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Class A G protein-coupled receptors assemble into functional higher-order hetero-oligomers

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Abstract

Although class A seven-transmembrane-helix (7TM) receptor hetero-oligomers have been proposed, information on the assembly and function of such higher-order hetero-oligomers is not available. Utilizing bioluminescence resonance energy transfer (BRET), bimolecular luminescence/fluorescence complementation (BiLC/BiFC) and BiLC/BiFC BRET in HEK293T cells, we provide evidence that chemokine (C-X-C motif) receptor 4 (CXCR4), atypical chemokine receptor 3 (ACKR3), α_{1a}-adrenoceptor (α_{1a}-AR), and arginine vasopressin receptor 1A (AVPR1A) form hetero-oligomers composed of 2-4 different protomers. We show that hetero-oligomerization per se and ligand binding to individual protomers regulate agonist-induced coupling to the signaling transducers of interacting receptor partners. Our findings support the concept that receptor hetero-oligomers form supramolecular machineries with molecular signaling properties distinct from the individual protomers. These findings provide a mechanism for the phenomenon of context-dependent receptor function.

Keywords

receptor hetero-oligomerization; receptor dimer; receptor trimer; receptor tetramer; CXCR4; ACKR3; AVPR1A; alpha1-adrenoceptors; bioluminescence resonance energy transfer; bimolecular fluorescence/luminescence complementation

Introduction

Seven transmembrane helix (7TM) receptors, of which most are G protein-coupled receptors (GPCRs), play important roles in human physiology and pathology, and are targeted by a large proportion of drugs approved by the Federal Drug Administration [1, 2].

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Conflict of Interest

The authors declare no conflicts of interest in regard to this manuscript.

Crystallographic structures revealed several GPCRs as homodimers. Although crystallographic structures of 7TM receptor heteromers are not available, evidence suggests that many 7TM receptors may form heterodimers and higher-order homo- and hetero-oligomers, resulting in receptor complexes with distinct functional properties [3-7]. While the existence of class C 7TM receptor heteromers is well established, class A 7TM receptor hetero-dimerization and hetero-oligomerization is still controversial [5, 8], and information on the assembly and function of putative higher-order receptor hetero-oligomers is not available.

Recently, we provided evidence that the class A 7TM receptors chemokine (C-X-C motif) receptor 4 (CXCR4) and atypical chemokine receptor 3 (ACKR3) form heterodimers with α_1-adrenergic receptors (ARs) and arginine vasopressin receptor 1A (AVPR1A) in recombinant systems and in human vascular smooth muscle cells, through which activation of the chemokine receptors regulates vasoconstrictor responses mediated by the vasopressor receptor agonists [9-14]. While activation of CXCR4 enhanced α_1_AR-mediated vasoconstriction, activation of ACKR3 inhibited α_1_AR and AVPR1A-induced vasoconstriction [12, 15]. Surprisingly, we demonstrated that interference with chemokine receptor:vasopressor receptor heteromerization in human vascular smooth muscle cells inhibits vasopressor receptor function, suggesting that direct physical interactions between the receptor partners facilitates Gq coupling of α_1_AR and AVPR1A upon agonist stimulation [9, 12]. Furthermore, we provided biophysical evidence that the CXCR4 dimer can form hetero-trimeric and hetero-tetrameric complexes with the α_{1a}-AR proto- and dimer, respectively [14]. Based on these observations, we sought to evaluate whether CXCR4, ACKR3, AVPR1A and α_1-ARs form higher-order heteromeric complexes with each other and to assess the molecular signaling behavior of such putative receptor clusters. Here, we provide evidence that higher-order class A 7TM receptor heteromers form supramolecular signaling machineries with pharmacological properties distinct from individual protomers.

Materials and Methods

Reagents.

Phenylephrine (PE), phentolamine, arginine vasopressin (aVP), conivaptan and AMD3100 were purchased from Sigma-Aldrich. CXCL12 was purchased from Protein Foundry. Coelenterazine H was from Nanolight Technology.

Plasmids.

The coding sequences of CXCR4, ACKR3, α_{1a}-AR, α_{1b}-AR, α_{1d}-AR, AVPR1A and $mGlu_1R$ were from Addgene (CXCR4-TANGO, #66262; ACKR3-TANGO, #66265, α_{1a}-AR-TANGO, #66213, α_{1b}-AR-TANGO, #66214, α_{1d}-AR-TANGO, #66215, AVPR1A-Tango #66225, mGlu1-Tango, #66387). Upper and lower case subscripts are used to denote endogenous and recombinant α_1-ARs, respectively [16]. CXCR4-hRLuII was generously provided by Dr. Michel Bouvier and Gαi-91Venus was generously provided by Dr. Jonathan A. Javitch. The coding sequence of Renilla luciferase was from CXCR4-hRLuII, which was PCR amplified and ligated at the C-terminus of CXCR4, ACKR3, AVPR1A and mGlu1R at

the sites of Age I and Xba I. To produce CXCR4-enhanced yellow fluorescent protein (YFP), ACKR3-YFP, α_{1a}-AR-YFP, α_{1b}-AR-YFP, α_{1d}-AR-YFP, AVPR1A-YFP and $mGlu_1R$-YFP, 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 the above GPCRs fused with split luciferase, the coding sequence of Renilla luciferase II was PCR amplified into two segments, L1 (AA1-229) and L2 (AA230-311) [17], and inserted in-frame at the C-termini of those genes at the sites of Age I and Xba I. To construct the GPCRs fused with split-yellow fluorescent protein Venus (V1, AA1-155; V2, AA156-240), V1 and V2 were PCR amplified from D2R-V1 (Addgene, #19967) and D2R-V2 (Addgene, #19968) with a primer that carries the Age I site and matches D2R-V1 and V2 linker sequences and primer sp6 [17]. The amplicons were fused with the GPCRs in-frame at the C-termini at the Age I and Xba I sites, respectively. All plasmids were confirmed by sequencing.

Cell culture.

HEK 293T cells were as described [13] and cultured in high-glucose Dulbecco's Modified Eagle's Medium containing 1 mM sodium pyruvate, 2 mM L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified environment at 37ºC, 5% CO_2. The HTLA cell line, a HEK293 cell line stably expressing a tTA-dependent luciferase reporter and a β-arrestin2-TEV fusion gene, was generously provided by the laboratory of Dr. Bryan Roth [18] and maintained in high glucose Dulbecco's Modified's Eagle Medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL hygromycin B, and 2 μg/mL puromycin. All cells were cultured in a humidified environment at 37ºC, 5% CO_2.

Intermolecular bioluminescence resonance energy transfer (BRET) to measure receptor-receptor interactions.

BRET assays were performed as described previously [12, 14, 19, 20]. 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, AVPR1A-RLuc at a fixed amount of 50 ng was transfected alone or with increasing amounts of YFP or $\alpha_{1a/b/d}$-AR-YFP. 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 AVPR1A-RLuc was transfected alone. For titration experiments, net BRET ratios are expressed as a function of fluorescence/total luminescence.

