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Different Effects of the Different Natural CC Chemokine Receptor 2b Ligands on β-Arrestin Recruitment, $G\alpha_i$ Signaling, and Receptor Internalization

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ABSTRACT

The chemokine receptor CCR2, which has been implicated in a variety of inflammatory, autoimmune, 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 technology. Compared with 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 $G\alpha_{i1}$ 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: although CCL7-induced arrestin recruitment had a half-life of less than 15 min, 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-ligand 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. At first, different chemokines binding the same receptor were referred to as functionally redundant, although differences between their effects in vivo were recognized early on. Pharmacologic mechanisms that may in part account for these differences are difficult to assess on primary cells and in in vivo systems because of 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 proteoglycan binding, protease processing, and oligomerization of chemokines as well as chemokine receptors, adding several layers of complexity. However, the description of the nonredundant pharmacology of ligands to a given receptor in defined in vitro systems and 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 because it has been implicated 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

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ABBREVIATIONS: CCR, CC chemokine receptor; CCL, CC chemokine ligand; MCP, monocyte chemotactic protein; GPCR, G-protein-coupled receptor; BRET, bioluminescence resonance energy transfer; HEK, human embryonic kidney; PTX, pertussis toxin; YFP, yellow fluorescent protein; RLuc, *Renilla reniformis* luciferase; ANOVA, analysis of variance.

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, 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 extracellular signal-regulated kinase 1/2 and Akt as well as intracellular calcium signaling, have been reported.

Surprisingly, β-arrestin recruitment to CCR2 by its ligands has so far received 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) signaling 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 signaling, functioning as a scaffold protein with links to a confounding number of downstream pathways (Kendall and Luttrell, 2009). Moreover, it seems 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 signaling hub in its own right. The advent of quantitative proximity assays using bioluminescence resonance energy transfer (BRET) technology to measure β-arrestin recruitment, traditionally detected by microscopy through cytosol-to-plasma membrane translocation, permits us to quantify the pharmacological parameters of this signaling axis.

We here set out to investigate the recruitment of β-arrestin to CCR2 induced by its different natural ligands. We also investigate $G\alpha_i$ signaling 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 inhibited only in part 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) was from Biotinium (Hayward, CA). Pertussis toxin (PTX) was from Calbiochem (San Diego, CA), forskolin was from Sigma (St. Louis, MO), and the anti-CCR2 monoclonal antibody (clone 48607) directly coupled to phycoerythrin was from R&D Systems (Minneapolis, MN).

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 from Michel Bouvier (Université de Montréal, Montréal, QC, Canada).

Cell Culture and Transfection. HEK293 cells (passage number, ~10–30; Invitrogen, Carlsbad, CA) were maintained in Dulbecco's modified Eagle's medium, 1% penicillin-streptomycin (Invitrogen), and 10% fetal bovine serum (Wisent, Montreal, QC, Canada). 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 with the use of Quanti-Brite standardization beads (BD Biosciences) and typically reached approximately 2 to 4×10^3 antibody binding sites per cell.

Binding Studies. Cells were transiently transfected with 1 μg of unfused CCR2b cDNA or 1 μg of empty vector per well. Twenty-four hours after transfection the medium was removed, the cells washed twice with PBS, resuspended in binding buffer (50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 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 h using 60 pM ^{125}I-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-bottomed microplates (View Plate; PerkinElmer Life and Analytical Sciences) pretreated with 0.1% poly-D-lysine (Sigma) 24 h after transfection and left in culture for another 24 h. Forty-eight hours after transfection the culture medium was replaced by BRET buffer (PBS, 0.1% bovine serum albumin, and 0.5 mM $MgCl_2$) at room temperature. Coelenterazine h or coelenterazine 400A was added at the final concentration of 5 μM 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 cotransfected -eYFP and -Rluc fusion protein, as described previously (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). In brief, the BRET signal was measured in cells cotransfected with 1.5 μg of CCR2-eYFP and 0.02 μg of β-arrestin 2-Rluc or 0.003 μg of Rluc-β-arrestin 1, completed to 2 μg per well with empty vector. The β-arrestin recruitment kinetics was assessed by adding the ligands 10 min after the addition of coelenterazine h. The β-arrestin decay kinetics were assessed by incubating 10 min RT 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). In brief, 1 μg of unfused CCR2b cDNA and 0.03 μg of the Epac reporter were transiently cotransfected. Cells were stimulated with ligands in the presence of 20 μM forskolin at room temperature for 10 min, immediately after the addition of coelenterazine 400A. For experiments with PTX, the cells were treated for 16 h

with 100 ng/ml PTX at 37°C before stimulation with forskolin and chemokines.

Endocytosis and Flow Cytometry. CCR2b-expressing HEK293 cells were incubated with 100 nM concentrations 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, and 150 mM NaCl), and the cells were subsequently washed three times with ice-cold PBS. Labeling with phycoerythrin-conjugated monoclonal anti-CCR2 antibody was done for 30 min on ice, followed by three PBS washes. CCR2 was quantified by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). Surface expression of CCR2 after ligand incubation at 37°C was expressed as percentage of CCR2 expression compared with a sample drawn before addition of the ligands.

