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DIFFERENT EFFECTS OF THE DIFFERENT NATURAL CC CHEMOKINE RECEPTOR 2B (CCR2B) LIGANDS ON β-ARRESTIN RECRUITMENT, Gαi SIGNALLING, AND RECEPTOR INTERNALIZATION

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Abstract:

The chemokine receptor CCR2, which has been implicated in a variety of inflammatory, auto-immune and cardiovascular conditions, binds several natural chemokine ligands. Here, we assessed the recruitment of β-arrestin to CCR2 in response to these ligands using Bioluminescence Resonance Energy Transfer (BRET) technology. Compared to CCL2, which was considered as a full agonist, other CCR2 ligands were partial agonists with reduced efficacy and potency. Agonist potencies were not a function of their affinity for CCR2. Efficacy of arrestin recruitment matched that of agonist-induced CCR2 internalization. Although the potency and efficacy rank orders of the ligands in arrestin recruitment were similar to those observed for $G\alpha_i 1$ activation, arrestin recruitment was at least in part resistant to $G\alpha_{i/o}$-inactivating pertussis toxin, suggesting partial independence from $G\alpha_{i/o}$. The degree of pertussis toxin resistance of arrestin recruitment was different between the chemokines. Moreover, qualitative differences between the arrestin responses to the different ligands were identified in the stability of the response: while CCL7-induced arrestin recruitment had a half-life of less than 15 minutes, CCL8 and CCL13 induced stable CCR2-arrestin interactions. Finally, the ligands stabilized different conformations of the CCR2 homodimer. Our results support the validity of models for receptor-ligands interactions in which different ligands stabilize different receptor conformations also for endogenous receptor ligands, with corresponding implications for drug development targeting CCR2.

Introduction:

Receptor-ligand promiscuity is a hallmark of the inflammatory CC-chemokine/receptor system, where most ligands bind more than one receptor, and most receptors bind more than one ligand. Initially, different chemokines binding the same receptor were referred to as functionally redundant, although differences between their effects *in vivo* were early recognized. Pharmacologic mechanisms that may in part account for these differences are difficult to assess on primary cells and in *in vivo* systems due to the presence of alternative receptors and the confounding variety of regulatory mechanisms of the chemokine response. For example, binding preferences and activity of a given chemokine are modulated by proteolglycan binding, protease processing, and oligomerization of chemokines as well as chemokine receptors, adding several layers of complexity. However, the description of the non-redundant pharmacology of ligands to a given receptor in defined *in vitro* systems, and thus in the absence of most of the confounding *in vivo* mechanisms, is of prime interest for drug development, which has indeed been hampered by the complexity of the chemokine system. Knowledge about intrinsically different effects of different chemokines on the same receptor should help to better direct drug developing efforts targeting chemokine-receptors.

The chemokine receptor CCR2 is of significant clinical interest, due to its implication in inflammatory and cardiovascular disease. Moreover, potential roles of CCR2 in cancer and pain perception have emerged. The receptor is best investigated in cells of the monocyte/macrophage lineage, although it is also expressed on a variety of other immune cells and different tissues. In the human system, CCR2 has been reported to bind seven

natural ligands in recombinant systems, namely the chemokines CCL2, CCL8, CCL7, CCL13, and CCL11, CCL24, and CCL26 (also called MCP-1, MCP-2, MCP-3, MCP-4, Eotaxin-1, Eotaxin-2, and Eotaxin-3, respectively) (Combadiere et al., 1995; Myers et al., 1995; Berkhout et al., 1997; Gong et al., 1997; Ogilvie et al., 2001; Ogilvie et al., 2003; Parody and Stone, 2004). An additional CCR2-binding chemokine, CCL12 (or mMCP-5), seems to be specific to the murine system, and to have no human ortholog (Murphy et al., 2000), which may be indicative of the limited conservation of some CC chemokine/receptor systems across species. On the molecular level, CCR2 has been demonstrated to couple adenylate cyclase inhibiting $G\alpha_i$ subunits of heterotrimeric G-proteins. Moreover, the activation of kinase cascades including erk 1/2 and Akt, as well as intracellular calcium signalling have been reported.

Surprisingly, β-arrestin recruitment to CCR2 by its ligands has to date found little attention. β-arrestins (β-arrestin 1 and β-arrestin 2, also termed arrestin 2 and arrestin 3, respectively) were initially described as molecules that terminate G-protein-coupled receptor (GPCR) signalling by uncoupling the receptor from heterotrimeric G-proteins and by promoting receptor internalization. However, it became clear that the β-arrestins also initiate a second round of signalling, functioning as a scaffold protein with links to a confounding number of downstream pathways (Kendall and Luttrell, 2009). Moreover, it appears now that for some receptor-ligand combinations β-arrestin recruitment can be independent from the activation of heterotrimeric G-proteins. Therefore, arrestin emerges as a signalling hub in its own right. The advent of quantitative proximity assays Bioluminescence Resonance Energy Transfer (BRET) technology to measure β-arrestin recruitment, traditionally detected by microscopy through cytosol to plasma membrane

translocation, permits now to quantify the pharmacological parameters of this signalling axis.

We here set out to investigate the recruitment of β-arrestin to CCR2 induced by its different natural ligands. We also investigate $G\alpha_i$ signalling and its link to β-arrestin recruitment, and explore CCR2 homodimer conformations induced by the different chemokines. By using a recombinant HEK293 system, we isolate the pharmacologic properties of the different ligands and thus circumvent difficulties in data interpretation encountered in more natural systems. We find that most of the natural CCR2 ligands qualify as partial agonists, with similar efficacies in the tested readouts. Nevertheless, β-arrestin recruitment is only in part inhibited by pertussis toxin, suggesting $G\alpha_{i/o}$-independent mechanisms of β-arrestin recruitment. The stability of the receptor-β-arrestin interaction depends on the tested CCR2-ligands, suggesting that they induce qualitatively different responses, in line with the concept of functional selectivity.

Materials and methods :

Materials. Recombinant chemokines were from PeproTech Inc (Rocky Hill, NJ). Coelenterazine h (for BRET[1] experiments) was from NanoLight Technology, Pinetop, AZ), and coelenterazine 400A (for BRET[2] experiments) from Biotinium, Hayward, CA. Pertussis toxin (PTX) was from Calbiochem, forskolin from Sigma, and the anti-CCR2 monoclonal antibody (clone 48607) directly coupled to phycoerythrin from R&D Systems.

