRS1.5 site. Post-translational sulfation of two sites on the N-terminus of CXCR3A (Tyr 27 and Tyr 29) is necessary for receptor function following ligand stimulation (Colvin et al., 2006). The extended N-terminus of CXCR3B adds two additional sulfation sites (Tyr 6 and Tyr 40). We speculate that this extended N-terminus of CXCR3B binds to its ligands through the CRS1 site or an alternate surface on the chemokine, thereby allosterically coupling with CRS2 site to generate distinct receptor:ligand:transducer conformations.

In summary, CXCR3 splice variants CXCR3A and CXCR3B, differing by an extended N-terminus at CXCR3B, demonstrated significantly biased signaling responses. Unlike signaling observed at CXCR3A, βarr2, but not Gαi protein signaling, was detected at CXCR3B. Different patterns of receptor-βarr2 interaction were also observed between splice variants, with CXCR3A displaying a more stable interaction with arrestin compared to CXCR3B. siRNA knockdown of GRK5/6 attenuated βarr2
recruitment at CXCR3A, but not CXCR3B. In addition, the splice variants had distinct internalization profiles that correlated with differences in late-phase ERK activation and transcriptional activity. At this time, it is unclear as to the mechanisms underlying the phosphorylation ‘barcode’ of the CXCR3 C-terminus and which phosphorylation sites are critical for activation of G protein- and βarr-mediated pathways downstream of the receptor. Appreciating the signaling differences between these splice variants may provide clarification to conflicting reports of CXCR3 function and offer a compelling example of how the GPCR extracellular residues can dramatically change intracellular pathway activation.
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Author contributions

Designed the experiments: Smith, Pack, Freedman, and Rajagopal.

Performed the experiments: Smith, Alagesan, Desai, Wu, and Rajagopal

Contributed new reagents or analytic tools: Pack and Inoue.

Wrote or contributed writing of the manuscript: Smith, Alagesan, Desai, Pack, Freedman, and Rajagopal.
REFERENCES


Footnotes

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FIGURE LEGENDS

**Figure 1. CXCR3A and CXCR3B splice variants.** Schematic representation of CXCR3A (A) and CXCR3B (B). Light pink signifies the additional 47 amino acids present on the N-terminus of CXCR3B. There is no difference in intracellular residues between these CXCR3 splice variants. (C) Sequence alignment of CXCR3A and B.

**Figure 2. Differential G protein activation and beta-arrestin recruitment by CXCR3 splice variants** (A) cAMP signal following transient CXCR3A expression in HEK293 cells stably expressing the cAMP activated firefly luciferase. CXCL10 and CXCL11 are full agonists in their ability to inhibit cAMP production through CXCR3A, while CXCL4 and CXCL9 are partial agonists. (B) No G\textsubscript{ai} activity is observed after transient transfection with CXCR3B. CXCL11 recruited ßarr2-YFP to both CXCR3A-Rluc (C) and CXCR3B-Rluc (D) with higher efficacy than the other endogenous ligands. CXCL11 recruited ßarr1-YFP to CXCR3A-Rluc with a significantly greater efficacy than CXCL4, but not significantly greater than CXCL9 or CXCL10 (E). At CXCR3B, no ligands were observed to be significantly different in recruiting ßarr1-YFP (F). Best fit calculated by a 3 parameter fit, ±SEM, n≥3 biological replicates per treatment group. *, p < 0.05, significant effect of ligand by two-way ANOVA. n.s is not significant.