Data Analysis. Data obtained were analyzed using Prism 4.0 software (GraphPad Software, San Diego, CA). 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} values were determined with the Cheng-Prusoff equation using Prism. 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 $E_{max\_B}EC_{50\_A}/E_{max\_A}EC_{50\_B}$ [eq. 17 in (Ehlert, 2008)], and relative efficacies were estimated using the formula $K_BE_{max\_B}EC_{50\_A}/K_AE_{max\_A}EC_{50\_B}$ [eq. 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 (Fig. 1). The affinity of CCL2 was in the 0.2 nM range, as expected (Myers et al., 1995), whereas CCL8, CCL7, CCL13, and CCL11 displaced ^{125}I-CCL2 with IC_{50} values 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. It is noteworthy that although we performed heterologous displacement assays, the observed IC_{50} values 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. (2001) but at odds with that found by Ogilvie et al. (2001) (in the micromolar range). Moreover, we find high IC_{50} values for CCL24 and CCL26 (over 850 and 700 nM, respectively). Whereas Ogilvie et al. (2003) 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, Parody and Stone (2004) report IC_{50} values against the same radioligand of 3 nM for CCL26 and 580 nM for CCL24 on CCR2-transfected COS-7 cells. 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 (data not shown).

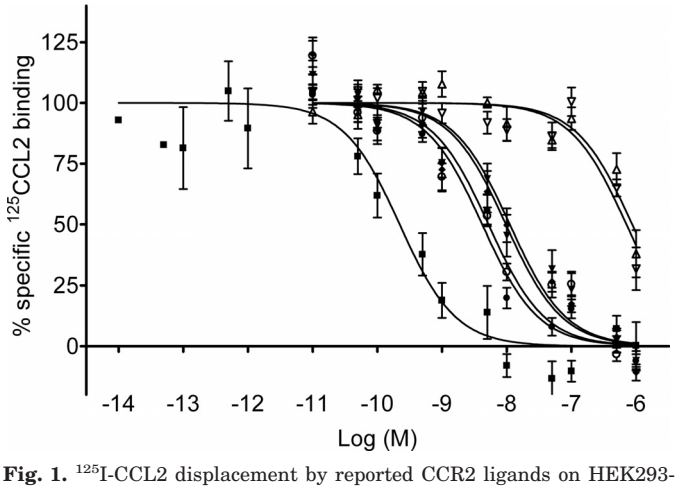

Fig. 1. ^{125}I-CCL2 displacement by reported CCR2 ligands on HEK293-CCR2B cells. ■, CCL2; ♦, CCL7; ●, CCL8; ▲, CCL11; ○, CCL13; open pyramids, CCL24; △, CCL26. Specific binding was defined as that displaced by 1 μM CCL2. All points are pooled data of four (CCL2, CCL8, CCL7, CCL13, and CCL11) or three (CCL24 and CCL26) independent experiments performed in duplicate ± S.E.M. For IC_{50} values, see Table 1. logIC_{50} for CCL24 was −6.06 ± 0.06 and, for CCL26, −6.15 ± 0.07.

***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) (Fig. 2A, Table 1). Similar observations were made using RLuc-β-arrestin 1 (Fig. 2B, Table 1), with the exception of CCL13, which showed exceedingly low potency in recruiting β-arrestin 1. CCL11 did not induce β-arrestin 2 recruitment. The efficacies of the BRET signals were also different among the chemokines (Fig. 2C), with a rank order of CCL2 > CCL7/CCL13 > CCL8. Therefore, although 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 β-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. A 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, compared with 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 (data not shown). To 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. Whereas CCL2 still reached 50% of the maximal response in less than 3 min, the response to CCL13 was considerably slower (Fig. 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, although 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 min 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 Fig. 3B, marked differences were seen between the chemokines. Although 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 half-life of less than 15 min. The CCL2 signal was somewhat more stable, with a half-life of >20 min. The CCL13-elicited signal was clearly biphasic, showing a transient increase during the first 10 min 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 with 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 an effect on receptor endocytosis, because 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 (García 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 Fig. 4, more than 50% of the surface CCR2 was

TABLE 1

Summary of the fitted curve parameters

Summary of the curve-fitting parameters of the experiments shown in Figs. 1 (radioligand displacement), 2 (arrestin recruitment), 5 (G-protein activation), and 7 (CCR2 homodimer BRET). pIC_{50} (radioligand displacement) and pEC_{50} (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. Errors are presented as S.E.M.

