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Characterization of heteromeric complexes between chemokine (C-X-C motif) receptor 4 and α_1-adrenergic receptors utilizing intermolecular bioluminescence resonance energy transfer assays

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Abstract

Recently, we reported that chemokine (C-X-C motif) receptor 4 (CXCR4) heteromerizes with α_1-adrenergic receptors (AR) on the cell surface of vascular smooth muscle cells, through which the receptors cross-talk. Direct biophysical evidence for CXCR4:α_1-AR heteromers, however, is lacking. Here we utilized bimolecular luminescence/fluorescence complementation (BiLC/BiFC) combined with intermolecular bioluminescence resonance energy transfer (BRET) assays in HEK293T cells to evaluate CXCR4:$\alpha_{1a/b/d}$-AR heteromerization. Atypical chemokine receptor 3 (ACKR3) and metabotropic glutamate receptor 1 ($mGlu_1R$) were utilized as controls. BRET between CXCR4-RLuc (*Renilla reniformis*) and enhanced yellow fluorescent protein (EYFP)-tagged ACKR3 or $\alpha_{1a/b/d}$-ARs fulfilled criteria for constitutive heteromerization. BRET between CXCR4-RLuc and EYFP or $mGlu_1R$-EYFP were nonspecific. $BRET_{50}$ for CXCR4:ACKR3 and CXCR4:$\alpha_{1a/b/d}$-AR heteromers were comparable. Stimulation of cells with phenylephrine increased $BRET_{max}$ of CXCR4:$\alpha_{1a/b/d}$-AR heteromers without affecting $BRET_{50}$; stimulation with CXCL12 reduced $BRET_{max}$ of CXCR4:α_{1a}-AR heteromers, but did not affect $BRET_{50}$ or $BRET_{max/50}$ for CXCR4:$\alpha_{1b/d}$-AR. A peptide analogue of transmembrane domain (TM) 2 of CXCR4 reduced $BRET_{max}$ of CXCR4:$\alpha_{1a/b/d}$-AR heteromers and increased $BRET_{50}$ of CXCR4:$\alpha_{1a/b}$-AR interactions. A TM4 analogue of CXCR4 did not alter BRET. We observed

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CXCR4, α_{1a}-AR and $mGlu_1R$ homodimerization by BiFC/BiLC, and heteromerization of homodimeric CXCR4 with proto- and homodimeric α_{1a}-AR by BiFC/BiLC BRET. BiFC/BiLC BRET for interactions between homodimeric CXCR4 and homodimeric $mGlu_1R$ was nonspecific. Our findings suggest that the heteromerization affinity of CXCR4 for ACKR3 and α_1-ARs is comparable, provide evidence for conformational changes of the receptor complexes upon agonist binding and support the concept that proto- and oligomeric CXCR4 and α_1-ARs constitutively form higher-order hetero-oligomeric receptor clusters.

Keywords

G protein-coupled receptor heteromers; homodimer; hetero-oligomer; stromal cell-derived factor 1α; bimolecular luminescence complementation assay; bimolecular fluorescence complementation assay

Introduction

Seven transmembrane (7TM) receptors, of which most are G protein-coupled receptors (GPCRs), form the largest class of membrane proteins in humans [1,2]. 7TM receptors are essential in human physiology, play major roles in a broad variety of disease processes and are the targets of more than one third of all drugs approved by the Federal Drug Administration [1,2].

Accumulating evidence suggests that many 7TM receptors can form homo- and hetero-oligomers, which is thought to alter their pharmacological behavior and affect receptor function [3–7]. While the function of the prototypical GPCR chemokine (C-X-C motif) receptor 4 (CXCR4) appears to depend on the formation of homo-oligomers (nano-clusters) on the cell surface [8,9], CXCR4 has been reported to heteromerize with several other 7TM receptors, such as chemokine (C-C motif) receptor (CCR)2, CCR5, CXCR3, atypical chemokine receptor (ACKR) 3, chemerin receptor 23, β_2-adrenergic receptor (AR), δ-opioid receptor or cannabinoid receptor 2 [10–17]. Recently, we provided evidence that heteromeric complexes between CXCR4 and α_1-adrenergic receptors (AR) are constitutively expressed in rat and human vascular smooth muscle cells (VSMCs), through which the receptor partners regulate each other [18–21]. In these studies, we employed biochemical methods, such as proximity ligation assays and co-immunoprecipitation experiments, in combination with functional assays to demonstrate heteromerization and functional cross-talk between recombinant and endogenously expressed CXCR4 and α_1-ARs. Biophysical evidence for interactions between CXCR4 and α_1-ARs, however, is lacking. Furthermore, information on the composition and molecular characteristics of such complexes is not available.

In the present study we utilized bimolecular luminescence and fluorescence complementation (BiLC, BiFC) and intermolecular bioluminescence resonance energy transfer (BRET) assays, which are considered a gold standard for the detection of direct physical interactions between GPCRs [22], to evaluate heteromerization between CXCR4 and α_1-ARs. Our findings support the concept that CXCR4 constitutively heteromerizes with α_1-ARs in higher-order hetero-oligomeric complexes comprised of proto- and

oligomeric receptor partners and provide initial information on the molecular behavior of such complexes.

Materials and methods

Reagents –

Phenylephrine was purchased from Sigma-Aldrich and (C-X-C motif) chemokine ligand 12 (CXCL12) from Protein Foundry. The peptide analogues derived from the transmembrane domains 2 (TM2, LLFVITLPFWAVDAVANWYFGN) and 4 (TM4, VYVGVWIPALLLTIPDFIFAN) of CXCR4 were as described previously [18,20]; their sequences and purity were confirmed by mass spectrometry. Coelenterazine H was from Nanolight Technology.