Bimolecular luminescence and fluorescence complementation BRET.

Bimolecular luminescence complementation assays (BiLC), bimolecular fluorescence complementation assays (BiFC) and BiLC/BiFC BRET was performed as previously described [14]. For bimolecular luminescence complementation assays (BiLC), two GPCRs, of which one is fused with L1 and the other with L2, were co-transfected in HEK293T cells, with one GPCR at a fixed amount and the other at increasing amounts. Similarly, for bimolecular fluorescence complementation (BiFC) assays, two GPCRs, of which one is fused with V1 and the other with V2, were co-transfected with one GPCR at a fixed amount and the other at increasing amounts. 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, cells transfected with fixed amounts of L1- and L2-tagged receptors were co-transfected with increasing amounts of a YFP-tagged receptor or with V1- and V2-tagged receptors. For the BiFC BRET assay, RLuc-tagged receptor or L1- and L2-tagged receptors at fixed amounts were co-transfected with increasing amounts of V1- and V2-tagged receptors. BRET was measured as described before.

BRET for monitoring Gαi engagement.

HEK293T cells were seeded in 6-well plates. The next day, cells were co-transfected with 0.15 μg CXCR4-RlucII and 1.2 μg Gαi-91Venus together with 0.15 μg each of DNAs expressing α_{1a}-AR, AVPR1A and/or ACKR3 using lipofectamine 3000, as indicated in the graphs. As a control, cells were co-transfected with CXCR4-RlucII, Gαi-91Venus plus pcDNA3.1. Forty-eight hours post-transfection, cells were detached with phosphate buffered saline (PBS)/5 mM EDTA and replated in 0.1% glucose/PBS to 96-well plates. To examine the effects of agonists of receptor heteromerization partners, cells were co-treated with various concentrations of CXCL12 together with either 1 μM phenylephrine or 0.1 μM arginine vasopressin for 5 min at room temperature. To test the effects of antagonists of receptor heteromerization partners on Gαi engagement by CXCR4, cells were pretreated with 1 μM of phentolamine or 0.1 μM conivaptan for 15 min, followed by incubation with various concentrations of CXCL12 for 5 min at room temperature. Luciferase substrate coelenterazine h was added to cells at a final concentration of 5 μM 5 min prior to agonist treatment. The BRET signal was calculated as the luminescence read at 525 nm divided by the luminescence measured at 460 nm. CXCL12-induced BRET changes are plotted as BRET net, which was calculated as the BRET ratios at various concentrations of CXCL12 subtracted by the ratio in the absence CXCL12.

PRESTO-Tango β-arrestin-2 recruitment assays.

PRESTO-Tango assays were performed as previously described [11-13, 18, 21, 22]. HTLA cells were seeded in 6-well plates. The next day, cells were transfected with 0.8 μg CXCR4- or AVPR1A-Tango together with either pcDNA3.1 or 0.4 μg each of DNA expressing either α_{1a}-AR, AVPR1A, ACKR3 or CXCR4 using lipofectamine 3000, as indicated in the graphs. Twenty-four hour later, cells were re-plated to poly-L-lysine precoated white solid 96-well plates. After 4h of incubation, cells were subjected to treatment. To examine the effects of agonists of receptor heteromerization partners, cells were co-treated with various

concentrations of CXCL12 together with either 1 μM PE or 0.1 μM aVP. To test the effects of antagonists of receptor heteromerization partners on β-arrestin recruitment, cells were pretreated with either 1 μM of phentolamine, 0.1 μM conivaptan or 1 μM of AMD3100 for 15 min, followed by addition of various concentrations of Tango-receptor agonists (CXCL12 or aVP). After overnight incubation, cells were incubated with 1:10 diluted Bright-Glow substrate for 10 min at room temperature prior to luminescence reading on a Biotek Synergy II plate reader.

Flow cytometry.

Flow cytometry was used to evaluate receptor expression, as described [9, 14]. Cells were incubated with mouse anti-CXCR4 (R&D, MAB172), mouse anti-AVPR1A (LSBio, LS-C196728), rabbit anti-α_{1A}-AR (Abcam Ab137123) or mouse anti-FLAG antibody (Sigma, F1804, for FLAG-ACKR3) followed by incubation with Alexa Fluor 488 conjugated goat anti-mouse or anti-rabbit antibody (Thermofisher Scientific, 1: 50 dilution). The fluorescence intensities of at least 10,000 cells were recorded and analyzed using the FlowJo software (Tree Star).

Data analyses.

Data are expressed as mean ± standard error. Titration curves were analyzed with nonlinear regression analyses. Best-fit values were compared with the extra-sum-of-squares F test. One- or Two-way analyses of variance (ANOVA) with Dunnett's multiple comparison post hoc test for multiple comparisons were used to assess statistical significance, as appropriate. A two-tailed $p<0.05$ was considered significant. All analyses were calculated with the GraphPad Prism 8, Version 8.4.0 software.

Results and Discussion

Heterodimerization between CXCR4, ACKR3, AVPR1A and α_1-ARs

We showed previously that CXCR4 heterodimerizes with $\alpha_{1a/b/d}$-ARs and ACKR3, and that ACKR3 heterodimerizes with AVPR1A [12, 14]. To evaluate whether $\alpha_{1a/b/d}$-ARs also heterodimerize with AVPR1A we performed intermolecular saturation bioluminescence resonance energy transfer (BRET) assays (Figure 1A). Consistent with a non-specific bystander BRET signal (open diamonds), the BRET signal was low and increased linearly with increasing energy acceptor : donor ratios in cells expressing AVPR1A-*Renilla luciferase* (RLuc) and enhanced yellow fluorescent protein (YFP). BRET signals between AVPR1A-RLuc and $\alpha_{1a/b/d}$-AR-YFP showed hyperbolic progressions with increasing energy acceptor : donor ratios, suggesting constitutive heterodimerization. To confirm these findings and to evaluate whether CXCR4, ACKR3, α_{1a}-AR and AVPR1A can heterodimerize with each other, we employed bimolecular fluorescence and luminescence complementation (BiFC/BiLC) assays. We observed robust luminescence signals in HEK293T cells expressing each combination of *Renilla luciferase* AA1-229 (L1)- and *Renilla luciferase* AA230-311 (L2)-fused receptor pairs, while luminescence signals after transfection with only one L1- or L2-fused receptor were negligible (Figure 1B-G). Furthermore, we detected in parallel experiments that luminescence signals in HEK293T cells transfected with metabotropic glutamate receptor 1 ($mGlu_1R$)-L1 and ACKR3-L2 were

more than 80% lower than luminescence signals in cells transfected with AVPR1A-L1 and ACKR3-L2 (Figure 1G/H). Luminescence signals in cells expressing $mGlu_1R$-L1 and $mGlu_1R$-L2 were comparable with those in cells expressing AVPR1A-L1 and ACKR3-L2 (Figure 1I). Similarly, fluorescence signals in cells expressing CXCR4-yellow fluorescent protein Venus AA1-155 (V1) and $mGlu_1R$- yellow fluorescent protein Venus AA156-240 (V2) were more than 77% lower than fluorescence signals in cells expressing CXCR4-V1 and α_{1a}-AR-V2 (Figure 1J/K). These data suggest selectivity of the interactions between the class A GPCRs CXCR4, ACKR3, α_{1a}-AR and AVPR1A, and confirm that the class C GPCR $mGlu_1R$ is not a heterodimerization partner [14, 23, 24].

