CXC Chemokine Receptor 3 Alternative Splice Variants Selectively Activate Different Signaling Pathways

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ABSTRACT

The G protein-coupled receptor (GPCR) C-X-C chemokine receptor 3 (CXCR3) is a potential drug target that mediates signaling involved in cancer metastasis and inflammatory diseases. The CXCR3 primary transcript has three potential alternative splice variants and cell-type specific expression results in receptor variants that are believed to have different functional characteristics. However, the molecular pharmacology of ligand binding to CXCR3 alternative splice variants and their downstream signaling pathways remain poorly explored. To better understand the role of the functional consequences of alternative splicing of CXCR3, we measured signaling in response to four different chemokine ligands (CXCL4, CXCL9, CXCL10, and CXCL11) with agonist activity at CXCR3. Both CXCL10 and CXCL11 activated splice variant CXCR3A, Whereas CXCL10 displayed full agonistic activity for Gαi activation and extracellular signal regulated kinase (ERK) 1/2 phosphorylation and partial agonist activity for β-arrestin recruitment, CXCL9 triggered only modest ERK1/2 phosphorylation. CXCL11 induced CXCR3B-mediated β-arrestin recruitment and little ERK phosphorylation. CXCR3Alt signaling was limited to modest ligand-induced receptor internalization and ERK1/2 phosphorylation in response to chemokines CXCL11, CXCL10, and CXCL9. These results show that CXCR3 splice variants activate different signaling pathways and that CXCR3 variant function is not redundant, suggesting a mechanism for tissue specific biased agonism. Our data show an additional layer of complexity for chemokine receptor signaling that might be exploited to target specific CXCR3 splice variants.

Introduction

Chemokine receptors are members of the G protein-coupled receptor (GPCR) family that bind peptidic chemotactic cytokines also known as chemokines. One hallmark of chemokine receptors is that they have multiple endogenous agonist ligands. Conversely, some chemokines can activate multiple separate chemokine receptor subtypes. Accumulating in vitro and in vivo evidence suggests that this promiscuity results in a diversity of specific chemokine receptor signaling, rather than mere functional redundancy (Schall and Proudfoot, 2011). Furthermore, chemokine receptor signaling is also controlled by chemokine binding to proteoglycans (Brady and Limbird, 2002; Proudfoot et al., 2003; Groom and Luster, 2011b; Zweemer et al., 2014), postranslational modifications, and chemokine dimerization (Ludeman and Stone, 2014). Another potential mechanism controlling receptor function is the expression of alternative splice variants, which remains poorly explored (Wise, 2012). Because many chemokine receptors and chemokines are coexpressed by the same cell types or are present in the same cellular environment during inflammation, it is challenging to assess differences in the function of chemokine receptors and their alternative splice variants in response to different ligands in in vivo settings. Inflammatory chemokine receptor CXCR3 is of increasing clinical interest because it controls leukocyte chemotaxis and is involved in inflammatory disorders such as atherosclerosis as well as in cancer metastasis (Hancock et al., 2000; Murphy et al., 2000; Groom and Luster, 2011a,b; Li et al., 2015; Lleo et al., 2015; Zhu et al., 2015). CXCR3 is expressed at the surface of a plethora of cells, including monocytes, lymphocytes, natural killer, and endothelial cells (García-López et al., 2001). CXCR3 displays classic receptor-ligand promiscuity and binds four chemokine ligands: CXCL4, CXCL9, CXCL10, and CXCL11 (Loetscher et al., 1996; Cole et al., 1998; Mueller et al., 2008). CXCR3 couples to Gαi/o heterotrimeric G proteins, which are pertussis toxin (PTX)-sensitive, and can also activate signaling pathways including MAPK, intracellular calcium flux, actin polymerization, and chemotaxis in response to chemokines (Loetscher et al., 1996; Smit et al., 2003; Kouroumalis et al., 2015; Zhu et al., 2015). CXCR3 is expressed at the surface of a plethora of cells, including monocytes, lymphocytes, natural killer, and endothelial cells (García-López et al., 2001). CXCR3 displays classic receptor-ligand promiscuity and binds four chemokine ligands: CXCL4, CXCL9, CXCL10, and CXCL11.
suggest that individual CXCR3 variants might prove to be profiles in response to different ligands, which could lead to showing that CXCR3 splice variants have different signaling and CXCR3B in a ligand-independent manner. In summary, we strongly suggest that poly-D-lysine were from Sigma (St. Louis, MO), and the anti-CXCR3 from Biotium (Hayward, CA). Forskolin, pertussis toxin (PTX), and

The expression patterns and function of CXCR3 splice variants in normal and disease states remain largely unexplored. Tissue specific expression of CXCR3 alternative variants has been mainly quantified using reverse-transcription polymerase chain reaction, and variant-specific antibodies that might allow measurement of expression levels remain unavailable. Nevertheless, in prostate cancer specimens CXCR3A mRNA levels are upregulated, whereas CXCR3B mRNA levels are downregulated (Wu et al., 2012). These differences receptor variant expression levels are reported to alter the migration and invasion capabilities of prostate cancer cells (Wu et al., 2012). In addition, mRNA levels of CXCR3A are decreased, whereas CXCR3Alt mRNA levels are increased in CD3+ peripheral blood lymphocytes in patients suffering from Crohn’s disease, suggesting a variant specific role of CXCR3Alt with Crohn’s disease (Manousou et al., 2008).

We set out to determine whether CXCR3 alternative splice variants activated different signaling pathways in response to their chemokine ligands. We dissected signaling profiles across multiple pharmacological assays when CXCR3 variants were individually expressed in an human embryonic kidney (HEK) 293T expression system. We carefully quantitated receptor expression levels and measured chemokine-induced Gai activity, β-arrestin recruitment, receptor internalization, and ERK1/2 phosphorylation to demonstrate that the nature of the activated signaling pathways depends on both the alternative receptor variant and the chemokine tested. For example, nearly all ligands induced CXCR3A internalization, whereas selected ligands induced β-arrestin recruitment in a PTX-insensitive manner, supporting a possible Gai-independent mechanism of β-arrestin recruitment to CXCR3A and CXCR3B. Moreover, our results strongly suggest that β-arrestin2 is recruited to both CXCR3A and CXCR3B in a ligand-independent manner. In summary, we show that CXCR3 splice variants have different signaling profiles in response to different ligands, which could lead to different pathophysiological roles. Our data support the concept of biased agonism in CXCR3 variant-mediated signaling and suggest that individual CXCR3 variants might prove to be viable drug targets.