Plasmids –

The coding sequences of CXCR4, ACKR3, α_{1a}-AR, α_{1b}-AR, α_{1d}-AR and $mGlu_1R$ were from Addgene (CXCR4-TANGO, #66262; ACKR3-TANGO, #66265, α_{1a}-AR-TANGO, #66213, α1b-AR-TANGO, #66214, α1d-AR-TANGO, #66215, mGlu1-Tango, #66387). Upper and lower case subscripts are used to denote endogenous and recombinant α_1-ARs, respectively [23]. The coding sequence of Renilla luciferase was from CXCR4-hRLuII, which was generously provided by Dr. Michel Bouvier, PCR amplified and ligated at the C-terminus of CXCR4 at the sites of Age I and Xba I (CXCR4-RLuc). To produce ACKR3-enhanced yellow fluorescent protein (EYFP), α_{1a}-AR-EYFP, α_{1b}-AR-EYFP, α_{1d}-AR-EYFP and $mGlu_1R$-EYFP, the cDNA of EYFP was PCR amplified and ligated in-frame with the receptor genes at the C-termini at the sites of Age I and Xba I, respectively. To construct CXCR4 fused with split luciferase, the coding sequence of Renilla luciferase was PCR amplified into two segments, L1 (N-Luc - AA1–229) and L2 (C-Luc - AA230–311) [24], and inserted in pcDNA3.1-CXCR4 at the sites of Age I and Xba I. To construct CXCR4 fused with split-yellow fluorescent protein Venus (V1, N- Venus - AA1–155; V2, C-Venus - AA156–240), V1 and V2 were PCR amplified from D2R-V1 and D2R-V2 (both from Addgene) with a primer that carries the Age I site and matches D2R-V1 and V2 linker sequences and primer sp6 [24]. The amplicons were fused with CXCR4 in-frame at the C-terminus at the Age I and Xba I sites, respectively. α_{1a}-AR and $mGlu_1R$ fused with split luciferase fragments (L1 or L2), α_{1a}-AR-L1, α_{1a}-AR-L2, $mGlu_1$-L1 and $mGlu_1$-L2, were generated as for CXCR4-L1 and CXCR4-L2. All plasmids were confirmed by sequencing.

Cell culture –

HEK 293T were as described [21] and cultured in high-glucose Dulbecco's Modified Eagle's Medium containing 10 mg/mL sodium pyruvate, 2 mM L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified environment at 37°C, 5% CO_2.

BRET assays –

BRET assays were performed as described previously [25,26]. In brief, HEK293T cells were seeded in 12-well plates and transfected with the plasmids indicated using the Lipofectamine 3000 transfection reagent (ThermoScientific). For BRET titration assays, CXCR4-RLuc at a

fixed amount of 50 ng was transfected alone or with increasing amounts of EYFP, $mGlu_1R$-EYFP, ACKR3-EYFP or $\alpha_{1a/b/d}$-AR-EYFP. For BRET assays at a constant energy donor : acceptor ratio [27], increasing amounts of both CXCR4-RLuc and ACKR3-EYFP or $\alpha_{1a/b/d}$-AR-EYFP were co-transfected at a ratio of 1:10. In all assays, empty vector pcDNA3.1 was added to maintain the total DNA amount for each transfection constant. After an overnight incubation, cells were seeded in poly-L-lysine coated 96-well white plates and incubated again overnight. Cells were then washed with PBS and fluorescence was measured in a Biotek Synergy HT4 plate reader (excitation 485 nm, emission 528 nm). For BRET measurements, coelenterazine H was added at a final concentration of 5 μM. After 10 min incubation at room temperature, luminescence was measured at 460 nm and 528 nm. The BRET signal was calculated as the ratio of the relative luminescence units (RLU) measured at 528 nm over RLU at 460 nm. The net BRET is calculated by subtracting the BRET signal detected when CXCR4-hRLuc was transfected alone. For titration experiments, net BRET ratios are expressed as a function of fluorescence/total luminescence. To test the effects of receptor agonists on BRET, cells were replaced with PBS after 48h of transfection, phenylephrine (final concentration 200 μM) or CXCL12 (final concentration 500 nM) were added and cells were incubated at 37°C for 5 min before the addition of coelenterazine H. To test the effects of TM-derived peptides on BRET, cells were replaced with PBS after 48h of transfection, the TM2 or TM4 peptide were added in a final concentration of 20 μM and cells were incubated at 37°C for 15 min before the addition of coelenterazine H.

Bimolecular luminescence and fluorescence complementation BRET –

For bimolecular luminescence complementation assays (BiLC), CXCR4-L1, α_{1a}-AR-L1 or $mGlu_1R$-L1 at a fixed amount were co-transfected with increasing amounts of CXCR4-L2, α_{1a}-AR-L2 or $mGlu_1R$-L2, respectively. For bimolecular fluorescence complementation (BiFC) assays, CXCR4-V1 at a fixed amount was co-transfected with increasing amounts of CXCR4-V2. After overnight transfection, cells were seeded in poly L-lysine precoated 96 well plates and incubated further overnight before detection of luminescence or fluorescence. For the BiLC BRET assay, CXCR4-L1 and CXCR4-L2 at fixed amounts were co-transfected with increasing amounts of α_{1a}-AR-EYFP. For the BiFC BRET assay, α_{1a}-AR-RLuc or $mGlu_1R$-RLuc at constant amounts were co-transfected with increasing amounts of CXCR4-V1 and CXCR4-V2. For combined BiLC and BiFC BRET, α_{1a}-AR-L1 and α_{1a}-AR-L2 or $mGlu_1R$-L1 and $mGlu_1R$-L2 at constant amounts were transfected with increasing amounts of CXCR4-V1 and CXCR4-V2. BRET was measured as described before.