CXCR4, ACKR3, AVPR1A and α_{1a}-AR form hetero-trimeric and hetero-tetrameric clusters

CXCR4 has been reported to exist as a monomer, dimer and within nanoclusters comprised of more than three protomers in cells [24, 25]. Consistent with CXCR4 dimerization, we observed robust luminescence and fluorescence signals for dimeric CXCR4 in BiLC (Figure 2A) and BiFC assays (Figure 2B). Moreover, saturation BiFC and BiLC BRET experiments showed hyperbolic progressions of the BRET signals in cells co-transfected with CXCR4-Rluc, CXCR4-V1 and CXCR4-V2 (Figure 2C) and in cells co-transfected with CXCR4-L1, CXCR4-L2, CXCR4-V1 and CXCR4-V2 (Figure 2D), suggesting formation of CXCR4 homo-trimers and homo-tetramers. In contrast, saturation BiFC and BiLC BRET signals for interactions between dimeric CXCR4 and protomeric or dimeric $mGlu_1R$ were non-specific (Figure 2C/D). Similar to our previous observation that the CXCR4 homodimer forms hetero-trimers with α_{1a}-AR [14], we observed hyperbolic progressions of saturation BiLC/BiFC BRET signals for interactions between dimeric CXCR4 and AVPR1A (Figure 2E) or ACKR3 (Figure 2F).

Next, we tested whether trimeric and tetrameric receptor complexes composed of different protomers can be detected. As shown in Figure 3A-C, saturation BiLC/BiFC BRET suggested that the AVPR1A:ACKR3 heterodimer can form trimers with α_{1a}-AR (Figure 3A) and CXCR4 (Figure 3B), and that the four protomers can assemble in a hetero-tetrameric cluster (Figure 3C).

To gain initial insights into the molecular behavior of the hetero-tetrameric receptor cluster, we exposed cells transfected with AVPR1A-L1, ACKR3-L2, CXCR4-V1 and α_{1a}-AR-V2 to their agonists and measured BiLC/BiFC BRET. Arginine vasopressin (aVP) and chemokine (C-X-C motif) ligand 12 (CXCL12), the cognate agonist of CXCR4 and ACKR3, dose-dependently increased BRET signals with an EC_{50} of 16±8 nM and 28±11 nM, respectively (Figure 3D/E). In contrast, the α_1-AR agonist phenylephrine (PE) dose-dependently reduced BRET signals with an IC_{50} of 16±5 μM (Figure 3F). The measured EC_{50}/IC_{50} of the agonists to induce BRET changes are in the order of magnitude of their EC_{50} to activate their receptors in other functional organ- and cell-based assays [12, 15, 21]. Thus, our findings that the agonists dose-dependently alter BRET signals at pharmacologically relevant concentrations suggest agonist-induced conformational changes of the heterotetrameric receptor cluster, and indicate specificity of the hetero-tetrameric receptor interactions because such agonist-induced changes would not be expected for non-specific bystander BiFC/BiLC BRET signals [14, 19]. While reports on 7TM receptor heterodimers

have exponentially increased during past decades, there are only few reports on the formation 7TM receptor clusters formed out of three different protomers, such as the hetero-trimer comprised of $mGlu_5R$, dopamine D2 and adenosine A2a receptors [26] or the hetero-trimer comprised of chemokine (C-C motif) receptor 2 (CCR2), CCR5 and CXCR4 [23]. The findings of the present study expand this list to include the AVPR1A:ACKR3:CXCR4 and AVPR1A:ACKR3:α_{1a}-AR hetero-trimers. Moreover, our findings provide initial evidence that a hetero-tetrameric class A 7TM receptor complex composed of AVPR1A, ACKR3, CXCR4 and α_{1a}-AR is formed in cells.

The presence of hetero-oligomerization partners alter the coupling of CXCR4 to its signaling transducers

While molecular signaling signatures of heterodimeric receptor complexes have been reported previously, the function of hetero-oligomeric complexes composed of three or more receptor partners is unknown. To address this question, we selected CXCR4 as a read-out receptor, and utilized BRET and the PRESTO-Tango cell system to measure and compare agonist-induced Gαi coupling (Figure 4B) and β-arrestin recruitment (Figure 4C), respectively. Because CXCR4 has previously been reported to form heterodimeric complexes with ACKR3 and α_{1a}-AR [9, 11, 14, 27, 28], we compared Gαi coupling of and β-arrestin recruitment to CXCR4 upon stimulation with CXCL12 in cells expressing CXCR4 alone, CXCR4 plus ACKR3 or α_{1a}-AR, and in cells expressing all four heteromerization partners. We confirmed by flow-cytometry that under our experimental conditions all receptor partners were expressed on the cell surface and that expression of CXCR4 in cells transfected with CXCR4 alone or with all four receptor partners was comparable (Figure 4A).

The EC_{50} of CXCL12 to induce engagement of CXCR4 with Gαi was 1.8 ± 0.7 nM in cells expressing CXCR4 alone. While co-expression of CXCR4 with α_{1a}-AR did not affect CXCL12-induced Gαi coupling (EC_{50}: 1.9 ± 0.5 nM), co-expression of CXCR4 with ACKR3 reduced the potency of CXCL12 to induce engagement with Gαi 5.9-fold (EC50: 10.6 ± 2.4 nM, $p<0.05$ vs. CXCR4 alone) (Figure 4B). The latter finding is in line with the previously described effects of heterodimerization between CXCR4 and ACKR3 on Gαi-mediated signaling events and now provides direct evidence for reduced CXCL12-induced Gαi coupling of CXCR4 when co-expressed with ACKR3 [27, 28]. However, co-expression of all four heteromerization partners did not significantly affect the potency of CXCL12 to induce engagement of CXCR4 with Gαi (EC_{50}: 3.2 ± 1.4 nM, $p>0.05$ vs. CXCR4 alone), as compared with cells expressing CXCR4 alone. The efficacy of CXCL12 to induce Gαi coupling to CXCR4 was indistinguishable under all conditions (Fig. 4B).