Materials and Methods

Materials. Recombinant chemokines were from PeproTech, Inc. (Rocky Hill, NJ). Coelenterazine 400A for BRET experiments was from Biotium (Hayward, CA). Forskolin, pertussis toxin (PTX), and poly-o-lysine were from Sigma (St. Louis, MO), and the anti-CXCR3 mAb (clone 1C6) directly coupled to phycerythrin was from R&D Systems (Minneapolis, MN). Dulbecco’s modified Eagle’s medium Glutamax, 1% penicillin-streptomycin, and Lipofectamine 2000 were from Life Technologies (Carlsbad, CA). bovine serum albumin (BSA) fraction V, fatty acid free was from EMD Millipore (Bedford, MA), and 96-well white microplates with clear bottom and 384-well black microplates with clear bottom plates were from Corning (Woburn, MA).

Plasmids. The CXCR3A (Uniprot identifier P49682-1) plasmid was purchased from the Missouri & T cdNA Resource Center (Rolla, MO; www.cdna.org). The cDNA of splice variants CXCR3B (Uniprot identifier P49682-2) and CXCR3Alt (Uniprot identifier P49682-3) were synthesized by Bio Basic (Markham, Canada) and subcloned into pcDNA3.1+ using with BatEII and XbaI restriction sites. The cAMP EPA2 sensor was a gift from Michel Bouvier (Université de Montréal, Canada). CXCR3A-GFP10, CXCR3B-GFP10, and CXCR3Alt-GFP10 were constructed by ligating the coding sequence of CXCR3 isoforms to GFP10, amplified by PCR from the cAMP EPAC reporter using XhoI and XbaI, or NotI and XhoI, or NotI and XbaI, respectively. The amino acid sequence of the linker region between the terminal receptor residue and the fluorophore was described elsewhere (Percherancier et al., 2005; Berchiche et al., 2011). β-Arrestin1-Rluc3 and β-arrestin2-Rluc3 sensors were constructed by C-terminally fusing the coding sequence of β-arrestin to Rluc3, amplified from the cAMP EPAC reporter including the linker region, using HindIII and XbaI unique restriction sites (Leduc et al., 2009). All sequences were verified by direct sequencing.

Cell Culture and Transfection. HEK293T cells (passage number 5 to 15, ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium Glutamax, 1% penicillin-streptomycin, and 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA). Transient transfection was performed in six-well plates using the polyethyleneimine method as described previously (Percherancier et al., 2005). Transient high-throughput in-plate transfections were performed in 384-well plates using Lipofectamine 2000 according to manufacturer’s instructions with some modifications. Briefly, cells were trypsinized, counted, and mixed with the DNA-Lipofectamine 2000 complex then directly plated at 0.01% poly-o-lysine coated 384-well plates at a density of 20,000 cells/well. The total amount of transfected DNA was kept constant at 2 μg/well for six-well plates and 30 ng/well for 384-well plates by adding empty vector pcDNA3.1+. Transfected DNA amounts were adjusted to obtain comparable cell surface expression among the transfected and GFP10- fused CXCR3 splice variants in all experiments.

Flow Cytometry. HEK293T cells transfected in six-well plates with CXCR3 alternative variants were detached in ice-cold phosphate-buffered saline. Human peripheral blood mononuclear cells (PBMC) obtained by leukapheresis (Gift from Prof. Michel Nussenzweig, The Rockefeller University) and purified T cells (Gift from Dr. Helen Su, National Institute of Health/National Institute of Allergy and Infectious Diseases, NIH/NAID) obtained using a Pan T cell isolation kit (Miltenyi Biotec, San Diego, CA) were stimulated with 10 μg/ml phytohemagglutinin and cultured for 10 days in RPMI containing 10% fetal bovine serum and 50 IU/ml interleukin-2. Cells were labeled with monoclonal 1C6 phycerythrin-conjugated antibody (BD Biosciences, San Jose, CA) that recognizes all three CXCR3 alternative variants for 30 minutes at 4°C in BRET buffer [phosphate buffered saline (PBS) containing 0.5 mM MgCl2 and 0.1% BSA]. Cells were then washed three times in ice-cold PBS. Cell surface expression was quantified by flow cytometry using the Accuri C6 flow cytometer (BD Biosciences). We also determined receptor surface expression of transfected CXCR3 variants in HEK293T and the total CXCR3 quantities in both activated PBMC and T cells using the Quanti-BRITE standardization beads (BD Biosciences). Receptor quantities typically reached around 104 antibody-binding sites per cell for both HEK293T transfected CXCR3 alternative variants and endogenous CXCR3 expressed in activated PBMCs and purified T cells.

BRET Measurements. HEK293T cells transfected in six-well plates were seeded in 0.01% poly-o-lysine coated 96-well, white microplates with clear bottom 24 hours after transfection at a density of 90,000 cells/well. Forty-eight hours posttransfection, media was replaced with BRET buffer. Coelenterazine 400A was added at a final
concentration of 5 μM followed by a 5 minutes incubation at room temperature (RT). Luminescence and fluorescence readings were collected using the Synergy NEO plate reader from Biotek (Winooski, VT) and Gen5 software. BRET² readings between Rlu3 and GFP10 were collected by sequential integration of the signals detected in the 365 to 435 nm (Rlu3) and 505 to 525 nm (GFP10) windows. BRET² ratios were calculated as described previously (Leduc et al., 2009; Berchiche et al., 2011). All BRET experiments were performed while cells remained attached to the 96-well plates.