Plasmids. The cloning of CCR2b-Rluc and CCR2b-YFP was described previously (Percherancier et al. 2005). β-arrestin 2-Rluc, Rluc-β-arrestin 1, $G\alpha_i1$-91Rluc, $G\alpha_i1$-122-Rluc and $G\alpha_i1$-60Rluc and the cAMP Epac BRET sensor were gifts of Michel Bouvier (Université de Montréal).

Cell culture and transfection. HEK293 (passage number ~ 10-30, Invitrogen, Carlsbad, CA) cells were maintained in Dulbecco's modified Eagle's medium, 1% penicillin-streptomycin (Invitrogen), and 10% fetal bovine serum (Wisent). Transient transfections were performed in six well plates using the polyethylenimine method; the total amount of transfected DNA was kept constant for all transfections by adding empty vector. Surface CCR2 quantities were determined by flow cytometry, using Quanti-Brite standardization beads (BD Biosciences), and typically reached around 2000-4000 antibody binding sites/cell.

Binding studies. Cells were transiently transfected with 1μg unfused CCR2b cDNA or 1μg of empty vector per well. 24 h after transfection the medium was removed, the cells washed twice with PBS, resuspended in binding buffer (HEPES 50mM pH 7.4, $MgCl_2$ 5 mM, $CaCl_2$ 1 mM, 0.2% BSA), and seeded in 96 well plates in the presence of increasing concentrations of unlabeled competitor ligand. The competition studies were carried out at 4°C for 4 hours using 60 pM of I^{125}-CCL2 (PerkinElmer Life and Analytical Sciences, Waltham, MA) as a tracer. A combi cell harvester was used to wash the excess tracer and capture samples on glass fiber filter paper (Molecular Devices, Menlo Park, CA). Binding

was measured using a gamma counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). Experiments were carried out in duplicate.

BRET Measurements. Transfected cells were seeded in 96 well, white, clear-bottom microplates (View Plate, PerkinElmer Life and Analytical Sciences, Waltham, MA) pre-treated with 0.1 % poly-D-lysine (Sigma) 24 hours after transfection and left in culture for another 24 hours. 48 hours after transfection the culture medium was replaced by BRET buffer (PBS, 0.1% bovine serum albumin, 0.5mM $MgCl_2$) at room temperature. Coelenterazine h, or coelenterazine 400A was added at the final concentration of 5μM, and fluorescence and luminescence reading were collected using the Mithras LB940 (Berthold Technologies) as described previously (Percherancier et al., 2005; Berchiche et al., 2007; Leduc et al., 2009). All BRET experiments were performed while the cells remained attached to the 96 well plates. To assess ligand-induced changes of the BRET[1] signal, we first established the assay conditions by performing BRET[1] titration experiments to determine the $BRET_{max}$ ratio for each co-transfected –eYFP and –Rluc fusion protein, as previously described (Berchiche et al., 2007; Kalatskaya et al., 2009). For dose-response experiments, cells expressing the –eYFP and –Rluc fusion proteins at $BRET_{max}$ ratios, were stimulated for 5 min at 37°C with increasing concentrations of the indicated ligand before the addition of the substrate, unless specified otherwise. The values were corrected to net BRET by subtracting the background BRET signal detected when the -RLuc construct was expressed alone.

Arrestin Recruitment. β-arrestin recruitment was measured by BRET[1] as described previously (Kalatskaya et al. 2009). Briefly, the BRET signal of cells co-transfected with 1.5μg CCR2-eYFP and 0.02 μg β-arrestin 2-Rluc or 0,003 μg Rluc-β-arrestin 1 completed to 2 μg with empty vector per well was measured. The β-arrestin recruitment kinetics was assessed by adding the ligands 10 minutes after the addition of coelenterazine h. The β-arrestin decay kinetics were assessed by incubating 10 min RT for and for various periods at 37°C, followed by BRET readings.

Adenylyl Cyclase Activity. cAMP was determined using Rluc3-Epac-GFP10, a BRET[2] reporter, as described previously (Leduc et al., 2009). Briefly, 1 μg of unfused CCR2b cDNA and 0.03 μg of the Epac reporter were transiently co-transfected. Cells were stimulated with ligands in the presence of 20 μM forskolin at room temperature for 10 minutes, immediately after the addition of coelenterazine 400A. For experiments with PTX, the cells were treated for 16h with 100 ng/ml PTX at 37°C prior to stimulation with forskolin and chemokines.

Endocytosis and Flow Cytometry. CCR2b expressing HEK293 cells were incubated with 100 nM of each ligand at 37°C with gentle shaking. The reaction was stopped by removing aliquots on ice. Surface-bound chemokine was removed with acid washing (50 mM Glycine buffer pH 2.7, 150 mM NaCl), and the cells were subsequently washed 3 times with ice cold PBS. Labelling with phycoerythrin conjugated monoclonal anti-CCR2 antibody was done for 30 minutes on ice, followed by three PBS washes. CCR2 was

quantified by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences). Surface expression of CCR2 after ligand incubation at 37°C was expressed in % of CCR2 expression compared to a sample drawn before addition of the ligands.

Data analysis. Data obtained were analyzed using Graphpad Prism 4.0 software. Statistical significance of the differences between the different conditions was determined using one-way analysis of variance with Tukey's post-test; when appropriate, multiple measures ANOVA was also used. IC_{50}s were determined with the Cheng-Prusoff equation using GraphPad. When indicated, differences of top or bottom values were also determined using simultaneous curve fitting. The intrinsic relative activities (RA_i) of CCL8, CCL7 and CCL13 were estimated using the formula $E_{max\_B}EC_{50\_A}/E_{max\_A}EC_{50\_B}$ (equation 17 in (Ehlert, 2008)), and relative efficacies using the formula $K_BE_{max\_B}EC_{50\_A}/K_AE_{max\_A}EC_{50\_B}$, equation 19 in (Ehlert, 2008).