Co-expression of CXCR4 with any combination of the heteromerization partners did not significantly affect the potency of CXCL12 to recruit β-arrestin to CXCR4 (Figure 4C). While the efficacy of CXCL12 to induce β-arrestin recruitment to CXCR4 was comparable in cells expressing CXCR4 alone and in cells expressing CXCR4 plus α_{1a}-AR, co-expression of CXCR4 with ACKR3 reduced the efficacy of CXCL12 to induce β-arrestin recruitment to CXCR4 by 20%, and co-expression of all four receptor partners by 58%, respectively (top plateau (fold increase in luminescence signals): CXCR4 alone – 5.8 ± 0.3;

CXCR4 plus α_{1a}-AR – 5.8 ± 0.35; CXCR4 plus ACKR3 – 4.4 ± 0.2 ($p<0.05$ vs. CXCR4 alone); all four heteromerization partners – 2.4 ± 0.1 ($p<0.05$ vs. CXCR4 alone)). Figure 4D summarizes how the presence of the various hetero-oligomerization partners alter the coupling of CXCR4 to its signaling transducers. Our findings demonstrate that agonist-induced coupling of CXCR4 to its signaling transducers is significantly altered by the presence of receptor hetero-oligomerization partners. Furthermore, our findings imply that the composition of the heteromeric complexes determine the balance between G protein and β-arrestin-mediated functions of CXCR4.

Recently, we showed that agonist stimulation of α_{1b}-AR within the α_{1b}-AR:CXCR4 heterodimer and of AVPR1A within the AVPR1A:ACKR3 heterodimer leads to cross recruitment of β -arrestin to the chemokine receptor heteromerization partners and reduces the efficacy of agonist-induced increases in luminescence signals in α_{1b}-AR and AVPR1A PRESTO-Tango assays [12, 13]. Furthermore, CXCL12 is also a cognate agonist of ACKR3 and ACKR3 activation results in β-arrestin recruitment to the receptor [28, 29]. Thus, besides changes in the intrinsic properties of CXCR4 to engage with β-arrestin when in complex with its heteromerization partners, cross-recruitment of β-arrestin to other receptor heteromerization partners could also account for the observed effects. Nevertheless, as β-arrestin recruitment is intimately involved in the termination of G protein signaling and mediates G protein-independent signaling, our observations imply that the formation of hetero-oligomeric complexes per se biases CXCR4 signaling in favor of Gαi-mediated signaling [30].

Ligand binding to one receptor partner modulates agonist-induced coupling to its signaling transducers of partnering receptors

Next, we tested whether ligand binding to α_{1a}-AR or AVPR1A affects agonist-induced coupling of CXCR4 to its signaling transducers. As expected, agonist (Figure 5A/C) and antagonist (Figure 5E/G) binding to either α_{1a}-AR or AVPR1A did not affect CXCL12-induced engagement with Gαi or β-arrestin in cells expressing CXCR4 alone. When co-expressed with the heteromerization partners, however, agonist binding to α_{1a}-AR or AVPR1A reduced the efficacy of CXCL12-induced engagement of CXCR4 with Gαi (Figure 5B) and increased the potency (EC_{50} (pretreatment with): 16.6 ± 3.7 nM (vehicle); 3.5 ± 1.0 nM (phenylephrine, 1 μM); 5.6 ± 1.3 nM (arginine vasopressin, 0.1 μM); $p < 0.01$ vs. vehicle for both) and efficacy of CXCL12 to induce β-arrestin recruitment of CXCR4 (Figure 5D). While antagonist binding to α_{1a}-AR or AVPR1A did not affect the efficacy of CXCL12 to induce engagement of CXCR4 with Gαi, phentolamine reduced the potency of CXCL12 to induce Gαi coupling of CXCR4 4.7-*fold* (EC_{50}: vehicle – 2.1 ± 0.7 nM; phentolamine – 9.9 ± 4.3 nM, $p=0.013$) and conivaptan 7-*fold* (EC_{50}: 14.9 ± 6.3 nM, $p<0.01$), respectively (Figure 5F). Both antagonists, however, showed differential effects on CXCL12-induced β-arrestin recruitment to CXCR4 (Figure 5H): phentolamine increased the efficacy of CXCL12 by 42% ($p<0.01$ vs. vehicle), whereas conivaptan reduced the potency of CXCL12 2.3-*fold* (EC_{50}: vehicle – 10.5 ± 1.7 nM; conivaptan – 24.0 ± 5.1 nM; $p<0.01$). Figure 5I shows a simplified schematic summarizing the observed effects. Our findings that agonist and antagonist binding to one of the heteromerization partners modulates agonist-induced coupling to signaling transducers of another receptor partner suggest that distinct

ligand-bound conformational states of the receptors allosterically regulate the signaling behavior of partner receptors within the hetero-oligomeric receptor complex.

To gain initial insight into the contribution of the individual heteromerization partners to the observed effects of their ligands on CXCR4, we selected phentolamine as one example ligand and studied CXCL12-induced engagement of CXCR4 with Gαi and β-arrestin in cells co-expressing various combinations of the heteromerization partners (Figure 6). As in cells expressing CXCR4 alone (Figure 5E/G), phentolamine did not affect Gαi coupling or β-arrestin recruitment to CXCR4 upon stimulation with CXCL12 when co-expressed with ACKR3 (Figure 6A/E). Similar to the effects of phentolamine in cells co-expressing all four heteromerization partners (Figure 5F/H), phentolamine reduced the efficacy of CXCL12 to induce Gαi coupling of CXCR4 (Figure 6B) and enhanced the efficacy to recruit β-arrestin to the receptor (Figure 6F) when CXCR4 was co-expressed with ACKR3 and α_{1a}-AR. However, when CXCR4 was co-expressed with α_{1a}-AR alone (Figure 6C/G) or with α_{1a}-AR plus AVPR1A (Figure 6D/H), phentolamine increased potency and/or efficacy of CXCL12 to induce Gαi coupling of CXCR4 (Figure 6C/D), and reduced its potency and/or efficacy for β-arrestin recruitment (Figure 6G/H). The effects of ACKR3 on CXCL12-induced CXCR4 activation in the presence of antagonist-bound α_{1a}-AR are summarized in Figure 6I. These findings demonstrate that the presence of ACKR3 result in opposite effects of antagonist binding to α_{1a}-AR on CXCL12-induced CXCR4 activation. Furthermore, in combination with our observations in cells co-expressing all four receptor partners (Figures 4 and 5), these data support the concept that each of the four receptor partners contributes to the regulation of CXCR4-mediated signaling.

To provide proof of principle that the observed regulation of CXCR4 is generalizable to the other receptor partners, we then tested whether antagonists of CXCR4 and α_{1a}-AR would also affect aVP-induced β-arrestin recruitment to AVPR1A in cells co-expressing all four receptor partners. As shown in Figure 6J, phentolamine and AMD3100 did not affect aVP-induced β-arrestin recruitment to AVPR1A in the absence of the heteromerization partners. In contrast, in cells co-expressing all four receptor partners, phentolamine increased whereas AMD3100 reduced the efficacy of aVP to recruit β-arrestin to AVPR1A (Figure 6K).