Adenylyl Cyclase Activity. cAMP was determined by using the Rlu3-EPAC-GFP10, a BRET² cAMP sensor, as described previously (Leduc et al., 2009). Briefly, cells cotransfected with 1.0 μg CXCR3A or 1.5 μg CXCR3B or 1.0 μg CXCR3Alt and 0.03 μg Rlu3-EPAC-GFP10 reporter were seeded into poly-L-lysine coated 96-well plates 24 hours after transfection. Coelenterazine 400A was added to the cells followed by a 5-minute incubation at RT. Cells were then stimulated with ligand in the presence of 5 μM of forskolin at RT for 3 minutes. For experiments involving PTX, 100 ng/ml of PTX was added to the media 20 minutes at RT. Detection of phosphor-ERK1/2 and total ERK2 activation was determined by InCell Western assay (Li-COR). Briefly, cells expressing -GFP10 ligand before the addition of the substrate, unless specified otherwise. The values were corrected to net BRET by subtracting the background BRET² signal detected when the -Rlu3 construct was expressed alone.

Endocytosis. HEK293T transfected with 1.0 μg CXCR3A or 1.5 μg CXCR3B or 1.0 μg CXCR3Alt were incubated with 100 nM of chemokines at 37°C with gentle agitation, followed by immediate incubation on ice to stop the reaction. Surface bound chemokines were removed with acid washing (50 mM glycine, pH 2.7, 150 mM NaCl) and cells subsequently washed twice with ice-cold PBS. Cells were then labeled with anti-CXC 1C6 phycoerythrin-conjugated antibody for 30 minutes on ice following manufacturer's instructions. Antibody incubation was followed by two washes with ice-cold PBS. Cell surface receptor expression was quantified by flow cytometry using the Accuri C6 flow cytometer (BD Biosciences). Surface expression of CXCR3 after ligand incubation at 37°C was expressed as percentage of CXCR3 expression compared with a sample drawn before addition of the ligands and kept on ice before the acid wash and antibody staining.

Erk1/2 Phosphorylation. Chemokine-induced MAPK pathway activation was determined by InCell Western assay (Li-COR). Briefly, cells were transfected in a high-throughput in-plate transfection manner with 10 ng of CXCR3A, 15 ng of CXCR3B, or 10 ng of CXCR3Alt. After 24 hours, the media was removed and replaced with 1× Hank’s Balanced Salt Solution-20 mM HEPES pH 7.4 supplemented with 0.2% BSA (HBSS-H 0.2% BSA). Transfected cells were then stimulated with 100 nM of chemokines at 37°C for different periods of time (2, 5, 7, 10, and 15 minutes) or in the presence of increasing concentration of ligand. ERK activity was stopped by fixing cells with a solution of PBS containing 3.7% formaldehyde for 20 minutes at RT. Detection of phospho-ERK1/2 and total ERK2 proteins were performed as described previously (Wong, 2004). Plates were scanned using the Li-COR Odyssey infrared reader.

Data Analysis. Data were analyzed using Prism 6.0 software (GraphPad Software, San Diego, CA). Statistical significance of the differences between the various conditions was determined using one-way analysis of variance with Tukey’s post t test when appropriate. When indicated, differences of top or bottom values were also determined using sigmoidal dose response simultaneous curve fitting.

Results

Gαi Activity of CXCR3 Is Limited to Specific Alternative Splice Variants. We evaluated the ability of CXCR3 alternative splice variants to activate Gαi signaling in response to chemokines (Fig. 1). Receptor cDNA quantities were adjusted to obtain comparable cell surface expression for all CXCR3 variants, which were kept constant across all assays (Supplemental Fig. 3). HEK293T cells coexpressing the EPAC cAMP biosensor previously described (Leduc et al., 2009) with CXCR3 alternative variants were stimulated with increasing concentrations of chemokines in the presence of forskolin in live cells and the inhibition of forskolin induced cAMP production as a consequence of Gαi activation was measured.

Chemokine receptor CXCR3A inhibited cAMP production when stimulated with CXCL10 and CXCL11, and both chemokines had similar efficacies and potencies (Fig. 1A, Table 1). These results are in line with previous reports (Scholten et al., 2012). Yet, chemokines CXCL9 and CXCL4 failed to trigger significant Gαi activity even at chemokine concentrations as high as 100 nM. Furthermore, CXCR3B-mediated Gαi activation was only detected after stimulation with 100 nM of CXCL11 but not at lower concentrations or in the presence of the other chemokine ligands (Fig. 1B). In addition, we assessed the ability of chemokines to trigger Gαi activation via CXCR3B using the EPAC biosensor in the absence of forskolin (Leduc et al., 2009). As opposed to previously reported Gαi activity in human microvascular endothelial cell line-1 (HMEC-1) stably expressing CXCR3B (Lasagni et al., 2003), we found that in our assays it failed to activate Gαi upon treatment with its chemokine ligands (data not shown). Chemokine receptor variant CXCR3Alt failed to induce significant Gαi activation in response to all chemokines tested (Fig. 1C), although the truncated receptor was expressed at the cell surface as measured by flow cytometry. Results obtained with CXCR3Alt were similar to our results obtained when cells were cotransfected with the EPAC biosensor and empty vector pDNA3.1+ (Fig. 1D).