Results

Radioligand displacement. To establish the binding characteristics of the reported CCR2 ligands to CCR2 in our assays system, we performed radioligand displacement assays using ^{125}I-CCL2 as a probe (Figure 1). The affinity of CCL2 was in the 0.2 nM range, as expected (Myers et al., 1995), while CCL8, CCL7, CCL13 and CCL11 displaced ^{125}I-CCL2 with IC_{50}s in the nanomolar range (220 pM for CCL2, 4.2 nM for CCL8, 10 nM for CCL7, 5 nM for CCL13 and 11 nM for CCL11, Table 1), suggesting that they are specific CCR2 ligands. Of note, while we performed heterologous displacement assays, the

observed IC_{50}s for CCL8 and CCL7 are nearly identical with those reported previously in homologous binding competitions (Sozzani et al., 1994; Combadiere et al., 1995; Gong et al., 1997), suggesting that they indeed represent the affinities of these chemokines for CCR2. For CCL11, our IC_{50} is in agreement with that reported by Martinelli et al. (Martinelli et al., 2001), but at odds with that found by Oglivie et al. (in the micromolar range) (Ogilvie et al., 2001). Moreover, we find high IC_{50}s for CCL24 and CCL26 (over 850 nM and 700 nM, respectively). While Ogilvie et al reported that CCL26 competed with the binding of ^{125}I-CCL2 on monocytes with an IC_{50} in the 100 nM range, and on CCR2-transfected 300.19 murine pre-B cells with an IC_{50} in the 20 nM range (Ogilvie et al., 2003), Parody et al report IC_{50}s against the same radioligand of 3 nM for CCL26 and 580 nM for CCL24 on CCR2-transfected COS-7 cells (Parody and Stone, 2004). The reason for such discrepancies may lie in the different cell backgrounds used, especially considering potential effects of CCR2 heterodimerization with unidentified endogenous receptors of the competing chemokine (Springael et al., 2006). Indeed, cooperative effects could be inferred from the atypical competition slopes reported by these authors (Ogilvie et al., 2003; Parody and Stone, 2004). The investigation of CCL24 and CCL26 has not been expanded in the present study, but they were ineffective in initial experiments in all experimental system used (not shown).

β-arrestin recruitment. To test β-arrestin recruitment to CCR2, we used a BRET-based proximity assays system, in HEK cells transiently coexpressing the BRET donor β-arrestin 2-Rluc and CCR2 fused to the BRET acceptor YFP. No basal BRET was detected, suggesting the absence of constitutive interaction of CCR2 with β-arrestin 2. Dose-

response experiments revealed the following potency rank order of the chemokines: CCL2<CCL8/CCL7<CCL13 (EC_{50} of approximately 1 nM for CCL2, 55 nM for CCL8, 15 nM for CCL7, and over 140 nM for CCL13) (Figure 2A, Table 1). Similar observations were made using RLuc-β-arrestin 1 (Figure 2B, Table 1), with the exception of CCL13, which showed exceedingly low potency in recruiting beta-arrestin 1. CCL11 did not induce β-arrestin 2 recruitment. The efficacies of the BRET signals were also different among the chemokines (Figure 2C), with a rank order of CCL2>CCL7/CCL13>CCL8. Therefore, while CCL8 was a potent recruiter of β-arrestin to CCR2, it did so with low efficacy. CCL13 induced arrestin responses did not reach saturation even at highest chemokine doses, leaving some uncertainty about the EC_{50} values that were determined by curve fitting. However, the saturation for β-arrestin 1 recruitment is clearly less than for beta-arrestin 2, and thus the underestimation of the true EC_{50} value for β-arrestin 1 recruitment by CCL13 is probably even stronger than for β-arrestin 2. This may indeed suggest a β-arrestin 2 over β-arrestin 1 bias of CCL13. No preferences for either arrestin were found with the other ligands.

The different BRET efficacies in this proximity assay might reflect quantitative differences in arrestin recruitment, for example owing to variations in affinity for arrestin of the receptor-ligand complex. Alternatively, and perhaps more likely, they might reflect different conformations of the resulting receptor-arrestin complex, which result in different distances and/or orientations of the fluorophores and thus BRET efficiency. In both cases, we detect significant differences between the responses to the different chemokines. In general, the potency to recruit β-arrestins was much lower than ligand affinity

(approximately 1 log, see Table 1), in line with the independence of both parameters. An

notable exception in this respect was CCL7, for which the potency to recruit β-arrestin

almost matched the affinity for CCR2, suggesting that CCL7 forms with CCR2 a receptor-

ligand complex with a higher propensity to recruit β-arrestin, as compared to the other

ligands.

To further explore CCR2 recruitment of β-arrestin in response to the different

chemokines, we performed time-course experiments. Addition of chemokines led to very

rapid arrestin recruitment at 37°C (not shown). In order obtain higher resolution of the

kinetics, these experiments were performed at room temperature, thus decelerating β-

arrestin 2 recruitment to the receptor. Significant differences were seen when the rapidity

of the response was considered: while CCL2 still reached 50% of the maximal response in

less than 3 minutes, the response of CCL13 was considerably slower (Figure 3A). Overall,

the rapidity of the response was CCL2<CCL8<CCL7<CCL13. We then investigated the

stability of the β-arrestin 2 response over time. Indeed, while some GPCRs interact only

transiently with β-arrestin 2 (also termed class A receptors), others show sustained

interactions (also termed "class B receptors" (Kendall and Luttrell, 2009). To address this

issue, cells were incubated for 10 minutes with the ligands at room temperature as before,

followed by shifting to 37°C for the indicated periods and measurement of the remaining

signal. As shown in Figure 3B, marked differences were seen between the chemokines.

While the weak signal elicited by CCL8 was of great stability over time, the CCL7-elicited

signal rapidly diminished and its decrease fitted one-phase exponential decay ($R^2 = 0.76$)

with a halflife of less than 15 minutes. The CCL2 signal was somewhat more stable, with a

half life of >20 minutes. The CCL13-elicited signal was clearly biphasic, showing a

transient increase during the first 10 minutes at 37°C, followed by a stable signal. Similar observations were made with β-arrestin 1 (data not shown). In sum, CCL7, who had a particularly high potency to recruit arrestin (compared to its binding affinity), also showed the shorter half-life of the signal. Inversely, CCL13, a weak arrestin recruiter, induced signals that were stable over time. It thus seems that CCR2 does not *per se* belong to class A or class B receptors, but can adopt different arrestin recruitment characteristics depending on the bound chemokine.

CCR2 internalization. The observed differences in arrestin recruitment may have impact on receptor endocytosis, since arrestins are key molecules for chemokine receptor endocytosis. Indeed, a recent report finds that upon stimulation with CCL2, CCR2 colocalizes with β-arrestin 2 in endocytic compartments (Garcia Lopez et al., 2009), suggesting that receptor endocytosis is mediated by β-arrestin 2. We thus studied the effect of the chemokines on receptor internalization.