	Ligand				
	CCL2	CCL8	CCL7	CCL13	CCL11
^{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} ± S.E.M.	−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[a]	N.D.
pEC_{50} ± S.E.M.	−8.88 ± 0.06	−7.27 ± 0.18	−7.84 ± 0.09	−6.85 ± 0.09[a]	N.D.
$BRET_{max}$ ± S.E.M.	0.228 ± 0.005	0.063 ± 0.005	0.177 ± 0.007	0.185 ± 0.01[a]	N.D.
RLuc β-arrestin 1					
N	3	3	3	3	N.D.
EC_{50} (nM)	1.68	31.02	42.41	1102[a]	N.D.
pEC_{50} ± S.E.M.	−8.72 ± 0.07	−7.51 ± 0.50	−7.37 ± 0.13	−5.96 ± 0.12[a]	N.D.
$BRET_{max}$ ± S.E.M.	0.143 ± 0.004	0.010 ± 0.002	0.085 ± 0.005	0.075 ± 0.01[a]	N.D.
$G\alpha_{i1}$-122-RLuc					
N	3	3	3	3	3
EC_{50} (nM)	0.86	8.74	4.40	29.76	1.3
pEC_{50} ± S.E.M.	−9.07 ± 0.10	−8.06 ± 0.25	−8.36 ± 0.12	−7.53 ± 0.20	8.88 ± 0.30
E_{max} ± S.E.M. (%)	56 ± 3	24 ± 3	47 ± 3	37 ± 1	13 ± 1
$G\alpha_{i1}$-91-RLuc					
N	3	3	3	3	3
EC_{50} (nM)	0.51	19.3	3.60	54.8	N.D.
pEC_{50} ± S.E.M.	−9.30 ± 0.13	−7.71 ± 0.21	−8.44 ± 0.15	−7.25 ± 0.19	N.D.
E_{max} ± S.E.M. (%)	59 ± 2	32 ± 5	52 ± 5	49 ± 3	N.D.
Homodimer BRET					
N	3	5	3	4	N.D.
EC_{50} (nM)	6.39	N.D.	23.6	181.2[a]	N.D.
pEC_{50} ± S.E.M.	−8.20 ± 0.13	N.D.	−7.62 ± 0.22	−6.74 ± 0.42[a]	N.D.
$BRET_{max}$ ± S.E.M.	0.10 ± 0.002	N.D.	0.087 ± 0.002	0.080 ± 0.004[a]	N.D.

N.D., not determined.

[a] Theoretical value; experimental curves did not reach saturation.