Data analyses –

Data are expressed as mean ± standard error. Titration curves were analyzed with nonlinear regression analyses. One-way analyses of variance (ANOVA) with Dunnett's multiple comparison post hoc test for multiple comparisons were used to assess statistical significance. A two-tailed $p<0.05$ was considered significant. All analyses were calculated with the GraphPad Prism 8.0.2 software.

Results and Discussion

We performed saturation BRET experiments in cells expressing CXCR4-RLuc and $\alpha_{1a/b/d}$-ARs-EYFP. Saturation BRET experiments in cells expressing CXCR4-RLuc and ACKR3-EYFP were used as a positive control, and in cells expressing CXCR4-hRLuc and EYFP or $mGlu_1R$-EYFP as negative controls [8,11,15,28]. As shown in Fig. 1A/B, the BRET signals between CXCR4-RLuc and ACKR3-EYFP or $\alpha_{1a/b/d}$-AR-EYFP showed hyperbolic progressions with increasing energy acceptor : donor ratios. Consistent with a non-specific bystander BRET signal (open squares), the BRET signal was low and increased linearly with increasing energy acceptor : donor ratios in cells expressing CXCR4-RLuc and EYFP (Fig. 1A) or CXCR4-RLuc and mGluR1-EYFP (Fig. 1B). The BRET signals for interactions between CXCR4 and ACKR3 or $\alpha_{1a/b/d}$-ARs were independent of the concentrations of BRET partners when tested at fixed energy acceptor : donor ratios (Fig. 1C). Thus, the observed BRET between CXCR4-RLuc and ACKR3-EYFP or $\alpha_{1a/b/d}$-AR-EYFP indicate constitutive heteromerization of the receptor partners and support the assumption of direct physical interactions between CXCR4 and α_1-ARs [18–21,27]. Comparison of the $BRET_{50}$ values for each heteromerization pair did not show significant differences ($BRET_{50}$ (EYFP/Lum): CXCR4:ACKR3 – 0.062 ± 0.004; CXCR4:α_{1a}-AR - 0.055 ± 0.011; CXCR4:α_{1b}-AR - 0.072 ± 0.017; CXCR4:α_{1d}-AR - 0.051 ± 0.018; n=4 for all, $p>0.05$), suggesting that CXCR4 has a comparable interaction affinity for ACKR3 and $\alpha_{1a/b/d}$-AR.

Net $BRET_{max}$ (n=4 for all combinations) was significantly higher for CXCR4:ACKR3 (528nm/460nm: 0.080 ± 0.011) and CXCR4:α_{1a}-AR (528nm/460nm: 0.093 ± 0.006), as compared with net $BRET_{max}$ for CXCR4:α_{1b}-AR (528nm/460nm: 0.053 ± 0.004, $p<0.05$ vs. CXCR4:ACKR3 and CXCR4:α_{1a}-AR) and CXCR4:α_{1d}-AR (528nm/460nm: 0.052 ± 0.008, $p<0.05$ vs. CXCR4:ACKR3 and CXCR4:α_{1a}-AR). These observations, however, are difficult to interpret as the $BRET_{max}$ signal between CXCR4-RLuc and the EYFP-tagged receptor partners depends on the relative orientation or distance between the energy donor and acceptor, which may differ among the various receptor constructs [27].

Because BRET for various GPCR homo- and heteromers has been reported to be modulated upon agonist binding, we tested whether the selective α_1-AR agonist phenylephrine and the cognate CXCR4 agonist CXCL12 affect BRET between CXCR4 and $\alpha_{1a/b/d}$-AR. Fig. 2 shows typical titration BRET experiments for interactions between CXCR4 and $\alpha_{1a/b/d}$-ARs (top) and summarizes $BRET_{50}$ (center) and $BRET_{max}$ (bottom) values from four independent experiments when cells were exposed to phenylephrine or CXCL12. As compared with untreated cells, phenylephrine significantly increased $BRET_{max}$ of CXCR4:$\alpha_{1a/b/d}$-AR heteromers. CXCL12 reduced $BRET_{max}$ of CXCR4:α_{1a}-AR heteromers but did not affect $BRET_{max}$ between CXCR4 and $\alpha_{1b/d}$-AR. Both agonists did not significantly alter $BRET_{50}$ values. These findings are similar to previous observations on the CXCR4:CCR2 heteromer, which have been interpreted to reflect agonist-induced conformational changes of the receptor complex [25]. Whether the ligand-induced changes in $BRET_{max}$ that we observed for the CXCR4:α_1-AR heteromers also reflect increases in CXCR4:$\alpha_{1a/b/d}$-AR heteromerization upon binding of phenylephrine to α_1-ARs and reduced CXCR4:α_{1a}-AR heteromerization upon binding of CXCL12 to CXCR4, remains unclear because the apparent interaction affinity was not affected by agonist binding. Nevertheless,

these observations further support the concept that the BRET signals for CXCR4:α_1-AR heteromers are specific because such agonist-induced changes would not be expected for non-specific bystander BRET signals [25].