Taken together, our observations indicate that hetero-trimeric and hetero-tetrameric complexes composed of different 7TM receptor protomers can be formed and that hetero-oligomeric receptor clusters form signaling machineries with pharmacological properties distinct from individual protomers. It should be noted, however, that conclusive evidence for the existence of higher-order hetero-oligomeric receptor clusters among endogenously expressed 7TM receptors is currently not available because of the absence of methods to visualize endogenously expressed higher-order receptor complexes with confidence. Nevertheless, cross-talk between α_1-ARs and AVPR1A has been well established [31-33] and our previous findings provided evidence that endogenously expressed CXCR4, ACKR3, AVPR1A and α_1-ARs cross-talk via interactions within their hetero-dimeric or hetero-trimeric complexes in vascular smooth muscle cells [9-13]. In these studies, we showed that pharmacological interference with receptor heteromerization and manipulation of the expression levels of one heteromerization partner alters the formation of endogenously expressed heteromeric complexes between other receptor partners. This suggests that the

endogenously expressed receptors exist within a dynamic network in the plasma membrane and supports the concept of a supramolecular organization of these receptors into distinct signaling machineries. Our findings from the present study indicate that coupling of each receptor partner to signaling transducers is regulated by allosteric interactions among receptor partners and further modulated by ligand-induced conformations of partnering receptors. These findings provide a molecular mechanism for the phenomenon of context-dependent receptor signaling and function, as observed for CXCR4 and ACKR3 [34], and help to explain biological variability of 7TM receptor mediated pharmacological responses.

It is obvious that our findings on the effects of the receptor antagonists do not simulate the regulation of receptor signaling under physiological conditions. These studies, however, provide an additional glimpse into the roles of GPCR dynamics and the effects of transitioning into distinct GPCR conformations. Moreover, these findings could explain unexpected off-target effects of GPCR antagonists in the clinical setting and discrepancies between pharmacological properties of such drugs in vitro and in vivo. From a general physiological standpoint, the formation of higher order hetero-oligomeric receptor complexes provides the advantages that receptor function can be fine-tuned, and diverse biological processes integrated. As such, the hetero-tetrameric complex composed of CXCR4, ACKR3, AVPR1A and α_1-ARs offers a molecular mechanism by which the innate immune system, i.e. chemokine release, interacts with the vasoactive neurohormonal system, i.e. catecholamine and aVP release, to regulate cell and organ function in health and disease processes.

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Data Availability

The data that support the findings of this study are available from the corresponding majetschak@usf.edu] upon reasonable request.

Abbreviations

AR	adrenergic receptor
ACKR	atypical chemokine receptor
aVP	arginine vasopressin
AVPR1A	arginine vasopressin receptor 1A
BiFC	bimolecular fluorescence complementation
BiLC	bimolecular luminescence complementation
BRET	bioluminescence resonance energy transfer

CCR	chemokine (C-C motif) receptor
CXCL	(C-X-C motif) chemokine ligand
CXCR	chemokine (C-X-C motif) receptor
GPCR	G protein-coupled receptor
HEK	human embryonic kidney
hVSMC	human vascular smooth muscle cell
L1	Renilla luciferase AA1-229
L2	Renilla luciferase AA230-311
$mGlu_1R$	metabotropic glutamate receptor 1
PE	phenylephrine
RLuc	Renilla luciferase
7TM	seven transmembrane
V1	yellow fluorescent protein Venus AA1-155
V2	yellow fluorescent protein Venus AA156-240
YFP	enhanced yellow fluorescent protein

References

1. Alexander SP, Christopoulos A, Davenport AP, Kelly E, Marrion NV, Peters JA, Faccenda E, Harding SD, Pawson AJ, Sharman JL, Southan C, Davies JA & Collaborators C (2017) THE CONCISE GUIDE TO PHARMACOLOGY 2017/18: G protein-coupled receptors, Br J Pharmacol. 174 Suppl 1, S17–S129. [PubMed: 29055040]
2. Sharman JL, Benson HE, Pawson AJ, Lukito V, Mpamhanga CP, Bombail V, Davenport AP, Peters JA, Spedding M, Harmar AJ & Nc I (2013) IUPHAR-DB: updated database content and new features, Nucleic Acids Res. 41, D1083–8. [PubMed: 23087376]
3. Quitterer U & AbdAlla S (2019) Discovery of Pathologic GPCR Aggregation, Front Med (Lausanne). 6, 9. [PubMed: 30761305]
4. Gaitonde SA & Gonzalez-Maeso J (2017) Contribution of heteromerization to G protein-coupled receptor function, Curr Opin Pharmacol. 32, 23–31. [PubMed: 27835800]
5. Franco R, Martinez-Pinilla E, Lanciego JL & Navarro G (2016) Basic Pharmacological and Structural Evidence for Class A G-Protein-Coupled Receptor Heteromerization, Front Pharmacol. 7, 76. [PubMed: 27065866]
6. Ferre S, Baler R, Bouvier M, Caron MG, Devi LA, Durroux T, Fuxe K, George SR, Javitch JA, Lohse MJ, Mackie K, Milligan G, Pfleger KD, Pin JP, Volkow ND, Waldhoer M, Woods AS & Franco R (2009) Building a new conceptual framework for receptor heteromers, Nat Chem Biol. 5, 131–4. [PubMed: 19219011]
7. Gomes I, Ayoub MA, Fujita W, Jaeger WC, Pfleger KD & Devi LA (2016) G Protein-Coupled Receptor Heteromers, Annu Rev Pharmacol Toxicol. 56, 403–25. [PubMed: 26514203]
8. Lambert NA (2010) GPCR dimers fall apart, Sci Signal. 3, pe12. [PubMed: 20354223]
9. Tripathi A, Vana PG, Chavan TS, Brueggemann LI, Byron KL, Tarasova NI, Volkman BF, Gaponenko V & Majetschak M (2015) Heteromerization of chemokine (C-X-C motif) receptor 4

with alpha1A/B-adrenergic receptors controls alpha1-adrenergic receptor function, Proc Natl Acad Sci U S A. 112, E1659–68. [PubMed: 25775528]