Cells were incubated with the respective ligands (100 nM) at 37°C, and aliquots placed on ice at different time points. After acid wash to remove surface-bound chemokines, remaining CCR2 receptor was quantified by flow cytometry. As shown in Figure 4, over 50% of the surface CCR2 was internalized during the first 10 minutes after incubation with CCL2. CCL7-induced internalization was somewhat slower, and did not reach the same extent as with CCL2. CCL8 and CCL13 induced even less CCR2 internalization. The plateau reached after 60 minutes of incubation with the chemokine is significantly different between CCL2 and CCL13, as well as between CCL7 and CCL8. These observations are compatible with the vision that CCR2 internalization is a function of arrestin recruitment.

Activation of Ga$_i$ via CCR2 by its natural ligands. Given that the different chemokines showed different intrinsic properties in β-arrestin signalling, we asked whether this might reflect signalling events upstream of β-arrestin recruitment. To investigate the potential correlations between β-arrestin recruitment and G-protein signalling in CCR2, we measured CCR2-Gα$_i$1 signalling, taking advantage of a previously described BRET-based assay (Gales et al., 2005; Gales et al., 2006). This system uses the receptor fused to YFP in combination with Gα$_i$1-RLuc constructs, where the BRET donor is fused at different permissive locations within the Gαi_1 amino acid sequence (Gα$_i$1-RLuc-60, Gα$_i$1-RLuc-91, and Gα$_i$1-RLuc-122). Using this system, Galés et al demonstrated the existence of GPCRs in preformed signalling complexes containing the receptor and heterotrimeric G-proteins. Moreover, they were able to detect ligand-induced BRET changes between various GPCRs and the Gα$_i$ subunit, indicating conformational changes within preformed signalling complexes that correlate with Gα$_i$ activation (Gales et al., 2005).

Using this system, we detected specific basal BRET signals between Gα$_i$1-RLuc fusions and CCR2-YFP, in line with the existence of preformed and persistent complexes, as has been described for a number of 7TMRs (Dupre et al., 2006). Overall, Gα$_i$1-RLuc122 yielded the strongest signal, but similar albeit weaker signals were also obtained with the Gα$_i$1-RLuc91 fusion, while Gα$_i$1-RLuc60 only yielded weak BRET (Figure 5A). Incubation with agonists led to strong decreases in BRET (>50%), regardless whether Gα$_i$1-RLuc122 or Gα$_i$1-RLuc91 were used. This is different from observations made in the α2$_A$-adrenergic receptor system, where BRET increases upon stimulation with Gα$_i$1-

RLuc91, but decreases with $G\alpha_i1$-RLuc122 (Gales et al., 2006). Of note, the amplitude of ligand-induced BRET changes that we observe is much stronger than that reported with the $\alpha2_A$-adrenergic receptor. These divergences likely reflect different the position of the $G\alpha_i$ subunit with respect to the receptor C-terminus of different receptors.

Experiments with various concentrations of the different ligands yielded sigmoidal dose-response curves (Figures 5A and 5B). In general, there was good agreement between the potencies measured with $G\alpha_i1$-RLuc91 and $G\alpha_i1$-RLuc122, while the different efficacies reported by $G\alpha_i1$-RLuc91 failed to reach statistical significance due to the weaker signal (Table 1). Overall, both potency and efficacy rank orders with $G\alpha_i1$ strikingly matched those observed in the arrestin readout. The potency rank order was again CCL2>CCL8=CCL7>CCL13 (EC_{50} with $G\alpha_i1$-RLuc122 and $G\alpha_i1$-RLuc91, respectively: 860 and 510 pM for CCL2, 9 and 20 nM for CCL8, 4.4 and 3.6 nM for CCL7, 30 and 55 nM for CCL13, and 1 nM with CCL11 (determined with $G\alpha i_1$-RLuc122 only)), while the efficacy ranking was again different from potency ranking, due to strikingly low efficacy of CCL8 (CCL2>CCL7>CCL13>CCL8, Figures 5D and 5E). Surprisingly, using the $G\alpha_i1$-RLuc122 sensor, CCL11 did yield weak, but significant BRET changes, which, while they did not exceed 10-15% of BRET reduction, were observed with high potency. Also, we observed again that EC_{50}s were higher that binding competition IC_{50}s for all ligands except CCL7. This might be surprising given that the EC_{50}s of strong agonists (like CCL2) in functional assays lie normally below their affinity, reflecting that the activation of a fraction of the receptors is sufficient to saturate the response. However, the EC_{50}s

revealed by the BRET assays, unlike functional assays, record the entirety of receptor/G-protein complexes, and saturation is therefore not expected before full receptor occupancy.

Importantly, Galés et al found that efficacies measured by BRET and cAMP production correlate (Gales et al., 2005). To confirm this conclusion, we conducted measurement of the generated cAMP levels using a BRET-Epac cAMP sensor (Jiang et al., 2007; Leduc et al., 2009). As shown in Figure 5F, dose-response experiment conducted with CCL2 and CCL8 revealed different maximal inhibition of forskolin-induced cAMP production by both chemokines (63.5 ± 1.3% for CCL2 and 40.2 ± 2.1% for CCL8). This result supports that, indeed, the different CCR2 ligands lead to different changes in the receptor/G-protein complex that result in differences in the efficiency of $G\alpha_i1$ activation. These conclusion are in line with those drawn by Galés et al (Gales et al., 2005) and Nikolaev et al (Nikolaev et al., 2006) concerning the molecular basis of partial agonism.

Pertussis toxin sensitivity of arrestin recruitment. The agreement of potency and efficacy rank orders between $G\alpha_i1$ signalling and arrestin recruitment is striking, and compatible with a causal link between the two readouts. To further investigate this question, we tested their respective sensitivity to $G\alpha_{i/o}$-inactivating pertussis toxin (PTX) treatment. Indeed, if dependent on $G\alpha_i$ signalling, it would be expected that β-arrestin 2 recruitment is PTX sensitive. To test this, transfectants for β-arrestin 2 and Epac BRET were incubated in parallel with PTX, and stimulated with the respective agonists. As shown in figure 6A, all ligands reduced forskolin-induced cAMP production, representing $G\alpha_i$ activation. This reduction was entirely abolished by PTX treatment. Intriguingly, in the presence of both chemokine and PTX, we consistently observed cAMP production levels superior to those

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observed with forskolin alone. Potentially, this might hint at adenylate cyclase stimulation *via* marginal $G\alpha_s$ activation by the chemokines, which would indeed be expected to be unaffected by PTX treatment. Simultaneous coupling to PTX-sensitive $G\alpha_i$ and PTX-insensitive $G\alpha_s$ pathways has been reported for several 7TMRs. Alternatively, the excess cAMP inhibition by PTX might result from inhibition of the basal activity of endogenous $G\alpha_s$ coupling GPCRs.