We showed previously in proximity ligation assays that a peptide analogue of TM2 of CXCR4 selectively reduces signals corresponding to endogenously expressed CXCR4:$\alpha_{1A/B}$-AR heteromers in human vascular smooth muscle cells, signals corresponding to recombinant CXCR4:α_{1b}-AR heteromers in HEK293T cells and inhibits β-arrestin cross-recruitment to CXCR4 within the CXCR4:α_{1b}-AR heteromeric complex upon phenylephrine stimulation in Presto-Tango assays in HTLA cells [18,19,21]. Consistent with these observations, the TM2 peptide analogue of CXCR4 significantly increased $BRET_{50}$ and reduced $BRET_{max}$ of CXCR4:$\alpha_{1a/b}$-AR heteromers (Fig. 3A/B), which provides direct evidence that the TM2 peptide analogue interferes with CXCR4:$\alpha_{1a/b}$-AR heteromerization. While the observation that the TM2 peptide analogue reduced $BRET_{max}$ but did not increase $BRET_{50}$ of CXCR4:α_{1d}-AR heteromers implies conformational changes of the heteromeric receptor complex (Fig. 3C), interference with the formation of CXCR4:α_{1d}-AR heteromers cannot be directly inferred from these findings. Furthermore, complimentary data from other assays, such as proximity ligation assays, on the effects of this peptide on CXCR4:α_{1d}-AR heteromers are not available. Thus, we can currently not exclude that CXCR4:$\alpha_{1a/b}$-AR and CXCR4:α_{1d}-AR heteromerization differs in regard to the underlying structural determinants. Our finding that the TM4 peptide analogue of CXCR4 did not interfere with BRET between CXCR4 and $\alpha_{1a/b/d}$-ARs is in agreement with the previously described effects of this peptide on CXCR4:α_{1A}-AR heteromers in human vascular smooth muscle cells and indicates selectivity of the effects of the TM2 peptide analogue [19].

Because TM-derived peptide analogues of GPCRs can affect receptor dimerization/oligomerization through interference with the correct assembly of the target membrane protein [29,30], the effects of the TM2 peptide analogue that we observed previously and its effects on BRET in the present study may point towards TM2 of CXCR4 as a possible heteromerization interface for α_1-ARs [18,19,21]. Crystallographic structures revealed CXCR4 as a homodimer with main interfaces at TM5 and TM6 [31]. Accordingly, we have previously *in silico* developed a structure-based heteromeric receptor model, in which TM2 of the CXCR4 homodimer serves as heteromerization interface for α_{1A}-AR [19]. In this model heteromerization of CXCR4 with α_{1A}-AR would not interfere with the formation of CXCR4 homodimers.

To test whether the CXCR4 homodimer is able to heteromerize with α_{1a}-AR, we employed BiLC and BiFC assays to detect of CXCR4 homodimerization and tested BiLC and BiFC BRET to assess interactions with α_{1a}-AR. In agreement with homodimerization of CXCR4, we observed strong and robust luminescence and fluorescence signals in cells transfected with CXCR4-L1 and CXCR4-L2 (Fig. 4A) and in cells transfected with CXCR4-V1 and CXCR4-V2, respectively (Fig. 4B). Luminescence and fluorescence signals in cells transfected with CXCR4-L1 and CXCR4-L2 alone or with CXCR4-V1 and CXCR4-V2 alone, respectively, were negligible. As shown in Fig. 4C, in cells transfected with constant amounts of CXCR4-L1 and CXCR4-L2 and with increasing amounts of α_{1a}-AR-EYFP, the

BRET signal showed a hyperbolic progression, whereas BRET in cells transfected with constant amounts of CXCR4-L1 and CXCR4-L2 and with increasing amounts of EYFP was low and increased linearly. Similarly, the BRET signal showed hyperbolic progression when cells were transfected with constant amounts of α_{1a}-AR-RLuc and with increasing amounts of CXCR4-V1 and CXCR4-V2 (Fig. 4D). In contrast, the BRET signal in cells expressing constant amounts of $mGlu_1R$-RLuc and increasing amounts of CXCR4-V1 and CXCR4-V2 was low and increased linearly, indicating that neither the CXCR4 protomer nor the CXCR4 homodimer heteromerize with $mGlu_1R$ (Fig. 4D). These findings provide evidence that the CXCR4 homodimer heteromerizes with α_{1a}-AR. This implies that α_{1a}-AR interacts with CXCR4 via a heteromerization interface that is different from the homodimerization interface of CXCR4 and experimentally supports our *in silico* interaction model.

Because α_{1a}-AR and $mGlu_1R$ are known to form homodimers [32,33], we then tested whether the CXCR4 homodimer may interact with homodimeric α_{1a}-AR or $mGlu_1R$. BiLC assays showed strong luminescence signals in cells co-expressing $mGlu_1R$-L1 and $mGlu_1R$-L2 (Fig. 5A) or α_{1a}-AR-L1 and α_{1a}-AR-L2 (Fig. 5B), respectively, which confirms homodimerization of both receptors. While BiLC and BiFC BRET signals for interactions between homodimeric CXCR4 and homodimeric α_{1a}-AR showed hyperbolic progression with increasing energy acceptor-donor ratios, the signals for interactions between homodimeric CXCR4 and homodimeric $mGlu_1R$ were low and increased linearly. These findings suggest that CXCR4 and α_{1a}-AR interact in higher order receptor complexes, whereas oligomeric CXCR4 does not form higher order complexes with oligomeric $mGlu_1R$.

In conclusion, in the present study we provide biophysical evidence that CXCR4 constitutively heteromerizes with α_1-ARs, thus supporting our previous observations from other test systems. Our findings further suggest that the interaction affinities of CXCR4 for ACKR3 and α_1-ARs are comparable and that agonist-binding results in conformational changes of the CXCR4:α_1-AR complexes. Moreover, we provide evidence that proto- and oligomeric CXCR4 and α_{1a}-AR constitutively form higher-order hetero-oligomeric receptor clusters, which provides initial insights into the molecular composition of these GPCR hetero-oligomers.