CXCR3 Splice Variants Differentially Recruit β-Arestrins. We measured β-arrestin recruitment to CXCR3 in live cells using a BRET proximity assay, which was extensively used to study receptor interaction with arrestins, ligand-induced receptor/arrestin conformational changes, as well as receptor dimerization (Hamdan et al., 2005; Kalatskaya et al., 2009; Berchiche et al., 2011). We performed acceptor/donor titration experiments by coexpressing increasing quantities of CXCR3-GFP10 acceptors with fixed quantities of β-arrestin1-Rlu3 or β-arrestin2-Rlu3 donors (Fig. 2). Surprisingly, we measured a significant and saturating basal BRET signal in the absence of ligands for CXCR3A with both β-arrestins and CXCR3B with β-arrestin2, which further increased after stimulation with 100 nM CXCL11 (Fig. 2, A–C). As for CXCR3B/β-arrestin1, acceptor/donor titration in the absence of ligand resulted in a bystander curve, most likely due to random collision of coexpressed BRET pairs (Bonneterre et al., 2016). However, stimulation with CXCL11 resulted in a saturating curve supporting ligand-induced β-arrestin1 recruitment to CXCR3B (Fig. 2D). This suggests a preference of CXCR3B for β-arrestin2.
in the absence of ligand. Curve fitting of the data obtained from the titration experiments allowed us to determine both BRET\textsubscript{max} and BRET\textsubscript{50} values in the absence and presence of 100 nM CXCL11 (Table 2). BRET\textsubscript{max} corresponds to the best acceptor and donor concentrations determined at saturation with optimal sensitivity, which we used to assess ligand-induced CXCR3/\beta-arrestin interaction. Furthermore, BRET\textsubscript{50} corresponds to the acceptor/donor ratio giving 50% of the maximal response.

### TABLE 1
Summary of fitted curve parameters for results shown in Figs. 1 (G\textsubscript{ai} activity), 5 (\beta-arrestin recruitment), and 8 (ERK1/2 phosphorylation).
pEC\textsubscript{50} values are given and maximal responses are shown in absolute (\beta-arrestin recruitment) and relative (ERK1/2 phosphorylation and G\textsubscript{ai} activity) units. Errors are presented as ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>CXCR3A</th>
<th>CXCR3B</th>
<th>CXCL11</th>
<th>CXCL11</th>
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<tbody>
<tr>
<td>cAMP inhibition</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>N</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>N.D.</td>
</tr>
<tr>
<td>IC\textsubscript{50} (nM)</td>
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<td>0.5</td>
<td>N.D.</td>
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<tr>
<td>pEC\textsubscript{50} ± S.E.M.</td>
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<td>(-9.4 ± 0.2)</td>
<td>N.D.</td>
<td></td>
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<tr>
<td>E\textsubscript{max} ± S.E.M.</td>
<td>89 ± 11</td>
<td>70 ± 7</td>
<td>N.D.</td>
<td></td>
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<tr>
<td>\beta-arrestin2</td>
<td></td>
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<td></td>
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<tr>
<td>N</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>EC\textsubscript{50} (nM)</td>
<td>8</td>
<td>71</td>
<td>61</td>
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<tr>
<td>pEC\textsubscript{50} ± S.E.M.</td>
<td>(-8.2 ± 0.1)</td>
<td>(-7.3 ± 0.2)</td>
<td>(-7.2 ± 0.1)</td>
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<tr>
<td>BRET\textsuperscript{2} \textsubscript{max} ± S.E.M.</td>
<td>0.11 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.026 ± 0.001\textsuperscript{a}</td>
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<tr>
<td>\beta-arrestin1</td>
<td></td>
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<tr>
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<td>3</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>EC\textsubscript{50} (nM)</td>
<td>16</td>
<td>32</td>
<td>33</td>
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<tr>
<td>pEC\textsubscript{50} ± S.E.M.</td>
<td>(-7.8 ± 0.1)</td>
<td>(-7.5 ± 0.1)</td>
<td>(-7.6 ± 0.2)</td>
<td></td>
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<tr>
<td>BRET\textsuperscript{2} \textsubscript{max} ± S.E.M.</td>
<td>0.06 ± 0.01\textsuperscript{a}</td>
<td>0.025 ± 0.003\textsuperscript{a}</td>
<td>0.01 ± 0.001</td>
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<tr>
<td>ERK1/2 phosphorylation</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>N</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>EC\textsubscript{50} (nM)</td>
<td>2</td>
<td>3</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>pEC\textsubscript{50} ± S.E.M.</td>
<td>(-8.9 ± 0.1)</td>
<td>(-8.5 ± 0.1)</td>
<td>N.D.</td>
<td></td>
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<td>E\textsubscript{max} ± S.E.M.</td>
<td>368 ± 32</td>
<td>293 ± 32</td>
<td>N.D.</td>
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\textsuperscript{a}Theoretical value, experimental curves did not reach saturation.
N.D., not detectable.
signal and reflects the propensity of BRET partners to interact.

Stimulation of CXCR3A/β-arrestin2, CXCR3A/β-arrestin1, as well as CXCR3B/β-arrestin2 pairs with 100 nM CXCL11 resulted in an increased BRETmax (Fig. 2, A–C, and Table 2) compared with basal conditions. This increase is a direct consequence of changes in the distance and/or orientation between the -Rluc donor and -GFP10 acceptor fusions. This strongly suggests that CXCL11 induces conformational changes in these receptor/β-arrestin preformed complexes. In addition, CXCL11 stimulation also decreased BRET50 values, shifting the saturation curve to the left for these receptor/arrestin pairs, which indicates a higher propensity of CXCR3A and B variants to interact with β-arrestins in the presence than in the absence of CXCL11 (Table 2).

In contrast, titration of the CXCR3Alt-GFP10 acceptor with both β-arrestins donors showed no ligand-dependent or -independent association, indicating that CXCR3Alt does not interact with β-arrestins (Fig. 2, E and F).

Moreover, we assessed the specificity of the interactions we measured to ascertain whether select CXCR3 variants interact with β-arrestins in the absence of ligand. We cotransfected cells expressing the BRET2 β-arrestin donor and CXCR3 acceptor fusions with the corresponding FLAG-β-arrestin and measured its impact on the basal BRET signal. We observed a transfected FLAG-β-arrestin quantity-dependent decrease of the basal BRET signal for CXCR3A and both β-arrestins as well as CXCR3B and β-arrestin2, whereas CXCR3Alt and β-arrestin1 remained unaffected (Fig. 3). A decrease in a dose-dependent manner of the basal BRET signal indicates a competition between the donor fused β-arrestin and the corresponding FLAG-tagged β-arrestin, which in turn supports a specific ligand-independent

Fig. 2. CXCR3 variants constitutively interact with select β-arrestins. Acceptor/donor titration curves of CXCR3 splice variants fused with GFP10 and β-arrestin-Rluc3 were obtained after transfection of HEK293T cells with increasing quantities of CXCR3-GFP10 fusion with 0.05 μg β-arrestin-Rluc3 fusions as indicated: A, CXCR3A/β-arrestin2; B, CXCR3A/β-arrestin1; C, CXCR3B/β-arrestin2; D, CXCR3B/β-arrestin1; E, CXCR3Alt/β-arrestin2; F, CXCR3Alt/β-arrestin1. Measurements were carried out at room temperature in the absence or presence of 100 nM CXCL11. Data were plotted and fitted to a one-site binding hyperbola equation. BRET50 and BRETmax values were calculated for each BRET2 couple. Results obtained from at least three independent experiments, carried out in triplicate ± S.E.M are shown.
interaction between select CXCR3 alternative variants and β-arrestin.