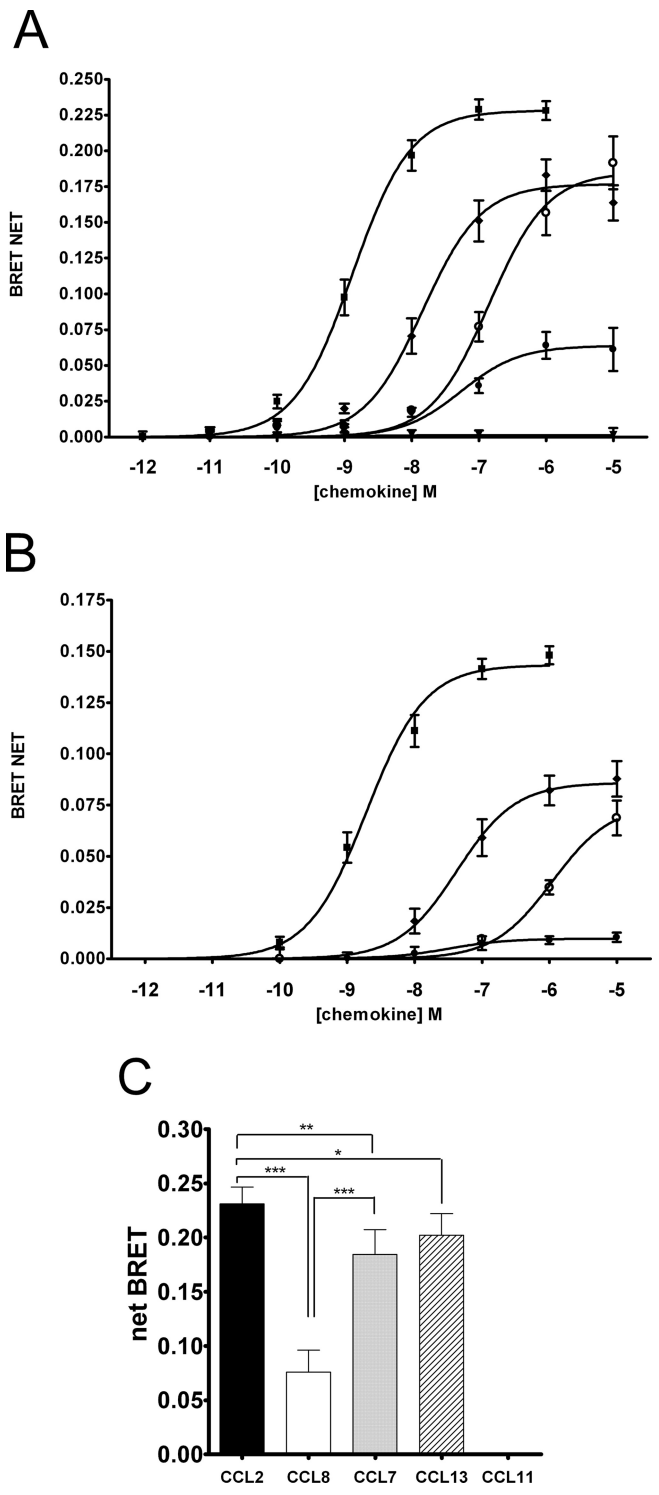

Fig. 2. Dose-response experiments of arrestin recruitment to CCR2 using different CCR2 ligands. HEK293 cells transiently coexpressing CCR2-YFP and β-arrestin 2-Rluc or Rluc-β-arrestin 1 were incubated with indicated concentrations of ligand and resulting BRET measured after 10 min at room temperature. ■, CCL2; ●, CCL8; ♦, CCL7; ○, CCL13; ▲, CCL11. A, β-arrestin 2-Rluc; B, Rluc-β-arrestin 1. Data are means of five (β-arrestin 2-RLuc) or three (Rluc-β-arrestin 1) independent experiments performed in triplicate ± 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.

internalized during the first 10 min 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 plateaus reached after 60 min of incubation with the chemokine are significantly different in 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 $G\alpha_i$ via CCR2 by Its Natural Ligands. Given that the different chemokines showed different intrinsic properties in β-arrestin signaling, we sought to determine whether this might reflect signaling events upstream of β-arrestin recruitment. To investigate the potential correlations between β-arrestin recruitment and G-protein signaling in CCR2, we measured CCR2-$G\alpha_{i1}$ signaling, taking advantage of a previously described BRET-based assay (Galés et al., 2005, 2006). This system uses the receptor fused to YFP in combination with $G\alpha_{i1}$-RLuc constructs, where the BRET donor is fused at different permissive locations within the $G\alpha_{i1}$ amino acid sequence ($G\alpha_{i1}$-RLuc-60, $G\alpha_{i1}$-RLuc-91, and $G\alpha_{i1}$-RLuc-122). Using this system, Galés et al. (2005) demonstrated the existence of GPCRs in preformed signaling complexes containing the receptor and heterotrimeric G-proteins. Moreover, they were able to detect ligand-induced BRET changes between various GPCRs and the $G\alpha_i$ subunit, indicating conformational changes within preformed signaling complexes that correlate with $G\alpha_i$ activation.

Using this system, we detected specific basal BRET signals between $G\alpha_{i1}$-RLuc fusions and CCR2-YFP, in line with the existence of preformed and persistent complexes, as has been described for a number of seven-transmembrane receptors (Dupré et al., 2006). Overall, $G\alpha_{i1}$-RLuc122 yielded the strongest signal, but similar albeit weaker signals were also obtained with the $G\alpha_{i1}$-RLuc91 fusion, whereas $G\alpha_{i1}$-RLuc60 only yielded weak BRET (Fig. 5A). Incubation with agonists led to strong decreases in BRET (>50%), regardless of whether $G\alpha_{i1}$-RLuc122 or $G\alpha_{i1}$-RLuc91 were used. This is different from observations made in the $\alpha 2_A$-adrenergic receptor system, where BRET increases upon stimulation with $G\alpha_{i1}$-RLuc91 but decreases with $G\alpha_{i1}$-RLuc122 (Galés et al., 2006). It is noteworthy that the amplitude of ligand-induced BRET changes that we observe is much stronger than that reported with the $\alpha 2_A$-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 (Fig. 5, A and B). In general, there was good agreement between the potencies measured with $G\alpha_{i1}$-RLuc91 and $G\alpha_{i1}$-RLuc122, whereas the different efficacies reported by $G\alpha_{i1}$-RLuc91 failed to reach statistical significance as a result of 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 $G\alpha_{i1}$-RLuc122 only)], whereas the efficacy ranking was again different from potency ranking as a result of strikingly low efficacy of CCL8 (CCL2 > CCL7 > CCL13 > CCL8; Fig. 5, D and E). Surpris-