10. Evans AE, Tripathi A, LaPorte HM, Brueggemann LI, Singh AK, Albee LJ, Byron KL, Tarasova NI, Volkman BF, Cho TY, Gaponenko V & Majetschak M (2016) New Insights into Mechanisms and Functions of Chemokine (C-X-C Motif) Receptor 4 Heteromerization in Vascular Smooth Muscle, Int J Mol Sci. 17, 971.
11. Albee LJ, Eby JM, Tripathi A, LaPorte HM, Gao X, Volkman BF, Gaponenko V & Majetschak M (2017) alpha1-Adrenergic Receptors Function Within Hetero-Oligomeric Complexes With Atypical Chemokine Receptor 3 and Chemokine (C-X-C motif) Receptor 4 in Vascular Smooth Muscle Cells, J Am Heart Assoc. 6, e006575. [PubMed: 28862946]
12. Albee LJ, LaPorte HM, Gao X, Eby JM, Cheng YH, Nevins AM, Volkman BF, Gaponenko V & Majetschak M (2018) Identification and functional characterization of arginine vasopressin receptor 1A : atypical chemokine receptor 3 heteromers in vascular smooth muscle, Open Biol. 8, 170207. [PubMed: 29386406]
13. Gao X, Albee LJ, Volkman BF, Gaponenko V & Majetschak M (2018) Asymmetrical ligand-induced cross-regulation of chemokine (C-X-C motif) receptor 4 by alpha1-adrenergic receptors at the heteromeric receptor complex, Sci Rep. 8, 2730. [PubMed: 29426850]
14. Gao X, Enten GA, DeSantis AJ, Volkman BF, Gaponenko V & Majetschak M (2020) Characterization of heteromeric complexes between chemokine (C-X-C motif) receptor 4 and alpha1-adrenergic receptors utilizing intermolecular bioluminescence resonance energy transfer assays, Biochem Biophys Res Commun. 528, 368–375. [PubMed: 32085899]
15. Bach HH, Wong YM, Tripathi A, Nevins AM, Gamelli RL, Volkman BF, Byron KL & Majetschak M (2014) Chemokine (C-X-C motif) receptor 4 and atypical chemokine receptor 3 regulate vascular alpha(1)-adrenergic receptor function, Mol Med. 20, 435–47. [PubMed: 25032954]
16. Bylund DB, Eikenberg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP, Molinoff PB, Ruffolo RR Jr. & Trendelenburg U (1994) International Union of Pharmacology nomenclature of adrenoceptors, Pharmacol Rev. 46, 121–36. [PubMed: 7938162]
17. Guo W, Urizar E, Kralikova M, Mobarec JC, Shi L, Filizola M & Javitch JA (2008) Dopamine D2 receptors form higher order oligomers at physiological expression levels, EMBO J. 27, 2293–304. [PubMed: 18668123]
18. Kroeze WK, Sassano MF, Huang XP, Lansu K, McCorvy JD, Giguere PM, Sciaky N & Roth BL (2015) PRESTO-Tango as an open-source resource for interrogation of the druggable human GPCRome, Nat Struct Mol Biol. 22, 362–9. [PubMed: 25895059]
19. Percherancier Y, Berchiche YA, Slight I, Volkmer-Engert R, Tamamura H, Fujii N, Bouvier M & Heveker N (2005) Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers, J Biol Chem. 280, 9895–903. [PubMed: 15632118]
20. Gao X, Cheng YH, Enten GA, DeSantis AJ, Gaponenko V & Majetschak M (2020) Regulation of the thrombin/protease-activated receptor 1 axis by chemokine (CXC motif) receptor 4, J Biol Chem. 295, 14893–14905. [PubMed: 32839271]
21. Cheng YH, Eby JM, LaPorte HM, Volkman BF & Majetschak M (2017) Effects of cognate, non-cognate and synthetic CXCR4 and ACKR3 ligands on human lung endothelial cell barrier function, PLoS One. 12, e0187949. [PubMed: 29125867]
22. Eby JM, Abdelkarim H, Albee LJ, Tripathi A, Gao X, Volkman BF, Gaponenko V & Majetschak M (2017) Functional and structural consequences of chemokine (C-X-C motif) receptor 4 activation with cognate and non-cognate agonists, Mol Cell Biochem. 434, 143–151. [PubMed: 28455789]
23. Sohy D, Yano H, de Nadai P, Urizar E, Guillabert A, Javitch JA, Parmentier M & Springael JY (2009) Hetero-oligomerization of CCR2, CCR5, and CXCR4 and the protean effects of "selective" antagonists, J Biol Chem. 284, 31270–9. [PubMed: 19758998]
24. Martinez-Munoz L, Rodriguez-Frade JM, Barroso R, Sorzano COS, Torreno-Pina JA, Santiago CA, Manzo C, Lucas P, Garcia-Cuesta EM, Gutierrez E, Barrio L, Vargas J, Cascio G, Carrasco YR, Sanchez-Madrid F, Garcia-Parajo MF & Mellado M (2018) Separating Actin-Dependent Chemokine Receptor Nanoclustering from Dimerization Indicates a Role for Clustering in CXCR4 Signaling and Function, Mol Cell. 70, 106–119 e10. [PubMed: 29625032]

25. Wu B, Chien EY, Mol CD, Fenalti G, Liu W, Katritch V, Abagyan R, Brooun A, Wells P, Bi FC, Hamel DJ, Kuhn P, Handel TM, Cherezov V & Stevens RC (2010) Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists, Science. 330, 1066–71. [PubMed: 20929726]

26. Cabello N, Gandia J, Bertarelli DC, Watanabe M, Lluis C, Franco R, Ferre S, Lujan R & Ciruela F (2009) Metabotropic glutamate type 5, dopamine D2 and adenosine A2a receptors form higher-order oligomers in living cells, J Neurochem. 109, 1497–507. [PubMed: 19344374]

27. Levoye A, Balabanian K, Baleux F, Bachelerie F & Lagane B (2009) CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling, Blood. 113, 6085–93. [PubMed: 19380869]

28. Decaillot FM, Kazmi MA, Lin Y, Ray-Saha S, Sakmar TP & Sachdev P (2011) CXCR7/CXCR4 heterodimer constitutively recruits beta-arrestin to enhance cell migration, The Journal of biological chemistry. 286, 32188–97. [PubMed: 21730065]

29. Balabanian K, Lagane B, Infantino S, Chow KY, Harriague J, Moepps B, Arenzana-Seisdedos F, Thelen M & Bachelerie F (2005) The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes, J Biol Chem. 280, 35760–6. [PubMed: 16107333]

30. Pierce KL & Lefkowitz RJ (2001) Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors, Nat Rev Neurosci. 2, 727–33. [PubMed: 11584310]

31. Bartelstone HJ & Nasmyth PA (1965) Vasopressin Potentiation of Catecholamine Actions in Dog, Rat, Cat, and Rat Aortic Strip, Am J Physiol. 208, 754–62. [PubMed: 14274809]

32. Steppan J, Nyhan SM, Sikka G, Uribe J, Ahuja A, White AR, Shoukas AA & Berkowitz DE (2012) Vasopressin-mediated enhancement of adrenergic vasoconstriction involves both the tyrosine kinase and the protein kinase C pathways, Anesth Analg. 115, 1290–5. [PubMed: 22886838]

33. Noguera I, Medina P, Segarra G, Martinez MC, Aldasoro M, Vila JM & Lluch S (1997) Potentiation by vasopressin of adrenergic vasoconstriction in the rat isolated mesenteric artery, Br J Pharmacol. 122, 431–8. [PubMed: 9351498]

34. Heuninck J, Perpina Viciano C, Isbilir A, Caspar B, Capoferri D, Briddon SJ, Durroux T, Hill SJ, Lohse MJ, Milligan G, Pin JP & Hoffmann C (2019) Context-Dependent Signaling of CXC Chemokine Receptor 4 and Atypical Chemokine Receptor 3, Mol Pharmacol. 96, 778–793. [PubMed: 31092552]