**Figure 3. CXCR3A and CXCR3B demonstrate equivalent agonist-induced phosphorylation.** HEK293 cells were transfected with plasmids encoding either no protein (“Vector”) or N-terminal FLAG-tagged constructs of CXCR3A or CXCR3B. After serum starvation and metabolic labeling with \textsuperscript{32}P\textsubscript{i}, cells were exposed to serum-free medium lacking (“-”) or containing (“+”) the CXCR3 agonist CXCL11 (100 nM) for 5 min (37 °C), and then solubilized. CXCR3 isoforms were immunoprecipitated (IP) via their N-terminal FLAG epitope, and immune complexes were resolved by SDS-PAGE. Proteins were
transferred to nitrocellulose and then subjected sequentially to autoradiography (\(^{32}\text{P}\)) and immunoblotting (IB) for CXCR3, as indicated. Shown are an autoradiogram and immunoblot from a single experiment, representative of 3 performed. The arrow indicates the cell-surface, mature-glycosylated isoform of CXCR3 (which has slower electrophoretic mobility than the cotranslationally glycosylated or immature-glycosylated bands). From the \(^{32}\text{P}\) signal for each CXCR3 band we subtracted the (nonspecific) signal in the cognate location of the lane from untransfected cells; the resulting specific \(^{32}\text{P}\) CXCR3 signal was normalized to the cognate CXCR3 immunoblot band signal (from which nonspecific pixels in the cognate untransfected cell lane had been subtracted). In each experiment, this ratio of \(^{32}\text{P}/\text{CXCR3}\) was normalized to that obtained for CXCR3B immunoprecipitated from unstimulated cells, to obtain “fold/control”, plotted as individual values and corresponding means ± SEM. Compared with unstimulated cells: *, \(p < 0.05\) indicates a significant effect of agonist by two-way ANOVA, with no significant effect of receptor isoform. \(n=3\) biological replicates per treatment group.

**Figure 4. CXCR3A and CXCR3B induce distinct \(\beta\text{arr2}\) trafficking patterns**

Confocal microscopy of HEK 293 cells transiently transfected with unlabeled CXCR3A or CXCR3B and \(\beta\text{arr2}-\text{GFP}\) pre- (left panels) and post- (right 3 panels) CXCL11 treatment (100nM). Three different cells are shown post treatment for each splice variant.

**Figure 5. CXCR3A, but not CXCR3B, stimulation with CXCL11 causes a change in \(\beta\text{arr2}\) conformation**

(A) Schematic of \(\beta\text{arr2}\) biosensor for probing \(\beta\text{arr2}\)- conformational changes following transfection with the biosensor and untagged CXCR3A or CXCR3B. HEK293 cells were stimulated for 15 minutes with the indicated ligand (250 nM) prior to BRET measurements. (B) CXCL11 stimulation of CXCR3A led to a significant conformational change in \(\beta\text{arr2}\) signified by a change in magnitude of the net BRET ratio compared to all ligands, while CXCL10 stimulation caused a significantly different signal from CXCL4. (C) Stimulation of CXCR3B resulted in no appreciable change in signal across all
comparisons. *, p < 0.05 by one-way ANOVA with Tukey’s post hoc comparison between all treatment groups. n≥3 biological replicates per condition.

**Figure 6. The internalization pattern of CXCR3 splice variants diverge.** (A) Confocal images of transiently transfected untagged mVenus (left panel), myr-palm-mVenus (center panel) localized to the plasma membrane, and 2x-FYVE-mVenus (left panel) localized in an endosomal distribution. Internalization efficacy measured by BRET of myr-palm-mVenus transiently transfected cells with (B) CXCR3A-Rluc or (C) CXCR3B-Rluc CXCL11 and treated with the indicated ligand for one hour as a measure of total receptor internalization. 2x-FYVE-mVenus transiently transfected cells (D) CXCR3A-Rluc or (E) CXCR3B-Rluc and treated with the indicated ligand for one hour as a measure of receptor-endosome association. Treatment CXCL11 resulted in greater βarrestin-mediated internalization efficacy compared to all other ligands in cells transiently transfected with CXCR3A (F), but not CXCR3B (G). Aside from panel G which lacked appreciable signal with any ligand, data are normalized to CXCL11 (1μM) stimulation and expressed as a percentage of maximal signal. *, p < 0.05, significant effect of ligand by two-way ANOVA. Scale bar is 10μm. n≥3 biological replicates per condition.