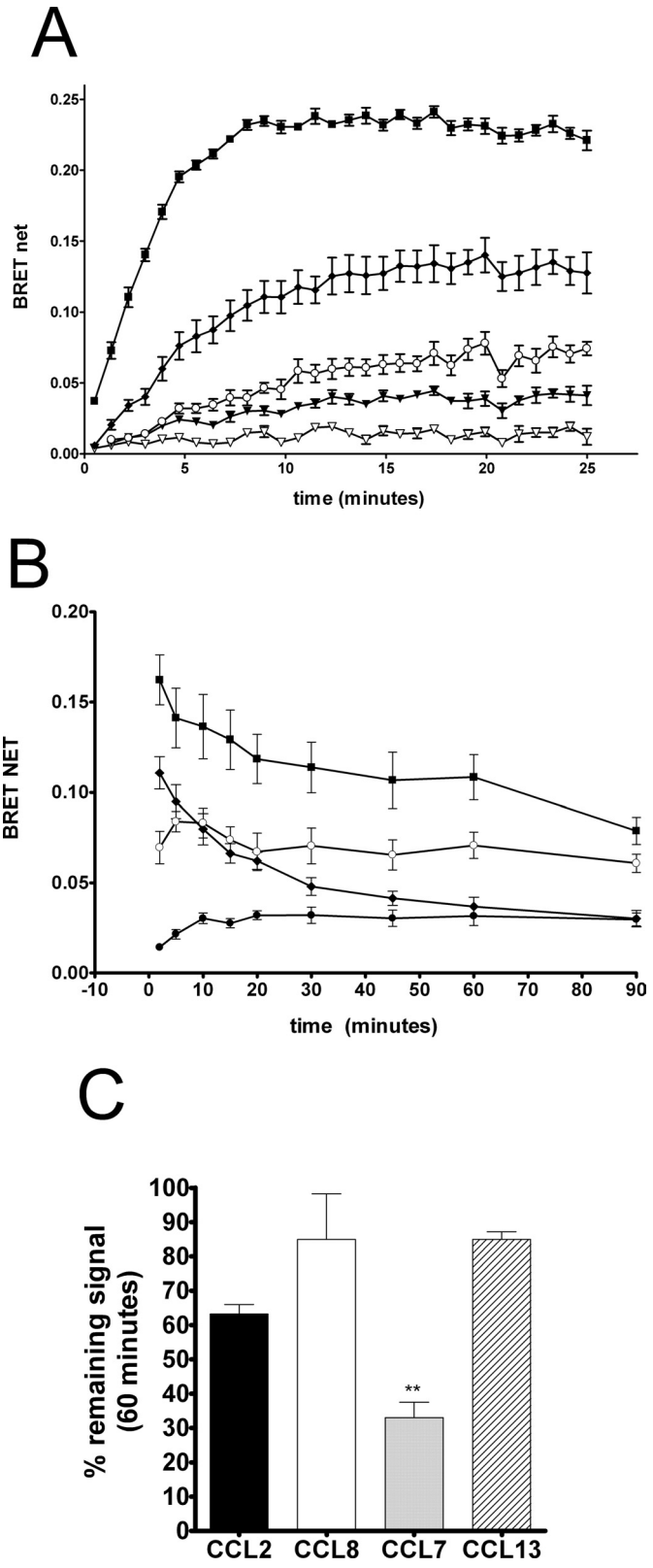

Fig. 3. Time-course experiments of β-arrestin-2 recruitment to CCR2 using different CCR2 ligands. ■, CCL2; ●, CCL8; ◆, CCL7; ○, CCL13; △, no ligand. A, time course of β-arrestin 2 recruitment. HEK293 cells transiently coexpressing CCR2-YFP and β-arrestin 2-Rluc were incubated with 100 nM concentrations of the indicated ligand, and resulting BRET was measured after 10 min at room temperature. Data are means of five independent experiments performed in triplicate ± S.E.M. B, stability of the CCR2-YFP/β-arrestin 2-RLuc signal over time. After 10 min of incubation, with 100 nM concentrations of the indicated ligand, plates were shifted at 37°C, and BRET was measured after the indicated time. Data are means of four (CCL2 and CCL8) or three (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 min), whereas the decay curves of the other ligands did not. C, remaining signal after 60 min 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 Fig. 2, D to F, were obtained in pilot experiments with Rluc-β-arrestin 1 (data not shown).

ingly, using the $G\alpha_{i1}$-RLuc122 sensor, CCL11 did yield weak but significant BRET changes, which, while they did not exceed 10 to 15% of BRET reduction, were observed with high potency. In addition, we observed again that EC_{50} values were higher than binding competition IC_{50} values for all ligands except CCL7. This might be surprising given that the EC_{50} values 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} values 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.

It is noteworthy that Galés et al. (2005) found that efficacies measured by BRET and cAMP production correlate. 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 Fig. 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. (2005) and 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 $G\alpha_{i1}$ signaling 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 PTX treatment. Indeed, if dependent on $G\alpha_i$ signaling, 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 Fig. 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 observed with forskolin alone. 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 seven-transmembrane receptors. Alternatively, the excess cAMP inhibition by PTX might result from inhibition of the basal activity of endogenous $G\alpha_i$ coupling GPCRs.

However, when evaluating β-arrestin 2 recruitment to CCR2, we found that recruitment induced by CCL2 was largely resistant to PTX (Fig. 6B). This reinforces the conclusion that CCL2-induced endocytosis is arrestin-dependent, as CCL2-induced endocytosis was also reported to be PTX-resistant (García 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, at present, we cannot 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 BRET changes yielded by the different CCR2 ligands in dose-response experiments.

As depicted in Fig. 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} values 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 (data 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 more than 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 signaling processes that affect receptor conformation. We envision that these events reflect rearrangements in the conformation and/or composition of receptor-associated signaling 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 $G\alpha_{i1}$ and β-arrestin and that this includes the observed unique match between IC_{50} and EC_{50} for CCL7. Although 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, as-yet-unidentified events, related or unrelated to these readouts.