However, when evaluating β-arrestin 2 recruitment to CCR2, we found that recruitment induced by CCL2 was largely resistant to PTX (Figure 6B). This reinforces the conclusion that CCL2-induced endocytosis is arrestin dependent, as CCL2-induced endocytosis was also reported to be PTX resistant (Garcia Lopez et al., 2009). However, stronger PTX inhibition was observed with other chemokines, ranging from 45% (CCL7) to 60% (CCL13) of the β-arrestin 2 response. The CCL8-induced signal was too weak to permit meaningful analysis. Our results suggest that β-arrestin 2 recruitment to CCR2 by CCL2 (and in part by the other chemokines) is indeed G-protein independent. Alternatively, we can at preset not exclude that other, endogenous, Gα subunits are also stimulated and play a role in β-arrestin recruitment. In sum, it seems that β-arrestin 2 recruitment is induced by a combination of different, simultaneously activated pathways, which are only in part $G\alpha_{i/o}$-dependent. However, the proportions by which these pathways account for β-arrestin 2 recruitment by the different ligands are not alike.

Ligand-induced changes in CCR2 homodimer BRET. The observed differences in the effects of the different ligands suggest that they stabilize different CCR2 conformations, as

has been shown for synthetic GPCR ligands. To directly assess this question, we used BRET within the CCR2 homodimer as a conformational sensor. We have indeed previously shown that CCR2 forms constitutive homodimers that can be detected by BRET signals between coexpressed CCR2-RLuc and CCR2-YFP as BRET donor and acceptor, respectively (Percherancier et al., 2005). Incubation of these dimers with CCL2 resulted in BRET changes, which we interpreted to reflect ligand-induced rearrangements in the CCR2 dimer conformation (Percherancier et al., 2005). Here, we compared in dose-response experiments BRET changes yielded by the different CCR2 ligands.

As depicted in Figure 7, only CCL2, CCL7, and CCL13 yielded appreciable changes in the constitutive CCR2 homodimer BRET. The changes induced by different chemokines show, indeed, different efficacies and potencies. The observed EC_{50}s were 6 nM for CCL2, 25 nM for CCL7, and 180 nM for CCL13. Maximal changes observed with CCL7 and CCL13 are clearly different from those observed with CCL2 ($p<0.0001$), but not from one another (although maximum levels with CCL13 were not attained at the chemokine doses used and had to be derived from curve fittings, Table 1). CCL8 and CCL11 did not induce changes even at highest doses (not shown). This could either mean that no changes in the CCR2 homodimer conformation is induced by these chemokines, or that such changes are not detected by this experimental system.

Perfect matches of radioligand displacement IC_{50} with homodimer BRET EC_{50} would be expected, if GPCR homodimer BRET changes directly reflected ligand binding associated conformational changes in the receptor dimer. Surprisingly, however, the potencies of the responses of CCL2 and CCL13 did not correspond to IC_{50} values of radioligand competitions, but were over 1 log higher for CCL2, whereas both values are

approximately in keeping for CCL7. This discrepancy suggests that the conformational change of the CCR2 dimer does not only relate to conformational changes induced by the ligand/receptor interaction. Rather, ligand-induced BRET change seems to be the result of more complex events that relate to early signalling processes that affect receptor conformation. We envision that these events reflect rearrangements in the conformation and/or composition of receptor associated signalling molecules or scaffold proteins. Following this line of interpretation, it is intriguing that the potencies of ligand-induced BRET changes in the homodimer are approximately in keeping with those observed with $G\alpha_i 1$ and β-arrestin, and that this includes the observed unique match between IC_{50} and EC_{50} for CCL7. While this might suggest that the conformational changes observed in the CCR2 homodimer result from G-protein activation and/or arrestin recruitment, rather than preceding it, it could also be that homodimer BRET reflects other, yet unidentified events, related or unrelated to these readouts.

Discussion

CCR2 is a well studied chemokine receptor that meets considerable clinical interest. Yet, few studies have been investigating recruitment of β-arrestin to CCR2, with CCL2 as the sole analyzed CCR2 ligand (Aragay et al., 1998; Garcia Lopez et al., 2009). Thus, we report the first study dedicated to the investigation of the effects of different CCR2 ligands on β-arrestin recruitment. We demonstrate that the effects of different CCR2 ligands on β-arrestin recruitment to the receptor are quantitatively and qualitatively distinct from one another. Differences were found in recruitment efficacy, identifying CCL7, CCL8, and

CCL13 as partial agonists, with submaximal responses despite full receptor occupancy. Comparing β-arrestin 1 or 2, we found potential bias towards β-arrestin 2 of CCL13 and CCL8, overall weak β-arrestin recruiters. Experiments addressing the decline of the CCR2/β-arrestin interaction over time revealed striking differences between the ligands. While CCL7-induced β-arrestin recruitment had the shortest half life (<15 minutes), CCL2-induced responses were longer lived, and CCL8 and CCL13 yielded β-arrestin recruitment that remained stable over 90 minutes. Our findings with CCL2 are in agreement with those reported for this chemokine (Aragay et al., 1998) (Garcia Lopez et al., 2009). Garcia Lopez et al. concluded that the stability of the signal places CCR2B in the class B receptor group that only slowly recycles. However, we show that the characteristics of the CCR2/arrestin interaction depend on the investigated ligand. Together with similar observations made in the CCR7 and CXCR7 systems (Kohout et al., 2004; Rajagopal et al., 2010), our data suggest that chemokine receptors may not *per se* belong to class A or B, but adopt different characteristics depending on the bound agonist. Of note, the efficacies of ligands in CCR7 endocytosis and β-arrestin recruitment correlate (Kohout et al., 2004; Byers et al., 2008).

The correlation of β-arrestin recruitment efficacy with maximally achieved endocytosis suggests that CCR2 endocytosis is an arrestin-dependent process. Our observation that CCL2-induced β-arrestin recruitment is PTX resistant, as is CCL2-induced CCR2 endocytosis (Garcia Lopez et al., 2009), supports this conclusion. While GPCR endocytosis has initially always been related to β-arrestin, more recent data have revealed that there are exceptions to this rule. Rather, β-arrestins fulfil additional roles in chemokine receptor function as scaffolds for a variety of molecules regulating kinase signalling pathways

(Kohout et al., 2004; Cheung et al., 2009). In this context, the observation that CCR2 chemokines induce distinct kinase signalling patterns (O'Boyle et al., 2007) is, in combination with our data on their distinct β-arrestin recruitment patterns, highly suggestive.