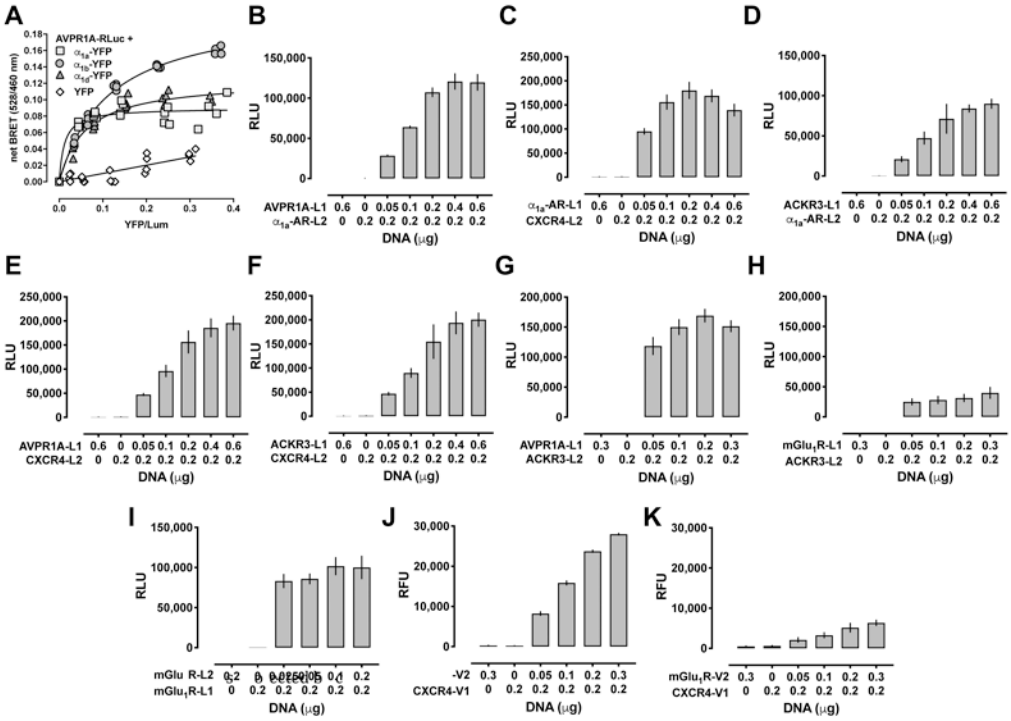

Figure 1.

Heterodimerization between recombinant CXCR4, ACKR3, AVPR1A and α_1-ARs. (**a**) BRET indicates that AVPR1A interacts with $\alpha_{1a/b/d}$-ARs. HEK293T cells were transfected with a fixed amount of AVPR1A-RLuc and increasing amounts of $\alpha_{1a/b/d}$-AR-YFP or YFP. 48 h after transfection, YFP fluorescence and luminescence were read as described in Methods. Net BRET (528nm/460nm) was plotted against YFP/luminescence (YFP/Lum). The graph is representative of at least three independent experiments. (**b-k**) BiLC or BiFC indicates heterodimerization between recombinant CXCR4, ACKR3, AVPR1A and α_1-ARs. HEK293T cells were transfected in triplicate with a pair of L1 and L2 tagged receptors for BiLC or with a pair of V1 and V2 tagged receptors for BiFC at the amounts indicated. $mGlu_1R$-L1 or $mGlu_1R$-V2 were used as negative controls. RLU: relative luminescence units. RFU: relative fluorescence units. Figures are representative of three independent experiments for each receptor pair.

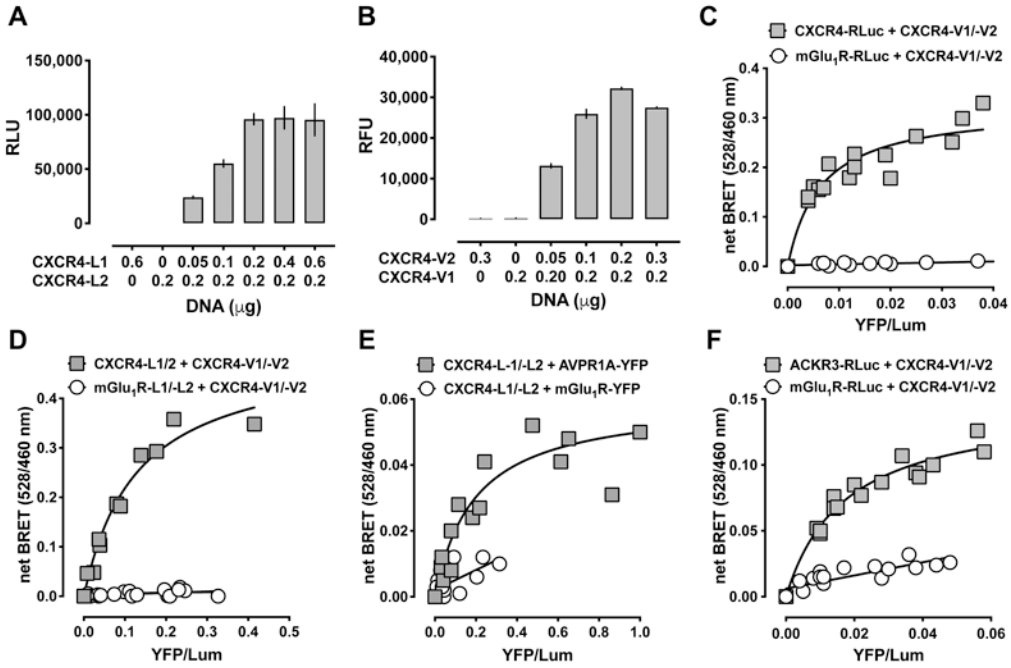

Figure 2.

CXCR4 homo-oligomerization and hetero-trimerization. (**a/b**) BiLC (A) and BiFC (B) indicates that CXCR4 forms homodimers. CXCR4-L1 and -L2 or CXCR4-V1 and –V2 were co-transfected at the amounts indicated. (**c**) BiFC BRET suggests that CXCR4 forms a homotrimer. Cells were transfected with CXCR4-RLuc or $mGlu_1R$-Rluc at a fixed amount, and with increasing amounts of CXCR4-V1/-V2. (**d**) CXCR4 forms a homo-tetramer. Cells were transfected with CXCR4-L1/-L2 or $mGlu_1R$-L1/-L2 at a fixed amount and with increasing amounts of CXCR4-V1/-V2. (**e**) The CXCR4 homodimer forms a heterotrimer with AVPR1A. Cells were transfected with CXCR4-L1/-L2 at a fixed amount and with increasing amounts of AVPR1A-YFP or $mGlu_1R$-YFP. (**f**) The CXCR4 homodimer forms a heterotrimer with ACKR3. Cells were transfected with ACKR3-RLuc or $mGlu_1R$-RLuc at a fixed amount and with increasing amounts of CXCR4-V1/-V2. BRET assays were performed as described in Methods. Net BRET was plotted against YFP fluorescence/luminescence (YFP/Lum). Figures are representative of three independent experiments for each condition.