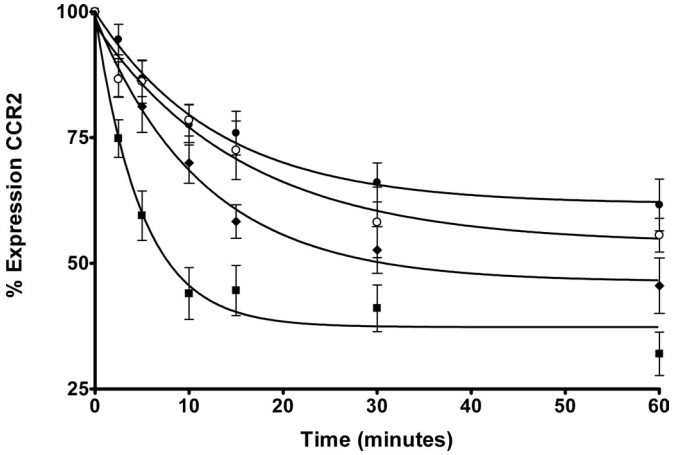

Fig. 4. CCR2 internalization induced by different CCR2 ligands. HEK293-CCR2B cells were incubated with 100 nM concentrations of the indicated ligand at 37°C, and aliquots removed on ice at the indicated times. Surface-bound ligand was removed by acid washing, and remaining surface CCR2 was measured by flow cytometry. ■, CCL2; ●, CCL8; ◆, CCL7; ○, CCL13. Data are means of seven 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 ($p < 0.05$).

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; García 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 toward 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. Although CCL7-induced β-arrestin recruitment had the shortest half-life (<15 min), CCL2-induced responses were longer lived, and CCL8 and CCL13 yielded β-arrestin recruitment that remained stable over 90 min. Our findings with CCL2 are in agreement with those