Despite the striking correlation between the respective efficacies in β-arrestin recruitment and Gαi activation, the PTX resistance of the CCL2 β-arrestin response leads to the conclusion that the former is not simply the consequence of the latter. Rather, in the case of CCL2, β-arrestin recruitment is either almost entirely G-protein independent, or due to the activation of alternative Gα subunits. Indeed, CCR2 can couple to different Gα subunits, and so far coupling of CCR2 to Gαq, Gα14, Gα16, has been reported. Given that β-arrestin 2 responses to the different ligands show various degrees of PTX sensitivity, it is tempting to speculate that different ligands have different preferences for CCR2 in association with different G-proteins. In support of this hypothesis, CCL8-induced chemotaxis was reported to be in part sensitive to cholera toxin (CTX), whereas chemotaxis induced by CCL2, CCL7 and CCL13 was not (Sozzani et al., 1994; O'Boyle et al., 2007).

We show that CCR2 binding chemokines other than CCL2 are partial agonists of CCR2 in the analyzed readouts. This is particularly striking for CCL8, a potent, but not very efficacious agonist in all studied readouts. To further describe the partial agonist properties of CCL8, CCL7, and CCL13, we estimated their intrinsic relative activity (RA$_i$) with respect to CCL2 (Table 2). Using the pIC$_{50}$ determined by ^{125}I-CCL2 displacement (Figure 1, Table 1) as an independent measure of ligand affinity, relative efficacies were also determined (Table 2). While the obtained values reasonably reflect the observed differences in efficacy, it should be borne in mind that efficacy in BRET

assays might indeed reflect quantitative differences in arrestin recruitment, but might also relate to qualitative parameters (the relative positioning of the fluorophores). In the latter case, no linear relationship between BRET and signalling efficacy can be expected. Therefore, the significance of the reported intrinsic relative activities might not be directly proportional to the respective ligand's effects. Nevertheless, this limitation might not be of great consequence in practice, as the resulting estimates of intrinsic relative activity are in good agreement with directly functional readouts (such as reported in figure 4 and 5F).

Of note, the functional implications of partial agonism will differ in different CCR2 expressing cell types, as they depend on receptor density and coupling efficiency, which in turn depend on the expression levels of the receptor and relevant downstream signalling molecules. CCR2 is mainly expressed at relatively low levels on monocytes (3-4000 molecules per cell, (Mine et al., 2006)), but can be drastically increased on NK cells (Hanna et al., 2005) and T-cells (Amoura et al., 2003) in disease. Here we used transfected HEK293 cells expressing approximately 2000 receptors per cell as determined with fluorescent standardization beads (not shown). It is attractive to envision that the partial agonism of CCR2 ligands differentially affects different CCR2 expressing cells, as partial agonists can convert into full agonists if a larger receptor reserve exists. Indeed, a link between receptor expression levels and responses to different chemokines has been drawn in the different responses of Th1 and Th2 cells to the CCR4-binding chemokines CCL17 and CCL22 (D'Ambrosio et al., 2002).

While different equilibria between one active and one inactive conformation of the receptor might explain, in principle, partial agonism, the stabilization of intrinsically

different receptor conformations by different ligands emerges as a new paradigm to explain partial agonism. Evidence for the latter model was for example presented for the $\alpha2_A$-adrenergic receptor (Nikolaev et al., 2006). The model is also in line with the concept of functional selectivity, which postulates that distinct receptor conformations stabilized by different ligands can not only lead to different response efficacies, but to qualitatively different responses in different signalling pathways (Kenakin, 2007; Kobilka and Deupi, 2007). While we did indeed observe different CCR2 conformations stabilized by the chemokine ligands, this was not directly due to the conformational effects of the sole receptor-ligand interaction, but involved also other components of the CCR2 signalling complex. A hallmark of functional selectivity is the reversal of relative rank orders of potency or efficacy of a set of ligands in different readouts. We have not found such rank order reversals. Moreover, our rank orders fit the efficacies reported in single-dose experiments for calcium signalling (Combadiere et al., 1995; Martinelli et al., 2001). Functional selectivity can thus not be inferred from the efficacy and potency rank orders of the CCR2 ligands. However, the differences in PTX sensitivity and decay rates of the β-arrestin response indeed point towards qualitative differences in CCR2 responses to the different chemokines. This conclusion is further supported by the atypical signalling profile of CCL11. CCL11 induced marginal Gαi activation that had eluded previous studies (Martinelli et al., 2001; Ogilvie et al., 2001; Ogilvie et al., 2004). However, CCL11 reportedly activates the mitogen activated protein kinase erk1/2 via recruitment of a p85/p110 PI3 kinase (unlike CCL2, which activates PI3Kγ) (Ogilvie et al., 2004). Our results suggest that these kinase cascade activations do not involve CCL11 recruitment of β-arrestin to the receptor.

Our data show that CCR2 responses to different ligands are not redundant on the pharmacological level. Similar observations have been reported in a reconstituted CCR5 system (Oppermann et al., 1999; Leach et al., 2007). Therefore, the absence of functional redundancy among chemokines binding the same receptor does not only result from complex *in vivo* regulatory mechanisms, but is also encoded in their intrinsic pharmacological properties. The investigation of only one natural "default"-ligand of chemokine receptors as a prototype agonist is thus insufficient. This is particularly relevant for drug screening campaigns that often rely on only one single agonist. Small molecules targeting chemokine receptors are likely to be allosteric, owing to the large size of the natural ligands, and the probe-dependent effects of such allosteric modulators are notorious (Kenakin, 2004). In consequence, CCR2-targeting small compounds might have given effects relative to one of its natural ligands, but different or no effects on the response to others. Whether this might be a disadvantage or rather a desired effect will depend on the implication of the different pathways activated by the different ligands in disease processes, and remains to be seen.

In sum, while much remains to be learned to fully characterize the functional differences of natural CCR2 ligands, our results provide further evidence that functional selectivity applies also to natural ligand/receptor combinations. Functional selectivity is now well-established as a drug phenomenon (Kenakin, 2007). Our results strengthen the case for functional selectivity as a general mechanism of GPCR activity.