Next, we quantified ligand-mediated β-arrestin recruitment to CXCR3 at BRETmax by performing kinetic experiments. These were carried out at RT to fully capture the details of recruitment kinetics after chemokine stimulation (100 nM). CXCR3A treatment with CXCL11 or CXCL10 resulted in a rapid β-arrestin2 recruitment reaching a plateau in 5 to 7 minutes (Fig. 4A). Similar results were obtained with β-arrestin1 (Supplemental Fig. 4). A small but statistically significant elevation in β-arrestin2 recruitment to CXCR3A after stimulation with CXCL9 or CXCL4 was also observed (Fig. 4A, Supplemental Fig. 5). In contrast, CXCL9 or CXCL4 failed to induce β-arrestin1 recruitment to CXCR3A (Supplemental Fig. 4A). Our data show that CXCR3A and CXCR3B acceptor fusions are functional and are able to recruit both β-arrestins in response to ligands. In addition, these results indicate a receptor preference for specific β-arrestins, which is selectively influenced by chemokines.

Also, CXCL11 increased β-arrestin2 recruitment to CXCR3B with the signal reaching a plateau within the first 5 minutes of stimulation (Fig. 4B). CXCL10, CXCL9, and CXCL4, a chemokine suggested to be a selective high affinity ligand of CXCR3B (Lasagni et al., 2003), failed to trigger β-arrestin2 recruitment to CXCR3B. Similar results were obtained with β-arrestin1 (Supplemental Fig. 4B). As for CXCR3Alt, all chemokines failed to induce both β-arrestin1 and β-arrestin2 recruitment to this C-terminally truncated alternative variant (Fig. 4C, Supplemental Fig. 4C).

To catalog the pharmacology of CXCR3 variants in response to their chemokines, we measured the effect of increasing concentrations of ligands on β-arrestin recruitment to CXCR3A and CXCR3B (Table 1). Our results obtained with CXCL11 and CXCL10 are consistent with previously reported

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**Table 2**

Fitted curve parameters of CXCR3 (acceptor)/β-arrestin (donor) titration curves. BRET50 and BRET maximal values are given in absolute units. Errors are presented as ± S.E.M.

<table>
<thead>
<tr>
<th>Receptor Variant</th>
<th>Basal</th>
<th>CXCL11 (100 nM)</th>
<th>Basal</th>
<th>CXCL11 (100 nM)</th>
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<tr>
<td>β-Arrestin2-Rluc3</td>
<td></td>
<td></td>
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<tr>
<td>N</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>BRET50 ± S.E.M</td>
<td>24 ± 5</td>
<td>5 ± 1</td>
<td>9.5 ± 0.4</td>
<td>7.9 ± 0.3</td>
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<td>BRETmax ± S.E.M</td>
<td>0.08 ± 0.001</td>
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<td>0.024 ± 0.002</td>
<td>0.036 ± 0.001</td>
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<td>β-Arrestin1-Rluc3</td>
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<td>N</td>
<td>4</td>
<td>2</td>
<td>ND</td>
<td>9 ± 3</td>
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<tr>
<td>BRET50 ± S.E.M</td>
<td>47 ± 12</td>
<td>2 ± 1</td>
<td>ND</td>
<td>0.023 ± 0.001</td>
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<tr>
<td>BRETmax ± S.E.M</td>
<td>0.034 ± 0.002</td>
<td>0.07 ± 0.01</td>
<td>0.023 ± 0.001</td>
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</table>

N.D., not detectable.

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Fig. 3. Constitutive β-arrestin interaction is receptor variant specific. HEK293T cells transfected with the indicated receptor variant/β-arrestin at BRETmax in the absence (control) or presence of increasing levels of C-terminally FLAG-tagged β-arrestin. A, CXCR3A/β-arrestin2; B, CXCR3A/β-arrestin1; C, CXCR3B/β-arrestin2; D, CXCR3B/β-arrestin1. The fold increase shown on the x-axis represents the ratio of micrograms of β-arrestin-Rluc3 to β-arrestin-FLAG transfected in cells coexpressing CXCR3 variant GFP10 fusion. Statistical significance of the differences between the control, in the absence of FLAG-tagged β-arrestin and increasing levels of coexpressed C-terminally FLAG-tagged β-arrestin: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one-way analysis of variance (ANOVA), Bonferroni’s multiple comparison test). Results obtained for three independent experiments carried out in triplicate ± S.E.M are shown.
observations obtained with a BRET1 arrestin proximity assay (Scholten et al., 2012). Indeed, CXCL11 acts as a full agonist with a potency of 8 nM, whereas CXCL10 acts as a partial agonist with a potency of 70 nM (Fig. 5A) on the β-arrestin2 recruitment to CXCR3A. A similar rank order of potencies was observed with β-arrestin1 (Table 1, Supplemental Fig. 6A). As for CXCR3B, CXCL11 caused arrestin recruitment with potencies of 61 and 33 nM for β-arrestin2 and β-arrestin1, respectively (Fig. 5B, Table 1, Supplemental Fig. 6B). These results suggest that the longer N terminus of CXCR3B is sufficient to change the receptor’s ability to respond to chemokines and recruit β-arrestins. Moreover, these data suggest that although the N-terminal tail of CXCR3Alt is identical to that of CXCR3A, not surprisingly differences in the C terminus of CXCR3Alt are sufficient to perturb its ability to recruit β-arrestins.