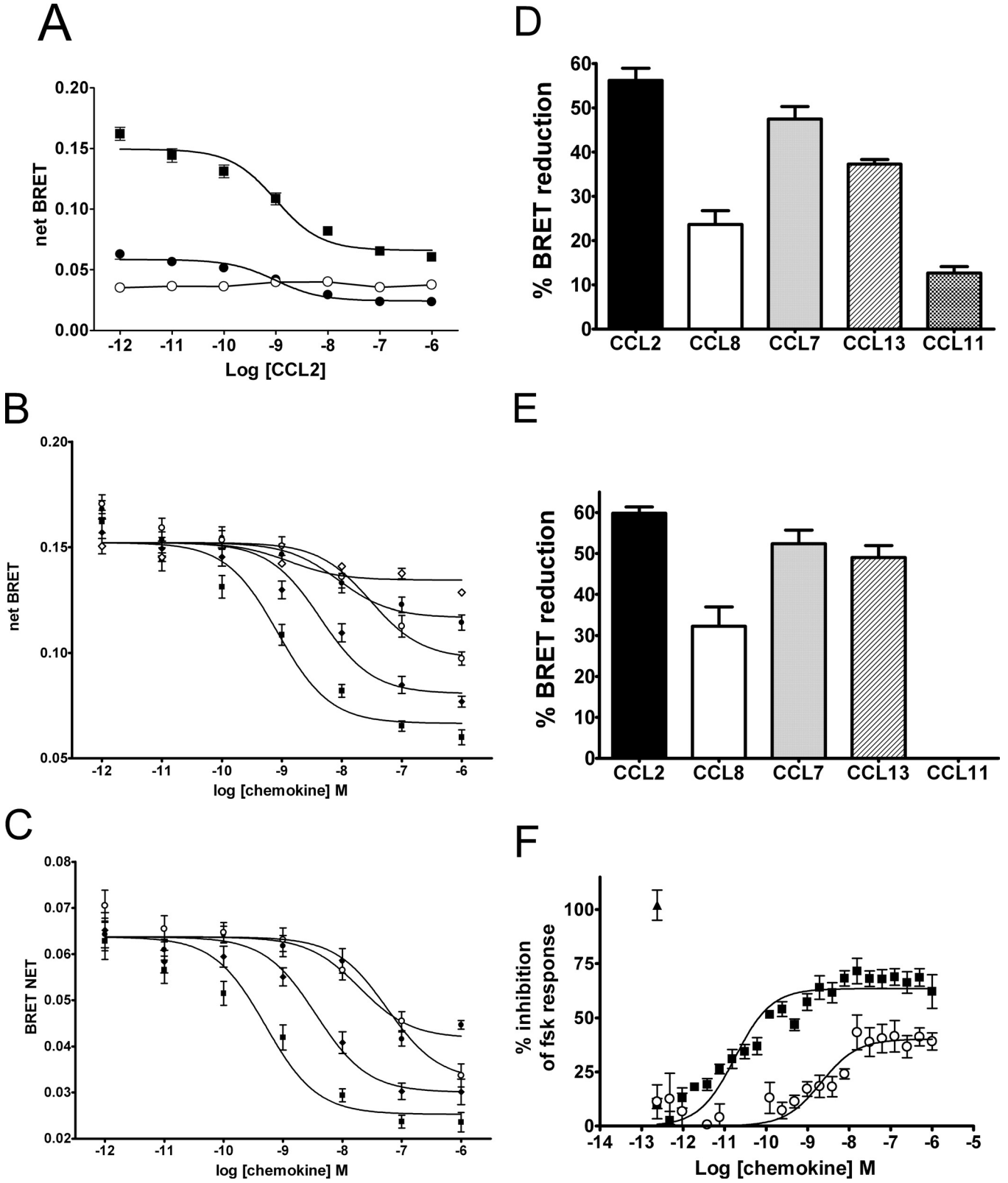

Fig. 5. BRET CCR2-YFP with different $G\alpha_{i1}$-RLuc constructs and modulation of the BRET signal by chemokines. A, dose-response experiments with CCL2 showing the responsiveness of the different $G\alpha_{i1}$-RLuc constructs. ■, $G\alpha_{i1}$-RLuc-122; ●, $G\alpha_{i1}$-RLuc91; ○, $G\alpha_{i1}$-RLuc60. Data are means of three independent experiments conducted in triplicate ± S.E.M. B and C, BRET CCR2-YFP with $G\alpha_{i1}$-RLuc-122 (B) or $G\alpha_{i1}$-RLuc91 (C). ■, CCL2; ●, CCL8; ◆, CCL7; ○, CCL13; ◇, CCL11. Data are means of three independent experiments conducted in triplicate ± S.E.M. For curve-fitting parameters, see Table 1. D and E, maximal BRET change induced by CCR2 ligands between CCR2-YFP and $G\alpha_{i1}$-RLuc-122 (D) and $G\alpha_{i1}$-RLuc91 (E). D, differences between all columns are statistically significant. E, only the differences between CCL2 and CCL8, as well as CCL7 and CCL8 reach significance (repeated measures ANOVA with Tukey's multiple comparison test). F, inhibition of forskolin-induced cAMP production by CCL2 and CCL8. To determine whether the different maxima observed in Fig. 2, D and E indeed corresponded to different maxima in adenylate cyclase inhibition,

reported for this chemokine (Aragay et al., 1998; García Lopez et al., 2009). García Lopez et al. (2009) 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. It is noteworthy that 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 (García Lopez et al., 2009), supports this conclusion. Although 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 signaling pathways (Kohout et al., 2004; Cheung et al., 2009). In this context, the observation that CCR2 chemokines induce distinct kinase signaling 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 $G\alpha_i$ 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 is due to the activation of alternative Gα subunits. Indeed, CCR2 can couple to different Gα subunits; so far, coupling of CCR2 to Gαq, Gα14, and 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 sensitive in part to cholera toxin, 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 with respect to CCL2 (Table 2). Using the pIC_{50} determined by ^{125}I-CCL2 displacement (Fig. 1, Table 1) as an independent measure of ligand affinity, relative efficacies were also determined (Table 2). Although 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 signaling 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 Figs. 4 and 5F).

It is noteworthy that 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 signaling molecules. CCR2 is mainly expressed at relatively low levels on monocytes [3–4 × 10^3 molecules per cell (Mine et al., 2006)] but can be drastically increased on natural killer cells (Hanna et al., 2005) and T cells (Amoura et al., 2003) in disease. Here we used transfected HEK293 cells expressing approximately 2 × 10^3 receptors per cell as determined with fluorescent standardization beads (data not shown). It is attractive to envision that the partial agonism of CCR2 ligands differentially affects different CCR2-expressing cells, because 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).

Although 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 $\alpha 2_A$-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 lead not only to different response efficacies but also to qualitatively different responses in different signaling pathways (Kenakin, 2007; Kobilka and Deupi, 2007). Although we did indeed observe different CCR2 conformations stabilized by the chemokine ligands, this was not due directly to the conformational effects of the sole receptor-ligand interaction but involved also other components of the CCR2 signaling 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 signaling (Combadiere et al., 1995; Martinelli et al., 2001). Functional selectivity thus cannot 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 toward qualitative differences in

forskolin-induced cAMP levels were measured in the presence of indicated concentrations of CCL2 (■) or CCL8 (○). The ▲ indicates the response to forskolin 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).

CCR2 responses to the different chemokines. This conclusion is further supported by the atypical signaling profile of CCL11. CCL11 induced marginal $G\alpha_i$ activation that had eluded previous studies (Martinelli et al., 2001; Ogilvie et al., 2001, 2004). However, CCL11 reportedly activates the mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 via recruitment of a p85/p110 phosphatidylinositol 3-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 not only results from complex in vivo regulatory mechanisms but also is 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.