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Authorship Contributions

Participated in research design: Berchiche and Heveker

Conducted experiments: Berchiche, Gravel, Pelletier, Saint-Onge

Performed data analysis: Berchiche, Gravel, Pelletier, Saint-Onge, Heveker

Wrote or contributed to the writing of the manuscript: Berchiche and Heveker

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Footnotes

Figure legends

Figure 1: ^{125}I-CCL2 displacement by reported CCR2 ligands on HEK293-CCR2B cells. CCL2: black squares, CCL7: black diamonds, CCL8: black circles, CCL11: black triangles, CCL13: open circles, CCL24: open pyramids, CCL26: open triangles. Specific binding was defined as that displaced by 1uM CCL2. All points are pooled data of 4 (CCL2, CCL8, CCL7, CCL13 and CCL11) or 3 (CCL24 and CCL26) independent experiments performed in duplicate, $\pm$ S.E.M. For IC$_{50}$s see Table 1. logIC$_{50}$ for CCL24 was -6.06 $\pm$ 0.06, and for CCL26 -6.15 $\pm$ 0.07.

Figure 2: Dose-response experiments of arrestin recruitment to CCR2 using different CCR2 ligands. HEK293 cells transiently coexpressing CCR2-YFP and β-arrestin 2-Rluc or Rluc-β-arrestin 1 were incubated with indicated concentrations of ligand and resulting BRET measured after 10 minutes at room temperature. CCL2: black squares, CCL8: black circles, CCL7: black diamonds, CCL13: open circles, CCL11 black triangles. A): β-arrestin 2-Rluc, B): Rluc-β-arrestin 1. Data are means of 5 (β-arrestin 2-RLuc) or 3 (Rluc-β-arrestin 1) independent experiments performed in triplicate $\pm$ S.E.M. C) maximal BRET values obtained with β-arrestin 2-RLuc. Statistical significance of the differences between the maxima: * $p < 0.05$, ** $p < 0.01$, and * $p < 0.001$ (repeated measures ANOVA with Tukey's multiple comparison test). Similar results were obtained with Rluc-β-arrestin 1, without reaching statistical significance (except with CCL8, not shown). For curve fitting parameters, see Table 1.

Figure 3: Time-course experiments of b-arrestin-2 recruitment to CCR2 using different CCR2 ligands. CCL2: black squares, CCL8: black circles, CCL7: black diamonds, CCL13: open circles, no ligand: open triangles. **3A)**: time-course of β-arrestin 2 recruitment. HEK293 cells transiently coexpressing CCR2-YFP and β-arrestin 2-Rluc were incubated with 100 nM of the indicated ligand and resulting BRET measured after 10 minutes at room temperature. Data are means of 5 independent experiments performed in triplicate ± S.E.M **3B)**: stability of the CCR2-YFP/β-arrestin 2-RLuc signal over time. After 10 minutes of incubation with 100 nM of the indicated ligand, plates were shifted at 37°C, and BRET measured after the indicated time. Data are means of 4 (CCL2 and CCL8) or 3 (CCL7 and CCL13) independent experiments performed in duplicate ± S.E.M. The decrease of the signal induced by CCL7 fitted one-phase exponential decay ($R^2 = 0.76$, with a half-life of 12 minutes), whereas the decay curves of the other ligands did not. **3C):** Remaining signal after 60 minutes of incubation expressed as percentage of the respective maximal signal. The strong decrease of the signal induced by CCL7 was significantly different from all other ligands ($p < 0.05$ against CCL2, and $p < 0.01$ against CCL8 and CCL13, by repeated measures ANOVA with Tukey's multiple comparison test). Similar results as in 2D-F were obtained in pilot experiments with Rluc-β-arrestin 1 (data not shown).

Figure 4: CCR2 internalization induced by different CCR2 ligands. HEK293-CCR2B cells were incubated with 100nM of the indicated ligand at 37°C, and aliquots removed on ice at the indicated times. Surface-bound ligand was removed by acid washing, and remaining surface CCR2 measured by flow cytometry. CCL2: black squares, CCL8: black

circles, CCL7: black diamonds, CCL13: open circles. Data are means of 7 independent

experiments ± S.E.M, and curve fitting follows a one-phase exponential decay (R^2 0.66-

0.8). The differences of the plateaus are significant between CCL2 and CCL8 ($p < 0.01$)

and CCL13 ($p < 0.05$), and between CCL7 and CCL8 ($p < 0.05$).

Figure 5: **BRET CCR2-YFP with different $G\alpha_{i1}$-RLuc constructs, and modulation of
the BRET signal by chemokines. 5A)**: Dose-response experiments with CCL2 showing

the responsiveness of the different $G\alpha_i1$-RLuc constructs. Filled squares: $G\alpha_i1$-RLuc-122,

filled circles: $G\alpha_i1$-RLuc91, open circles: $G\alpha_i1$-RLuc60. Data are means of 3 independent

experiments conducted in triplicate ± S.E.M. **5B) and 5C)**: BRET CCR2-YFP with $G\alpha_i1$-

RLuc-122 (5B) or $G\alpha_i1$-RLuc91 (5C). CCL2: black squares, CCL8: black circles, CCL7:

black diamonds, CCL13: open circles, CCL11: open diamonds. Data are means of 3

independent experiments conducted in triplicate ± S.E.M. For curve fitting parameters, see

Table 1. **5D) and 5E)**: maximal BRET change induced by CCR2 ligands between CCR2-

YFP and $G\alpha_i1$-RLuc-122 (5D) and $G\alpha_i1$-RLuc91 (5E). 4D: differences between all

columns are statistically significant. 4E: only the differences between CCL2 and CCL8, as

well as CCL7 and CCL8 reach significance (repeated measures ANOVA with Tukey's

multiple comparison test). **5F):** Inhibition of forskolin-induced cAMP production by CCL2

and CCL8. To determine if the different maxima observed in Figures 2D and 2E

corresponded indeed to different maxima in adenylate cyclase inhibition, forskolin-induced

cAMP levels were measured in the presence of indicated concentrations of CCL2 (black

squares) or CCL8 (open circles). The black triangle indicates the response to forskoline in

the absence of chemokine. cAMP levels were determined using a previously described

Epac cAMP BRET sensor (Jiang et al., 2007; Leduc et al., 2009). Pooled data of two independent experiments performed in triplicate ± S.E.M. are shown. Maximal inhibition of cAMP production is 63.5 ± 1.3% for CCL2 and 40.2 ± 2.1% for CCL8. Upon simultaneous curve fitting, curve maxima of CCL2 and CCL8 were significantly different ($p < 0.001$, for each experiment separately as well as across the two experiments).