**Chemokine Triggered β-Arrestin Recruitment to CXCR3 is PTX Insensitive.** To determine whether ligand-induced β-arrestin recruitment to CXCR3A and CXCR3B are linked to Gai signaling, we tested their sensitivity to Gai/o-inactivating PTX treatment (Fig. 6). Ligand-mediated β-arrestin2 (Fig. 6, A-C) and β-arrestin1 (Supplemental Fig. 7) recruitment to CXCR3A and CXCR3B were resistant to PTX treatment. As expected, PTX treatment inhibited ligand-induced Gai activity mediated by CXCR3A (Fig. 6A). This result suggests that β-arrestin recruitment to CXCR3A occurs independently from Gai signaling in response to CXCL11 and CXCL10. PTX treatment also impaired CXCL11 triggered
CXCR3B Gαi activity, even if it was only detected with 100 nM of chemokine (Fig. 6C) comparable to CXCR3A.

**Chemokines Induce Internalization of All CXCR3 Splice Variants.** We stimulated HEK293T cells expressing CXCR3 variants with chemokine ligands (100 nM) for various time periods at 37°C to assess ligand-induced receptor internalization. Excess chemokine was removed by acid wash and remaining surface receptors were quantified by flow cytometry. Stimulation with chemokines induced internalization of all receptor variants (Fig. 7). Chemokine CXCL11 induced CXCR3A internalization was modest in comparison with the other chemokines tested for this receptor variant, yet our results with CXCL11 remain comparable to previously reported observations (Scholten et al., 2012). Moreover, nearly 40% of CXCR3A receptors internalized within the first 10 minutes of stimulation with CXCL10 and CXCL4, compared with CXCL9, which required 30 minutes of stimulation to reach 40% internalization. Similarly, nearly 50% of CXCR3B receptors internalized during the first 10 minutes after incubation with CXCL10, CXCL9, and CXCL4.

Yet CXCL11 only induced moderate receptor internalization in the same fashion as CXCL11-induced CXCR3A

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** CXCR3A and CXCR3B recruit β-arrestin2 in the presence of increasing concentrations of chemokine. HEK293T cells transiently coexpressing A, CXCR3A-GFP10 or B, CXCR3B-GFP10 with β-arrestin2-Rluc3 at BRET<sub>max</sub> were incubated with increasing concentrations of the indicated ligands for 5 minutes at 37°C. BRET was measured 5 minutes after ligand addition. Data are reported as the mean values of three independent experiments performed in triplicate ± S.E.M (see Table 1 for curve fitting parameters).

![Figure 6](https://example.com/figure6.png)

**Fig. 6.** PTX treatment only affects chemokine induced Gαi activity of CXCR3A and CXCR3B. HEK293T cells transfected with CXCR3 and the EPAC cAMP biosensor or CXCR3-GFP10 fusion with β-arrestin2-Rluc3 were incubated with 100 ng/ml PTX for 16 hours at 37°C. Gαi activity of CXCR3A (A) and CXCR3B (C) are expressed as the % of forskolin-induced cAMP production in the presence of 100 nM of the indicated chemokine in the absence or presence of PTX. The forskolin induced cAMP production measured in the absence of chemokine and PTX was set to 100% (white bars). β-Arrestin2 recruitment to CXCR3A (B) and CXCR3B (D) induced with 100 nM of the indicated ligands in the absence or presence of PTX. Results represent data obtained from three to five independent experiments performed in triplicate ± S.E.M.
internalization. Interestingly, CXCL11, CXCL9, and CXCL4 induced a robust and rapid CXCR3Alt internalization, whereas CXCL10 had little to no effect. Surprisingly, in our assay CXCL4 (100 nM), a CXCR3B ligand, induced robust receptor internalization of all three CXCR3 variants. CXCL4 chemokine is reported to bind to CXCR3A and trigger chemotaxis at high chemokine concentrations of 500 to 750 nM (Mueller et al., 2008; Korniejewska et al., 2011). Our results indicate that all ligands (100 nM) are able to bind CXCR3 variants expressed at the cell surface and thus trigger their function.

**CXCR3 Splice Variants Display Different ERK1/2 Phosphorylation Profiles.** We quantitatively measured ligand-induced ERK1/2 phosphorylation in HEK293T cells expressing CXCR3 variants. Transfected cells were stimulated with chemokines (100 nM) at 37°C for various time periods (Fig. 8, A, C, and D). ERK1/2 phosphorylation was quantified using an InCell Western approach. This method was previously used to measure ERK1/2 phosphorylation of cannabinoid receptor CB1, a member of the GPCR family coupled to Gαi/o heterotrimeric G proteins (Daigle et al., 2008). Three out of the four chemokines tested induced CXCR3A-mediated ERK1/2 phosphorylation (Fig. 8A). In cells expressing CXCR3A, CXCL11 induced the longest and most significant response starting as early as 2 minutes after ligand addition (294 ± 37% of basal) and lasting 10 minutes (247 ± 21% of basal), with a peak phosphorylation measured at 5 minutes (402 ± 37% of basal). In comparison with CXCL11, CXCL10-mediated ERK1/2 phosphorylation through CXCR3A was shorter and more modest. Its signal was detected between 2 (250 ± 60% of basal) and 5 minutes (199 ± 25% of basal) after stimulation. Chemokine CXCL9 also triggered a moderate elevation of ERK1/2 phosphorylation starting at 5 minutes (225 ± 24% of basal), which declined after 10 minutes (217 ± 26% of basal). In contrast, CXCR3B activation by chemokines showed a different ERK1/2 phosphorylation profile than for CXCR3A (Fig. 8B). Indeed, CXCL11 triggered a modest ERK1/2 phosphorylation starting at 5 minutes (203 ± 18% of basal) and lasted 10 minutes (170 ± 13% of basal). Moreover, CXCL9 also induced a short ERK1/2 phosphorylation, peaking at 10 minutes (192 ± 22% of basal) and a short and weak, yet statistically significant, signal at 5 minutes (159 ± 22% of basal) after stimulation with CXCL4. Surprisingly, cells expressing CXCR3Alt variant also responded to ligands and we measured ERK1/2 phosphorylation after stimulation with CXCL11, CXCL10, and CXCL9 (Fig. 8D). Treatment with CXCL11 increased ERK1/2 phosphorylation between 5 to 10 minutes, with a peak
phosphorylation detected after 7 minutes (195 ± 16% of basal). We also quantified modest ERK1/2 phosphorylation from 5 to 15 minutes after treatment with CXCL10. In contrast, CXCL9 triggered ERK1/2 phosphorylation at a single time point of 10 minutes (155 ± 14% of basal).