We are currently unable to assess interactions of more than four receptor protomers. Nevertheless, previous observations suggest that α_1-ARs heterodimerize among each other, that ACKR3 dimerizes with α_{1a}-AR and that the CXCR4:ACKR3 heteromer interacts with $\alpha_{1b/d}$-ARs [20,32,34]. In combination with the finding from the present study that the interaction affinities of CXCR4 for ACKR3 and α_1-ARs are comparable, these data point toward a not anticipated complexity of the organizational structure of these 7TM receptor clusters, which may be comprised of multiple proto- and homo-oligomeric receptors. Further studies will be required to address this possibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Bimolecular luminescence/fluorescence complementation combined with intermolecular bioluminescence resonance energy transfer assays were utilized
- Proto- and oligomeric CXCR4 and α_1-adrenoceptors form higher-order hetero-oligomeric receptor complexes
- The heteromerization affinity of CXCR4 for ACKR3 and α_1-adrenoceptors is comparable
- CXCR4:α_1-adrenoceptor hetero-oligomers undergo conformational changes upon agonist binding

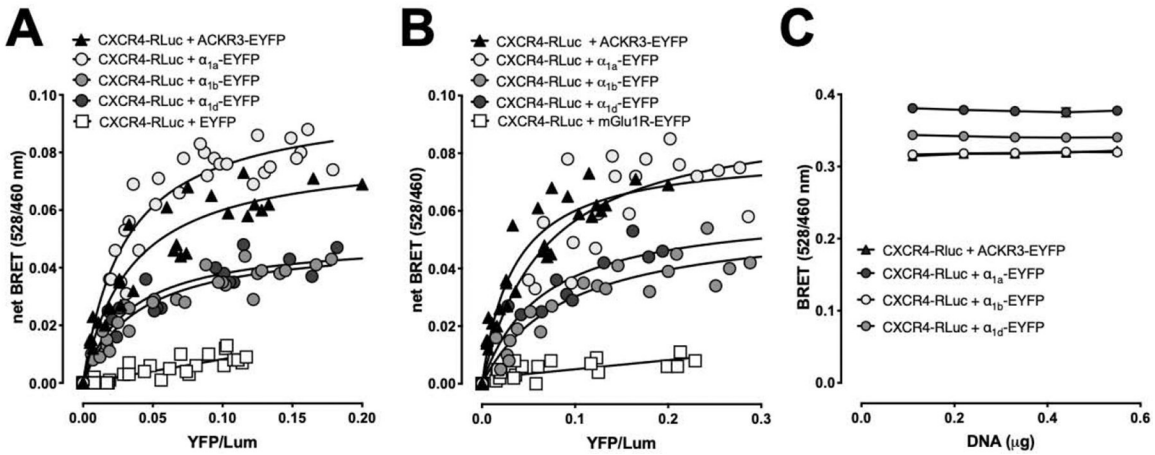

Figure 1:

BRET assays indicate that CXCR4 interacts with $\alpha_{1a/b/d}$-ARs. Graphs are representative of at least three independent experiments. **A/B**. HEK293T cells were transfected with a fixed amount of CXCR4-Rluc and increasing amounts of ACKR3-EYFP, $\alpha_{1a/b/d}$-AR-EYFP, EYFP (**A**) or mGlu1R-EYFP (**B**). 48 h after transfection, EYFP fluorescence and luminescence were read as described in Methods. Net BRET (528nm/460nm) was plotted against EYFP/luminescence (YFP/Lum). **C**. HEK293T cells were transfected with increasing amounts of both CXCR4-Rluc and ACKR3-EYFP or :$\alpha_{1a/b/d}$-AR-EYFP at a fixed ratio (1:10) in quadruplicate. Raw BRET (528nm/460nm) was plotted against total DNA amounts transfected.