**Figure 7. GRK5/6 knockdown differentially attenuates βarrestin2 recruitment.**

(A) Cartoon of CXCR3 truncation C-terminal mutants. (B) Truncation of either CXCR3A or CXCR3B reduced βarr2 recruitment as measured by BRET in response to CXCL11 (500nM). (C) siRNA knockdown of either GRK2/3 or GRK5/6 reduced CXCL11(500nM) induced βarr2 recruitment to CXCR3A, however, (D) only siRNA knockdown of GRK2/3, but not GRK5/6, reduced βarr2 recruitment to CXCR3B. For panel B, *, p < 0.05 by unpaired student’s t-test between respective WT and truncation mutant. For panels C and D, *, p < 0.05 by one-way ANOVA with Tukey’s post hoc comparison between all treatment groups. n≥3 biological replicates per condition.
Figure 8. Divergent ERK activation kinetics between CXCR3 splice variants. HEK293 cells were transfected with either CXCR3A or CXCR3B and stimulated for either 5 min (A) or 60 min (B) with the indicated ligand (100nM). The 60 minute, but not 5 minute, phospho-ERK 1/2 CXCL11 signal in cells transfected with CXCR3A was significantly greater than cells transfected with CXCR3B. (C, D) Quantification of phospho-ERK/ERK signal normalized to CXCR3A vehicle treatment. Data were normalized to vehicle treatment of each respective isoform. In panels C and D, *, p < 0.05 indicates a significant effect of transfected isoform at 60 minutes by two-way ANOVA. #, p < 0.05; by one-way ANOVA and Tukey post hoc comparisons within all CXCR3A treatment groups, with ‘*’ indicating a significant difference between of CXCR3A CXCL11 stimulated cells compared to vehicle and CXCL4 at both 5 and 60 minutes; ‡, p < 0.05, by one-way ANOVA and Tukey post hoc comparisons between all CXCR3B treatment groups, with ‘‡’ indicating a significant difference between CXCR3B CXCL11 stimulated cells compared to all treatment groups at 5 minutes. No significant differences between ligands were observed in CXCR3B transfected cells at 60 minutes. ±SEM, n≥3 biological replicates per condition. Western blots shown are representative of at least 3 separate experiments.

Figure 9. β-arrestin knockout differentially alters pERK signaling between CXCR3 splice variants

WT or ΔARRB1/2 cells transfected with CXCR3A (A), or CXCR3B (B), and stimulated with CXCL11 (100nM). A significant increase in phospho-ERK 1/2 signal was observed in both WT and ΔARRB1/2 cells transfected with CXCR3A. However, only WT, but not ΔARRB1/2, cells transfected with CXCR3B displayed a significant increase in phospho-ERK 1/2 signal. CXCR3A (C) and CXCR3B (D) phospho-ERK signal was quantified in WT and ΔARRB1/2 cells (please note the change in scale of the y-axis between panels C and D). In panels C and D, &, p < 0.05 indicates a significant effect of cell line by two-way ANOVA in cells transfected with CXCR3B; *, p < 0.05 indicates a significant difference of the 5 min time point by two-way ANOVA followed by Bonferroni post hoc comparison (corrected for all time points) in cells transfected with CXCR3B; #, p < 0.05 by one-way ANOVA and Dunnett’s post hoc comparison of CXCL11 stimulation to the respective isoform vehicle control at the indicated time point.
±SEM, n≥3 biological replicates per condition. Western blots shown are representative of at least 3 separate experiments.

**Figure 10. β-arrestin overexpression differentially regulates pERK signaling between CXCR3 splice variants at 5 minutes.** Either βarr1 and βarr2 or empty vector were transfected in ARRB1/2 KO cells expressing either (A) CXCR3A or (B) CXCR3B as a rescue overexpression experiment and stimulated for 5 minutes with either vehicle or CXCL11 (100nM). (C) Rescue of β-arrestin was confirmed (non-specific band in both lanes noted with an arrow). (D) Immunoblots were quantified by calculating phospho-ERK / total ERK signal. β-arrestin rescue resulted in decreased phospho-ERK signal in CXCR3A expressing cells, in contrast to an increased signal in CXCR3B expressing cells, relative to respective vehicle treatments. In panel D, #, p < 0.05 indicates a significant difference in pERK signal between pcDNA empty vector and β-arrestin rescue within isoform treatment by two-way ANOVA followed by Bonferroni post hoc comparison, while *, p < 0.05 indicates a significant difference in pERK signal between receptor isoforms treated with CXCL11 by two-way ANOVA followed by Bonferroni post hoc comparison. ±SEM, n≥3 biological replicates per condition. Western blots shown are representative of at least 3 separate experiments. IB is immunoblot.