Compared with CXCR3A, chemokines induced weaker ERK1/2 phosphorylation through CXCR3B and CXCR3Alt. However, these changes were statistically significant compared with cells transfected with the receptor and treated with assay buffer only. Furthermore, to ensure that the signals were indeed the result of CXCR3 alternative variants, all experiments were performed in parallel with cells transfected with empty vector pcDNA3.1. These cells failed to respond to chemokines (data not shown), indicating that the responses measured for CXCR3 were ligand induced and receptor-variant specific. We also collected pharmacological data for CXCR3A by taking advantage of favorable dynamic range and the quantitative nature of the ERK1/2 phosphorylation InCell Western assay. We measured ERK1/2 phosphorylation in cells transfected with CXCR3A in response to increasing concentrations of CXCL11 and CXCL10 (Fig. 8B). Our results indicate that both CXCL11 and CXCL10 induce ERK1/2 phosphorylation with similar efficacy and potency and are full agonists for this pathway (Table 1).

Discussion
The goal of this study was to explore the specific intracellular signaling pathways activated by CXCR3 splice variants in response to their chemokine ligands. We report that different CXCR3 splice variants induce quantitatively distinctive signaling responses after ligand stimulation and that receptor signaling efficacy depends on the splice variant/chemokine pair assessed. Receptor variant/chemokine pairs and the corresponding pathways activated are summarized in Table 3. The ability of ligands to activate different intracellular signaling pathways via the same receptor to different extents is a widely observed phenomenon and has been documented for many GPCRs, including chemokine receptors. This characteristic has been given several names including biased agonism, stimulus trafficking, and functional selectivity. Biased agonism of GPCRs supports the existence of multiple active receptor conformations that ligands may differentially stabilize, leading to the activation of specific signaling pathways (Kenakin, 2007, 2009). The nonredundant signaling responses we report for CXCR3 alternative splice variants could be explained if chemokines stabilized distinct receptor splice variant conformations leading to the activation of different intracellular signaling pathways.

Indeed, CXCR3A-mediated Gαi activity was only detectable in response to CXCL11 and CXCL10, which are in line with the Gαi activity previously reported for this canonical receptor (Scholten et al., 2012). Interestingly, CXCL11 also induced CXCR3B-mediated Gai activity, yet only at a saturating concentration of 100 nM. Sulfation of Tyr27 and Tyr29 in the N-terminal region of CXCR3A is essential for chemokine binding and thus receptor function (Colvin et al., 2006). The longer N terminus of CXCR3B contains two additional potential sulfation sites (Tyr6 and Tyr40). These Tyr residues could also be sulfated or influence the sulfation of the Tyr...
to the canonical CXCR3 receptor does not correlate with
that the internalization profiles of all three CXCR3 alterna-
tive and -independent fashion. Here, we demonstrate
internalization mechanism. Similarly, Rajagopal et al. (2013)
phosphorylation, favoring the interaction with
residue that might allow for some basal level of Ser/Thr
basal receptor phosphorylation. Indeed, the C-terminal tail of
CXCR3A and CXCR3B might also arise from low levels of
splice variants. Such constitutive
also differentially occur for the different CXCR3 alternative
vation was stronger and lasted
nal duration measured depend on the splice variant/chemo-
CXCR3-mediated ERK1/2 phosphorylation intensity and sig-

Another explanation for our results could be that the role of

It may be speculated that CXCR3Alt splice variant behaves as
an atypical chemokine receptor. Its role could be to
scavenge chemokines and contribute to the establishment of
a chemokine gradient, similarly to chemokine receptor
CXCR7, which does not activate Gai heterotrimeric G protein,
yet still recruits β-arrestin and induces ERK1/2 phosphoryla-
tion and internalization in response to CXCL12 (Boldajipour
et al., 2008; Kalatskaya et al., 2009; Levoye et al., 2009).
Another explanation for our results could be that the role of
CXCR3Alt is to modulate the function of a chemokine receptor
such as CXCR3A, because these variants can be coexpressed
in cells (Aksoy et al., 2006) and because truncated GPCRs

residues common to both variants, and therefore cotransla-
tional Tyr sulfation might explain the Gai activity measured for
CXCR3B.

As expected, Gai activation of CXCR3A was inhibited by
PTX treatment. Surprisingly, CXCR3B-induced Gai activation
was also inhibited by PTX treatment, suggesting that this
Gai activity is specific. Relatedly, stimulation of CXCR3A with
CXCL11 or CXCL10 and of CXCR3B with CXCL11 further
increased basal β-arrestin recruitment to the receptor and was
PTX insensitive. Taken together, our results indicate that
ligand-mediated Gai signaling and β-arrestin recruitment
occur independently from each other. Our findings also
suggest that chemokine receptor variant/ligand pairs select-
tively determine the receptor/β-arrestin conformation as well
as the efficacy of Gai activity. Similar to the ligand-initiated
β-arrestin recruitment and Gai signaling, we report that the
CXCR3-mediated ERK1/2 phosphorylation intensity and sig-