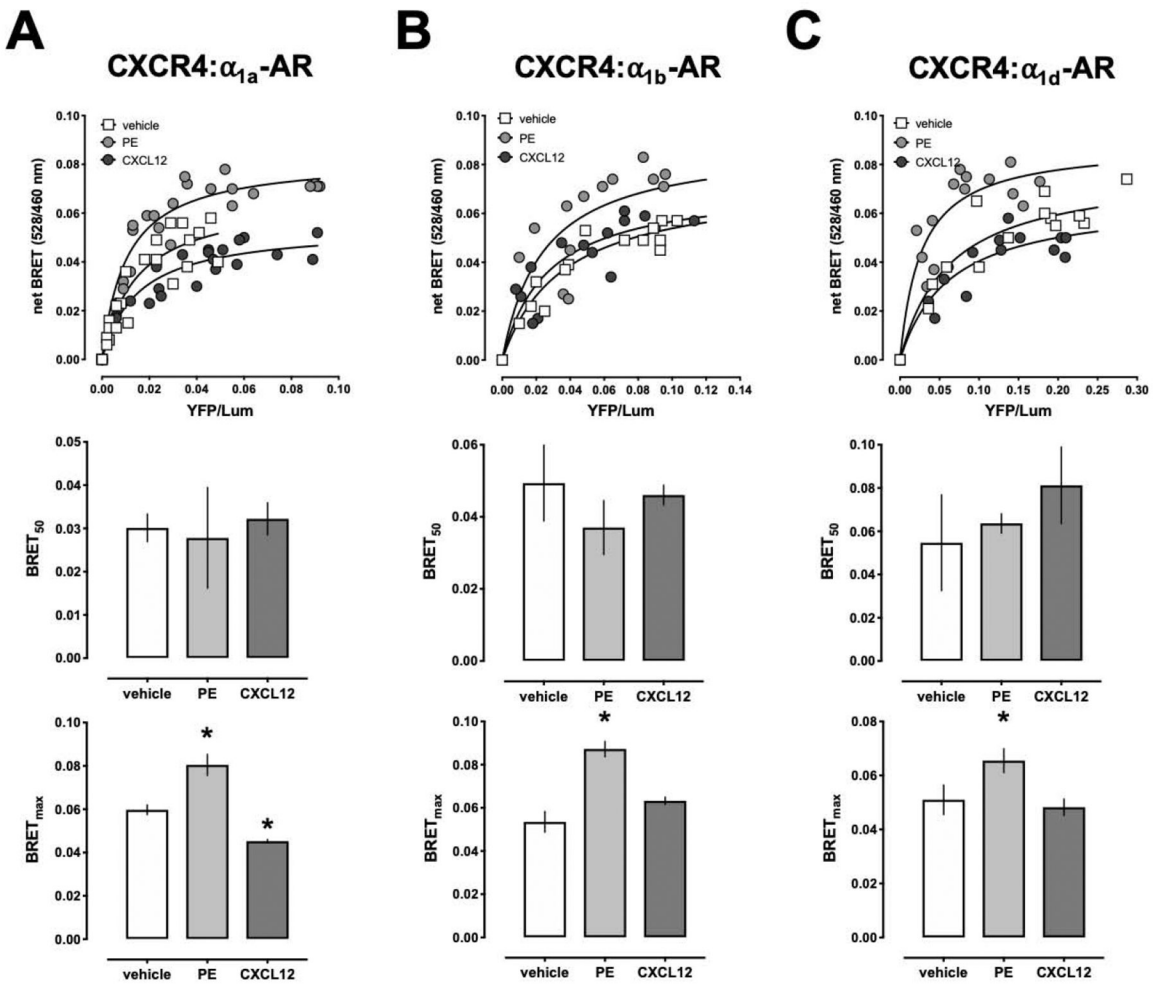

Figure 2:

Effects of phenylephrine and CXCL12 on BRET between CXCR4 and $\alpha_{1a/b/d}$-AR. HEK293T cells were co-transfected with a fixed amount of CXCR4-Rluc and increasing amounts α_{1a}-AR-EYFP (**A**), α_{1b}-AR-EYFP (**B**) or α_{1d}-AR-EYFP (**C**). 48 h after transfection, cells were treated with vehicle (=control), phenylephrine (PE, 200 μM) or CXCL12 (500 nM) for 5 min at 37°C before measuring BRET. *Top:* Representative measurements from a titration BRET experiment. *Center:* $BRET_{50}$ from n=4 independent titration BRET experiments. *Bottom:* $BRET_{max}$ from n=4 independent titration BRET experiments. *: $p<0.05$ vs. control.