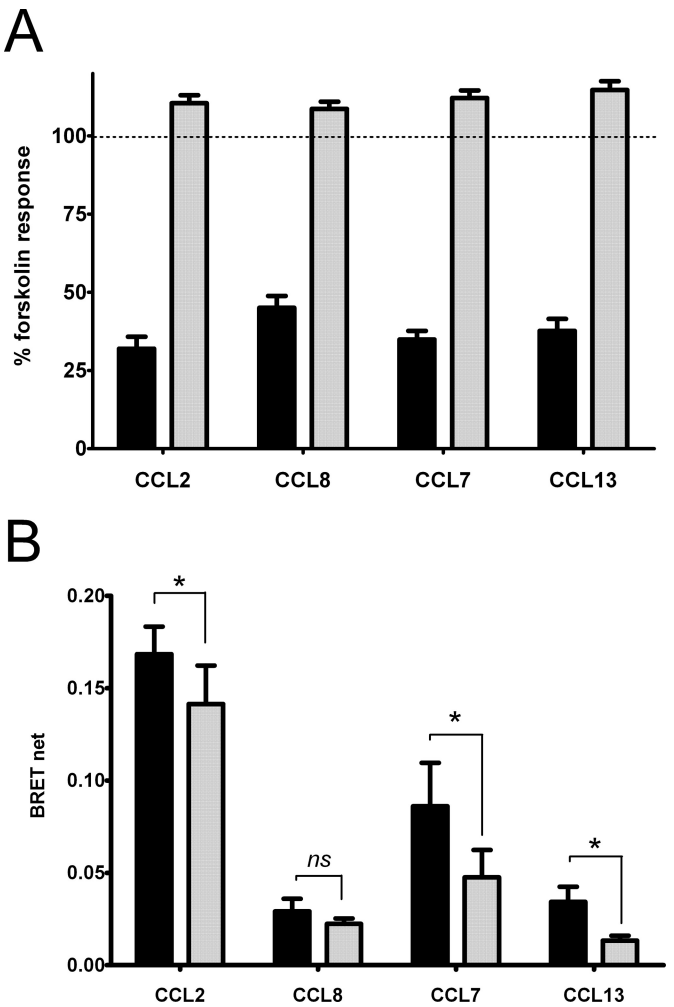

Fig. 6. Sensitivity to PTX of $G\alpha_i$ and arrestin responses. HEK293 cells coexpressing either CCR2 and the Epac BRET sensor or CCR2-YFP and β-arrestin 2-RLu, were incubated overnight with 100 ng/ml PTX. The results represent pooled data of four independent parallel experiment conducted in triplicate ± S.E.M. A, percentage of the remaining forskolin-induced cAMP production in the presence of 100 nM the indicated chemokine is shown, in the absence (black bars) and presence (gray bars) of PTX. The cAMP production in the absence of chemokines or PTX was set as 100%. B, β-Arrestin 2 recruitment to CCR2 by the ligands in the absence (black bars) and presence (gray 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 the decrease of the responses to CCL2 and CCL7, as well as to CCL2 and CCL13, was significant (paired t test, $p < 0.01$ and <0.05, respectively).

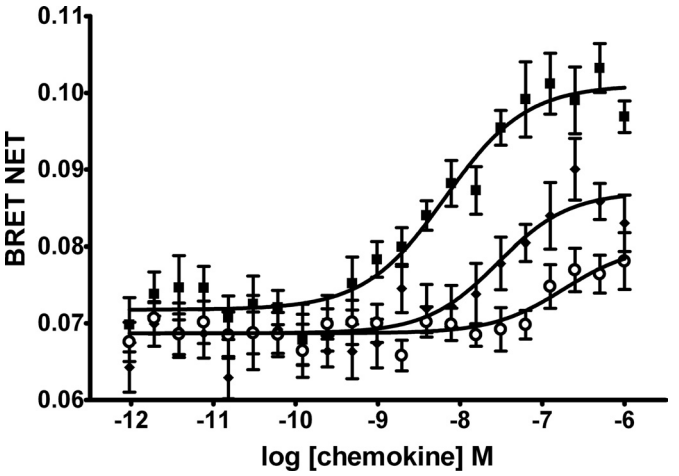

Fig. 7. Dose-response experiments of CCR2 homodimer BRET using different CCR2 ligands. HEK293 cells transiently coexpressing CCR2-YFP and CCR2-RLuc were incubated with indicated concentrations of ligand and resulting BRET measured after 10 min at room temperature. ■, CCL2; ♦, CCL7; ○, CCL13. CCL8 and CCL11 did not yield BRET changes (not shown). Data are means of three to five independent experiments performed in triplicate ± S.E.M. Maximally achieved BRET changes are significantly different among CCL2, CCL8, and CCL13. For curve-fitting parameters, see Table 1.

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 $E_{max\_B}EC_{50\_A}/E_{max\_A}EC_{50\_B}$ [eq. 25 in Ehlert (2005) and eq. 17 in Ehlert (2008)]. Using the pIC_{50} determined by heterologous displacement of ^{125}I-CCL2 (Fig. 1, Table 1) as an independent measure of ligand affinity, relative efficacies were also determined [using the formula $K_BE_{max\_B}EC_{50\_A}/K_AE_{max\_A}EC_{50\_B}$, eq. 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

RAi, intrinsic relative activities.

In sum, although 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.

Acknowledgments

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Authorship Contributions

Participated in research design: Berchiche and Heveker.

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

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

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

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