Figure 6: Sensitivity to pertussis toxin (PTX) of Gαi and arrestin responses. HEK293 cells coexpressing either CCR2 and the Epac BRET sensor, or CCR2-YFP and β-arrestin 2-RLuc, were incubated overnight with 100 ng/ml PTX. The results represent pooled data of four independent parallel experiment conducted in triplicate ± S.E.M. **6A):** Percent of the remaining forskolin-induced cAMP production in the presence of 100 nM of the indicated chemokine is shown, in the absence (black bars) and presence (grey bars) of PTX. The cAMP production in the absence of chemokines or PTX was set as 100%. **6B):** β-arrestin 2 recruitment to CCR2 by the ligands in the absence (black bars) and presence (grey bars) of PTX. Significant inhibition of the β-arrestin 2 response was observed for all chemokines ($p < 0.05$) except CCL8. The remaining β-arrestin 2 response was 82.7 ± 5.2% for CCL2, 55.6± 8.8% for CCL7, and 40.4± 6.7 % for CCL13, and the difference between remaining response to CCL2 and CCL7 and CCL2 and CCL13 was significant (paired t test, $p < 0.01$ and < 0.05, respectively).

Figure 7: Dose-response experiments of CCR2 homodimer BRET using different CCR2 ligands. HEK293 cells transiently coexpressing CCR2-YFP and CCR2-RLuc were incubated with indicated concentrations of ligand and resulting BRET measured after 10

minutes at room temperature. CCL2: black squares, CCL7: black diamonds, CCL13: open

circles. CCL8 and CCL11 did not yield BRET changes (not shown). Data are means of 3-5

independent experiments performed in triplicate ± S.E.M. maximally achieved BRET

changes are significantly different between CCL2 and CCL8 and CCL13, respectively. For

curve fitting parameters, see Table 1.

Table 1: Summary of the fitted curve parameters

Summary of the curve fitting parameters of the experiments shown in Figures 1 (radioligand displacement), 2 (arrestin recruitment), 5 (G-protein activation) and 7 (CCR2 homodimer BRET). pIC50 (radioligand displacement) and pEC50 (arrestin recruitment, G-protein activation, and homodimer BRET) values are given. Maximal responses are also given, in absolute (arrestin recruitment, homodimer BRET) or relative (G-protein activation) units. Some values are theoretical, since the experimental curves did not reach saturation, and are indicated by an asterisk (*). Errors are standard error of the mean (SEM). ND = not determined.

Table 1 (continued)

	CCL2	CCL8	CCL7	CCL13	CCL11
			Ligand		
^{125}I-CCL2 displacement					
n	*4*	*4*	*4*	*4*	*4*
IC_{50} (nM)	0.22	4.17	9.69	5.22	11.12
$pIC_{50} \pm SEM$	-9.66 ± 0.09	-8.38 ± 0.05	-8.01 ± 0.07	-8.28 ± 0.08	-7.95 ± 0.08
β-arrestin 2-RLuc					
n	5	5	5	5	*3*
EC_{50} (nM)	1.32	54.25	14.57	141.1 *	ND
$pEC_{50} \pm SEM$	-8.88 ± 0.06	-7.27 ± 0.18	-7.84 ± 0.09	-6.85 ± 0.09 *	ND
$BRET_{max} \pm SEM$	0.228 ± 0.005	0.063 ± 0.005	0.177 ± 0.007	0.185 ± 0.01 *	ND
RLuc-β-arrestin 1					
n	*3*	*3*	*3*	*3*	*ND*
EC_{50} (nM)	1.68	31.02	42.41	1102 *	ND
$pEC_{50} \pm SEM$	-8.72 ± 0.07	-7.51 ± 0.50	-7.37 ± 0.13	-5.96 ± 0.12*	ND
$BRET_{max} \pm SEM$	0.143 ± 0.004	0.010 ± 0.002	0.085 ± 0.005	0.075 ± 0.01 *	ND
Gα$_{i1}$-122-RLuc					
n	*3*	*3*	*3*	*3*	*3*
EC_{50} (nM)	0.86	8.74	4.40	29.76	1.3
$pEC_{50} \pm SEM$	-9.07 ± 0.10	-8.06 ± 0.25	-8.36 ± 0.12	-7.53 ± 0.20	8.88 ± 0.30
$E_{max} \pm SEM$ (%)	56 ± 3	24 ± 3	47 ± 3	37 ± 1	13 ± 1
Gα$_{i1}$-91-RLuc					
n	*3*	*3*	*3*	*3*	*3*
EC_{50} (nM)	0.51	19.3	3.60	54.8	ND
$pEC_{50} \pm SEM$	-9.30 ± 0.13	-7.71 ± 0.21	-8.44 ± 0.15	-7.25 ± 0.19	ND
$E_{max} \pm SEM$ (%)	59 ± 2	32 ± 5	52 ± 5	49 ± 3	ND
Homodimer BRET					
n	*3*	*5*	*3*	*4*	*ND*
EC_{50} (nM)	6.39	ND	23.6	181.2*	ND
$pEC_{50} \pm SEM$	-8.20 ± 0.13	ND	-7.62 ± 0.22	-6.74 ± 0.42*	ND
$BRET_{max} \pm SEM$	0.10 ± 0.002	ND	0.087 ± 0.002	0.080 ± 0.004*	ND

Table 2: Intrinsic relative activity (RAi) and relative efficacies of the partial agonist CCR2 ligands

The intrinsic relative activity (RA_i) was estimated, using the formula $E_{max\_B}EC_{50\_A}/E_{max\_A}EC_{50\_B}$ (equation 25 in (Ehlert, 2005) and equation 17 in (Ehlert, 2008)). Using the pIC50 determined by heterologous displacement of ^{125}I-CCL2 (Figure 1, Table 1) as an independent measure of ligand affinity, relative efficacies were also determined (using the formula $K_BE_{max\_B}EC_{50\_A}/K_AE_{max\_A}EC_{50\_B}$, equation 19 in (Ehlert, 2008)). For the analysis of $G\alpha_{i1}$- signaling, the data obtained with $G\alpha_{i1}$-122-RLuc have been used.

	Ligand		
	CCL8	CCL7	CCL13
β-arrestin 2			
RA_i	0.34	0.88	1.05
relative efficacy	0.29	0.73	0.90
β-arrestin 1			
RA_i	0.08	0.70	0.77
relative efficacy	0.07	0.58	0.66
$G\alpha_{i1}$			
RA_i	0.48	0.91	0.80
relative efficacy	0.42	0.76	0.68

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

B

C

Figure 5

D

E

F

Figure 6

Figure 7