Ligand-independent β-arrestin recruitment was previ-
ously reported for chemokine receptor CCR1 (Gilliland et al.,
2013), atypical chemokine receptor ACKR2/D6 (McCulloch
et al., 2008), as well as a chimeric receptor composed of
ACKR3/CXCR7 with its C-terminal tail swapped with CXCR4
(Gravel et al., 2010). C-terminal tails of both D6 and CCR1
show basal phosphorylation, which plays an important role in
their ligand independent activity. Our data are the first to
indicate that ligand-independent β-arrestin recruitment can
do not correlate with CXCL11-induced internalization. Likewise, CXCL11
and CXCL10 provoked receptor internalization in a β-arrestin-
independent fashion when CXCR3A was expressed in L1.2
murine cells (Meiser et al., 2008). Furthermore, β-arrestins
are well recognized for their role as scaffold proteins for
downstream signaling phosphorylation cascades (DeWire
et al., 2007). ERK1/2 phosphorylation induced by CXCR3
splice variants does not correlate either with ligand-induced
β-arrestin recruitment. This indicates that β-arrestin may
not be required for ERK1/2 phosphorylation provoked by
specific receptor variant/ligand pairs such as CXCR3B/CXCL9
and CXCR3Alt/CXCL10. Nevertheless, β-arrestins may
play a role in ERK1/2 phosphorylation provoked by the
CXCR3A/CXCL11 pair. Similarly, only CXCR3A/CXCL11 and
CXCR3A/CXCL10 pairs provoke Gai activity that correlates with
their ERK1/2 phosphorylation profile, supporting the
possibility that ERK1/2 phosphorylation may be a con-
sequence of Gai activation.

Many GPCRs with highly truncated alternative splice
variants lacking some TM helices are reported in the litera-
ture (Wise, 2012). Alternative splice variant CXCR3Alt is a
predicted five TM domain receptor with a short C-terminal
end (Ehler et al., 2004). CXCR3Alt lacks the third intracel-
ular loop important for G-protein interaction and subse-
quent activation of intracellular components (Thelen
and Thelen, 2008). Stimulation of this splice variant resulted in
weak but statistically significant ERK1/2 phosphorylation
and chemokine-induced receptor internalization. Neverth-
less, CXCR3Alt failed to induce Gai activation and β-arrestin
recruitment in the absence or presence of chemokines. Com-
pared with CXCR3A and CXCR3B, the C terminus of CXCR3Alt
lacks the Ser and Thr phosphorylation sites typically phos-
phorylated by GRKs and required for β-arrestin recruitment.
This fundamental difference of CXCR3Alt and its limited
signaling further supports the idea that CXCR3 splice vari-
ants play different roles in fine tuning chemokine-induced
signaling responses.

It may be speculated that CXCR3Alt splice variant behaves as
an atypical chemokine receptor. Its role could be to

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
 & CXCR3A & CXCR3B & CXCR3Alt \\
\hline
Goi activation & CXCL11, CXCL10 & CXCL11* & N.D. \\
\hline
β-arrestin2 recruitment & CXCL11, CXCL10 & CXCL11 & N.D. \\
\hline
β-arrestin1 recruitment & CXCL11, CXCL10 & CXCL11 & N.D. \\
\hline
Internalization & CXCL11, CXCL10, CXCL9, CXCL4 & CXCL11, CXCL10, CXCL9, CXCL4 & CXCL11, CXCL9, CXCL4 \\
\hline
ERK1/2 phosphorylation & CXCL11, CXCL10, CXCL9* & CXCL11, CXCL9, CXCL4* & CXCL11, CXCL10* CXCL9* \\
\hline
\end{tabular}
\caption{Summary of CXCR3 variant activated signaling pathways following stimulation with chemokines}
\end{table}
could act as dominant negative of CXCR3A (Wise, 2012). The later possibility is further supported by the atypical and predicted five TM arrangement of CXCR3Alt. An additional possibility could be that activation of CXCR3Alt leads to other functional consequences we did not assess. For example, coupling to Go_{12/13} (Kourounalis et al., 2005) or Gai proteins isoforms i2 and i3 (Thompson et al., 2007) are also reported for CXCR3.

We cannot rule out that our observations are cell-type specific, yet CXCR3 natural ligands behave as perfect biased ligands (Kenakin and Christopoulos, 2013), which activate only specific signaling pathways but not others. For example, CXCL4 induces internalization of all variants, modestly stimulates ERK1/2 phosphorylation via CXCR3B and yet has no other effects on any of the receptor variants in the pathways assessed. Lack of signaling in our assays of specific CXCR3 variant/chemokine pairs could also be explained by the need of a GPCR modifying protein such as CXCR3A splice variant to induce signaling, which in turn could contribute to further diversify CXCR3-mediated signaling. Another possibility is that specific CXCR3 alternative splice variants that do not signal in our assays play a role to internalize specific chemokines and help establish gradients in specific tissues. Nevertheless, the differences we report support that CXCR3 ligands stabilize different conformations of CXCR3 splice variants. As a consequence, each CXCR3 variant/chemokine pair is likely to fulfill different functions in vivo. Although other chemokines receptors, such as CCR2 and (Chao et al., 1994) CCR9 (Yu et al., 2000), possess alternative splice variants with some differences expression and signaling levels (Wong et al., 1997; Sanders et al., 2000), our work is the first to quantify the function of chemokine receptor CXCR3 alternative splice variants in response to its four natural ligands across multiple four signaling pathways.

In addition, our work provides insights into the differences in the signaling abilities of CXCR3 alternative splice variants that are a direct consequence of their differences in the N- and C-terminal regions and intracellular loops. Our finding that chemokine receptor CXCR3 splice variants are able to selectively stimulate specific signaling pathways in response to different chemokines supports the idea that the chemokine system is not functionally redundant. Instead, the different activation patterns of CXCR3 splice variants indicate that the chemokine system displays an additional layer of complexity fine-tuned by receptor splice variants. The differences in CXCR3 variant/chemokine pair signaling must be taken into account in the context of targeting CXCR3 with small molecules. Furthermore, these differences could form a basis to design small molecules that selectively modulate in a biased manner specific receptor alternative splice variants in disease settings.

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Authorship Contributions

Participated in research design: Berchiche and Sakmar. Conducted experiments: Berchiche. Performed data analysis: Berchiche. Wrote or contributed to the writing of the manuscript: Berchiche and Sakmar.

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