**Figure 11. CXCL11 robustly activates serum response element (SRE) and serum response factor (SRF) response element signaling at CXCR3A, but not CXCR3B.** HEK293T cells were transiently transfected with either the SRE or SRF reporter and either CXCR3A or CXCR3B. Prior to acquiring luminescence signal, cells were incubated for 5 hours with ligand (1μM). CXCL11 incubation caused a significant increase in luminescence signal in both SRE (A) and SRF (C) transfected cells. In cells transfected with CXCR3B, none of the endogenous ligands tested resulted in significant SRE (B) or SRF (D) signal, although cells still responded to the positive FBS control. In panels E-F, cells were pretreated either with vehicle or with MEK inhibitor PD98059 (20μM). The CXCL11 induced SRE and SRF signals
were sensitive to MEK inhibition (E, F). *, p < 0.05; for panels A-D, a one-way ANOVA and Tukey post hoc comparisons of treatment groups. The positive control of 10% FBS is included for reference, but not included in statistical analyses. For panels E and F, a one way ANOVA followed by a directed Bonferroni post hoc comparison of Veh+CXCL11 to PD98059+CXCL11 was conducted. n≥3 biological replicates per condition.

**Figure 12. Summary of observed CXCR3 isoform signaling.** Both CXCR3A and CXCR3B recruited β-arrestin2, became phosphorylated, and internalized in response to CXCL11. In contrast, only CXCR3A was observed to signal through Gαi, as CXCR3B transfected cells did not produce appreciable signal in either Gαi or Gαs assays. In further signaling divergence, CXCR3A and CXCR3B show distinct patterns of downstream signaling, with CXCR3A observed to display a stable ‘class B’ interaction with β-arrestin2 in contrast to CXCR3B which displayed a transient ‘class A’ interaction in confocal recruitment assays. GRK2/3 siRNA knockdown attenuated βarr2 recruitment to both receptor isoforms, however, only GRK5/6 knockdown was observed attenuate βarr2 recruitment to CXCR3A. Only CXCR3A was observed to show significant late phase (one hour) phospho-ERK activity, and overexpression rescue of β-arrestin attenuated early phase phospho-ERK activity mediated by CXCR3A while enhanced phospho-ERK activity mediated by CXCR3B. SRE and SRF transcriptional reporter activity was only observed downstream of CXCR3A.
TABLE 1. Efficacies and potencies for chemokines at CXCR3A and CXCR3B. Emax (expressed as % of CXCL11 signal) and EC50 values of CXCL4, CXCL9, CXCL10, and CXCL11 at CXCR3A and CXCR3B calculated from a 3 parameter fit (y=Min + (Max-min)/(1+10^((LogEC50-X))). If the 3 parameter fit of ligand-receptor interaction produced a poor fit, then the data was omitted from the table. Radioligand binding data is from Lasagni et al. (Lasagni et al., 2003), and their data was converted to log base 10. Standard error of the mean was propagated using the equation SEM=0.434(log10 EC50 error/log10 EC50).

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<th>Emax ± SEM</th>
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**CXCR3B**
| βarr2          | CXCL4       | -115 ± 10 | -6.5 ± 0.51 | 0.43 |
|               | CXCL11      | 100 ± 15  | -7.2 ± 0.30 | 0.47 |
| **Total Internalization** | **CXCL11** | 100 ± 24  | -6.7 ± 0.56 | 0.80 |
| **Endosome associated Internalization** | **CXCL11** | 100 ± 20  | -6.9 ± 0.24 | 0.71 |

**Radioligand Binding (Lasagni et al., 2003)**

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<td>CXCL11</td>
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Figure 1
Figure 2
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**Receptor Isoform**

- **CXCR3A**
- **CXCR3B**

**Phosphorylation (fold/control)**

- None
- CXCL11

*Significant difference*
Figure 4
Figure 5
Figure 7
**Figure 8**

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**A**

- **ERK**
  - 50kDa
  - 37kDa

**B**

- **ERK**
  - 50kDa
  - 37kDa

**C**

- **phospho-ERK Signal: 5 min**
  - CXCR3A
  - CXCR3B
  - n.s.

**D**

- **phospho-ERK Signal: 60 min**
  - CXCR3A
  - CXCR3B
  - *p
  - #
**Figure 9**

(A) Western blot of pERK in HEK 293 CRISPR WT and HEK 293 CRISPR ARRB 1/2 KO cells at 50kDa and 37kDa.

(B) Western blot of pERK in HEK 293 CRISPR WT and HEK 293 CRISPR ARRB 1/2 KO cells at 50kDa and 37kDa.

(C) Bar graph showing the normalised pERK/ERK signal for CXCR3A in WT and ARRB 1/2 KO cells.

(D) Bar graph showing the normalised pERK/ERK signal for CXCR3B in WT and ARRB 1/2 KO cells.
Figure 10
Figure 11
Figure 12