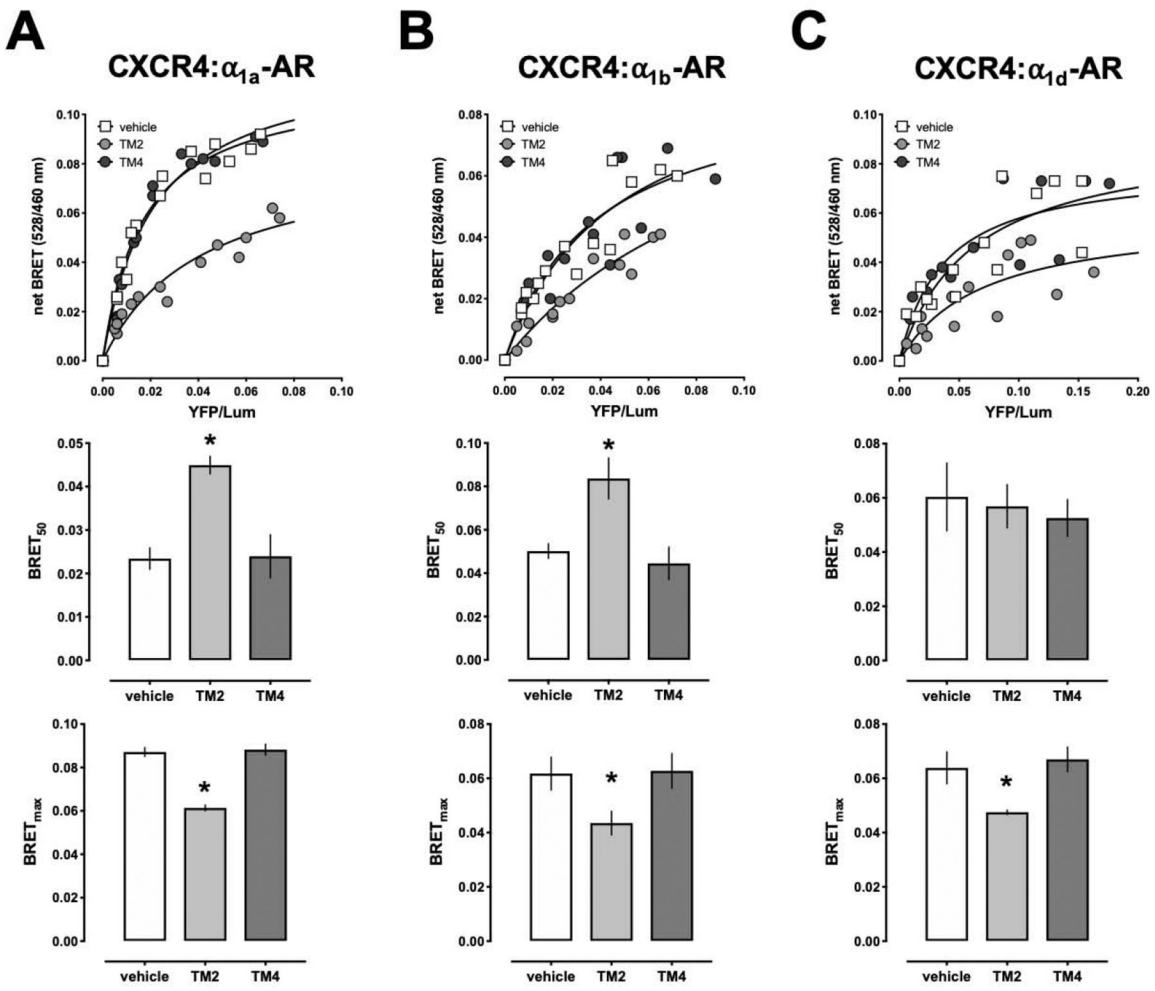

Figure 3:

A peptide analogue derived from TM2 of CXCR4 interferes with BRET between CXCR4 and $\alpha_{1a/b/d}$-ARs. HEK293T cells were co-transfected with a fixed amount of CXCR4-Rluc and increasing amounts of α_{1a}-AR-EYFP (**A**), α_{1b}-AR-EYFP (**B**) or α_{1d}-AR-EYFP (**C**). 48 h after transfection, cells were treated with vehicle (=control), TM2 or TM4 peptides (20 μM) for 15 min at 37°C before measuring BRET. *Top:* Representative measurements from a titration BRET experiment. *Center:* $BRET_{50}$ from n=4 independent titration BRET experiments. *: $p<0.05$ vs. control. *Bottom:* $BRET_{max}$ from n=4 independent titration BRET experiments. *: $p<0.05$ vs. control.

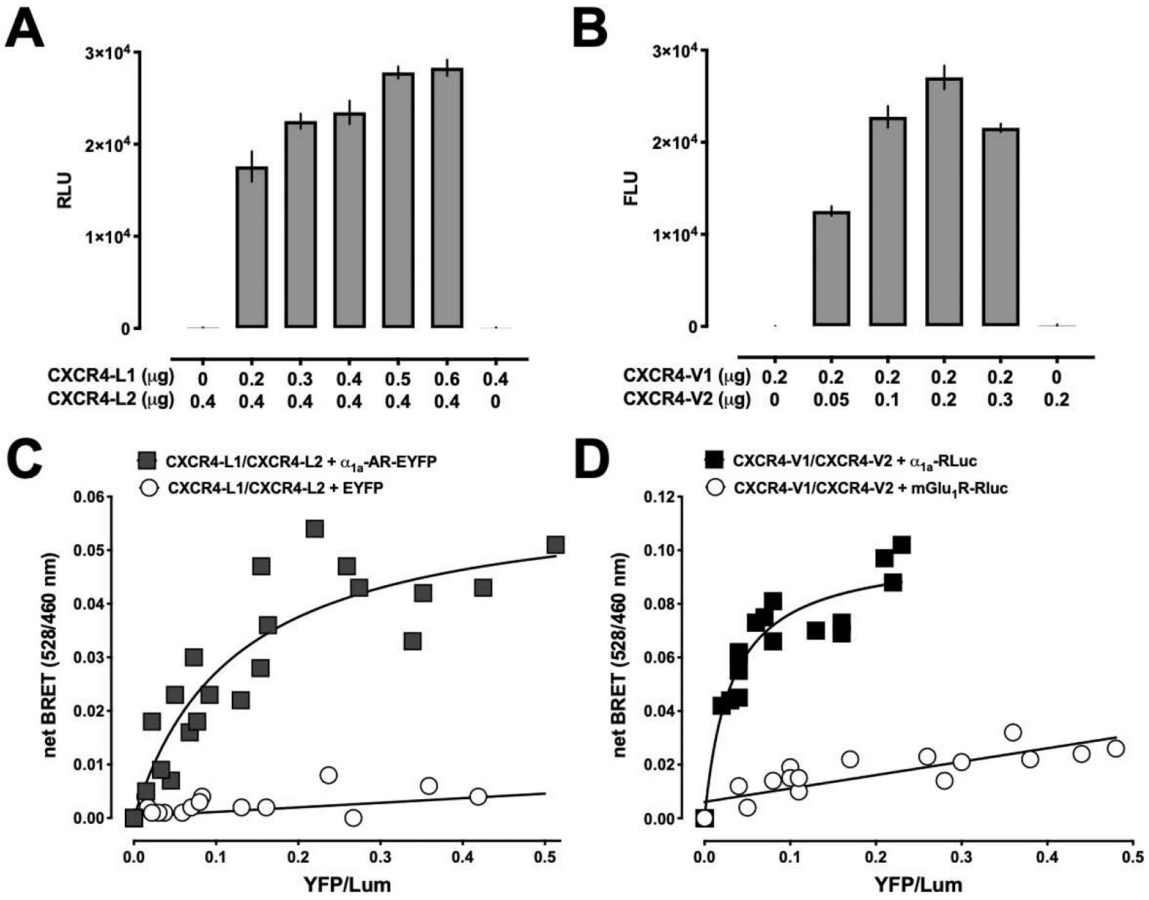

Figure 4:

The CXCR4 homodimer interacts with α_{1a}-AR. **A/B**. Bimolecular luminescence complementation (BiLC, **A**) and bimolecular fluorescence complementation (BiFC, **B**) assays to detect dimeric CXCR4. HEK293T cells were co-transfected with CXCR4-L1/2 or CXCR4-V1/2, as indicated. Luminescence was read after the addition of coelenterazine H. N=3. **C/D**. BiLC and BiFC BRET indicate that the CXCR4 homodimer interacts with α_{1a}-AR. HEK293T cells were co-transfected with a constant amount of CXCR4-L1/2 and with increasing amounts of α_{1a}-AR -EYFP or EYFP (control, **C**), or with a constant amount of α_{1a}-AR-RLuc or $mGlu_1R$-RLuc and with increasing amounts of CXCR4-V1/2 (control, **D**). 48 h after transfection, EYFP fluorescence and luminescence were read as described in the Methods. The net BRET (528/460) is plotted against YFP/Lum. The figure is representative of three independent experiments.

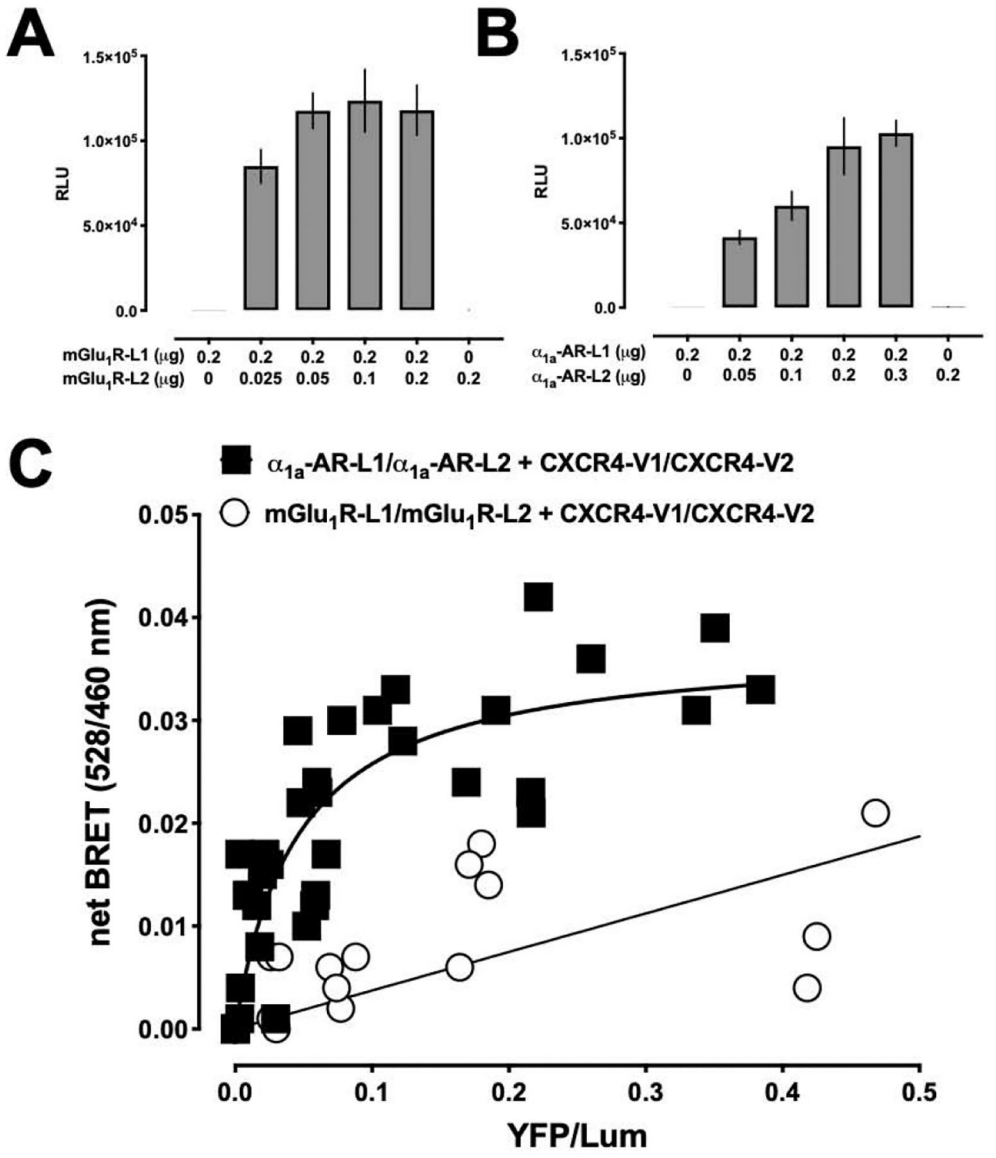

Figure 5:

Homodimeric CXCR4 interacts with homodimeric α_{1a}-AR. **A/B**. Bimolecular luminescence complementation (BiLC) assays to detect dimeric $mGLu_1R$ (**A**) and dimeric α_{1a}-AR (**B**). HEK293T cells were co-transfected with $mGLu_1R$-L1/2 or α_{1a}-AR-L1/2, as indicated. Luminescence was read after the addition of coelenterazine H. N=3. **C**. Combined BiLC and BiFC BRET indicates that the CXCR4 homodimer interacts with the α_{1a}-AR homodimer, but not with the $mGlu_1R$ homodimer. HEK293T cells were co-transfected with constant amounts of α_{1a}-AR-L1/2 or $mGlu_1R$-L1/L2 and with increasing amounts of CXCR4-V1/V2. 48 h after transfection, EYFP fluorescence and luminescence were read as described in the Methods. The net BRET (528/460) is plotted against YFP/Lum. The figure is representative of three independent experiments.