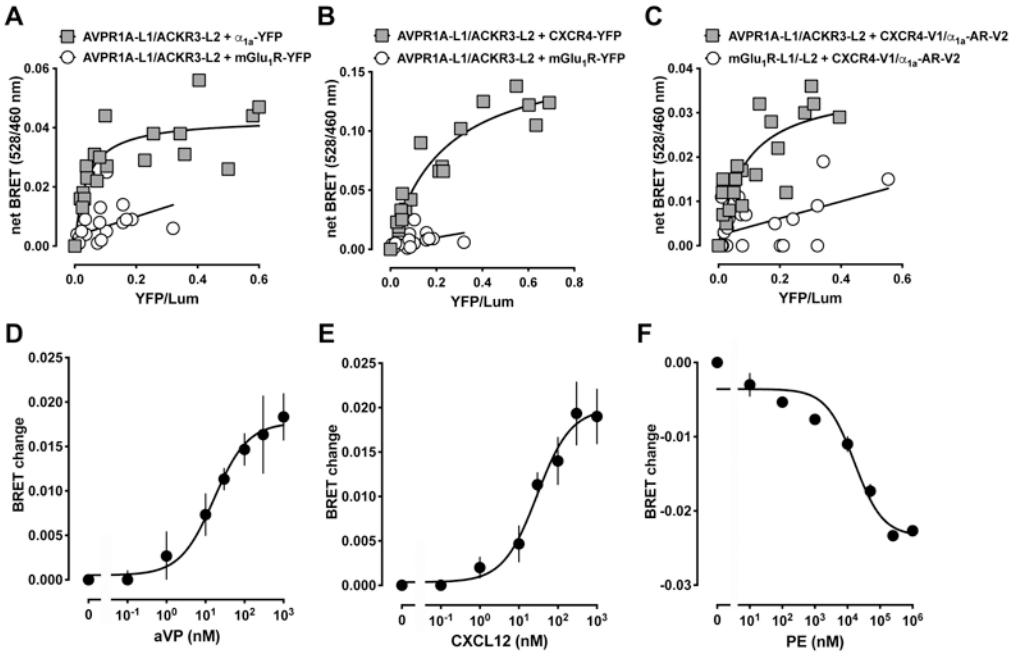

Figure 3.

Formation of hetero-oligomers comprised of CXCR4, ACKR3, α_{1a}-AR and AVPR1A.(**a-c**) BiLC and BiFC BRET suggests hetero-trimerization and hetero-tetramerization. HEK293T cells were co-transfected with a fixed amount of AVPR1A-L1/ACKR3-L2 and increasing amounts of α_{1a}-AR-YFP (**a**) or CXCR4-YFP (**b**) or CXCR4-V1/α_{1a}-AR-V2 (**c**). BRET assays were performed as described in Methods. Net BRET was plotted against YFP fluorescence/total luminescence (YFP/Lum). Figures are representative of three independent experiments. $mGlu_1R$ was used as a negative control. (**d-f**) Effects of agonists on BRET of the hetero-tetrameric receptor cluster. Cells were co-transfected with 0.6 μg of AVPR1A-L1/ACKR3-L2 and 1.8 μg of CXCR4-V1/α_{1a}-AR-V2. 48 h after transfection, cells were treated with different amounts of arginine vasopressin (aVP), CXCL12 or phenylephrine (PE) for 5 min before measuring BRET. The results shown are mean of BRET changes induced by agonists from three independent experiments.

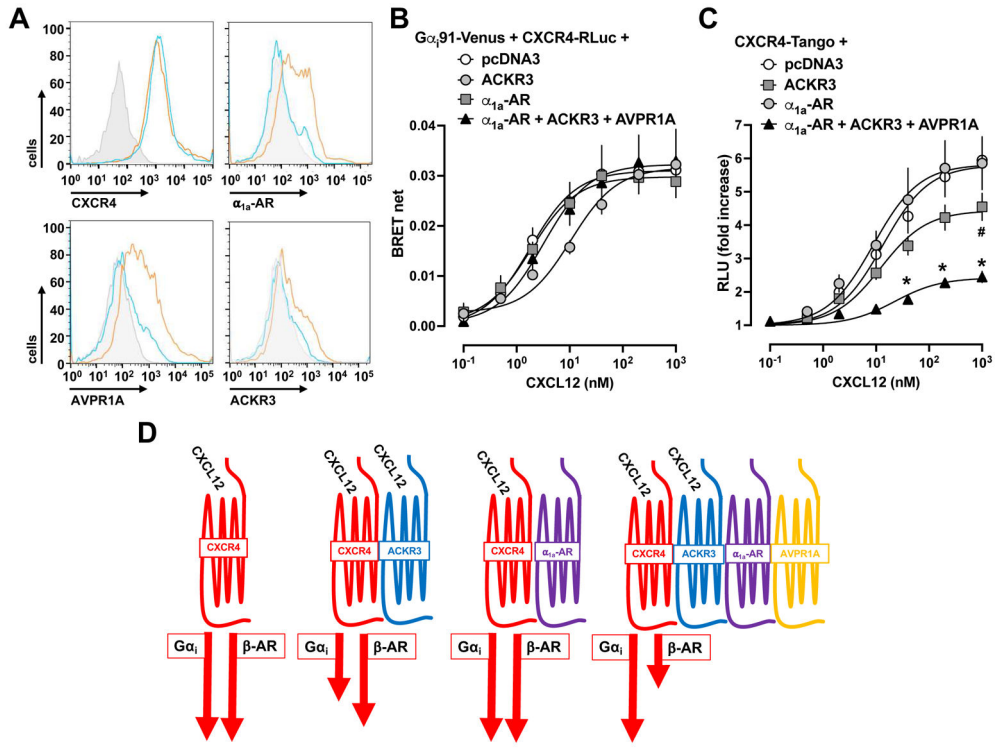

Figure 4.

Agonist-induced coupling of CXCR4 to signaling transducers is modulated by the presence of the heteromerization partners. (**a**) Quantification of receptor expression levels by flow cytometry in HTLA cells transfected with CXCR4-Tango alone (blue line) or with CXCR4-Tango plus α_{1a}-AR, AVPR1A and ACKR3 (orange lines). Grey areas: unstained cells. (**b**) BRET of CXCL12-induced engagement of CXCR4 with Gαi. Cells were co-transfected with CXCR4-RlucII, Gαi-91Venus plus pcDNA3 (control) or receptors as indicated. N=4. BRETnet: BRET ratios at various concentrations of the agonist subtracted by the ratios in the absence of agonist. (**c**) PRESTO-Tango assay to measure CXCL12-induced recruitment of β-arrestin to CXCR4. Cells were transfected with CXCR4-Tango plus pcDNA3 (control) or receptors as indicated. N=4. */#: $p<0.05$ vs. control. RLU (fold increase): Relative luminescence units (RLU) measured after stimulation with CXCL12 / RLU of unstimulated cells. (**d**) Simplified schematic summarizing the regulation of CXCL12-induced CXCR4 coupling to its signaling transducers within hetero-oligomeric receptor complexes. The lengths of the red arrows represent the general tendency of the changes in potency and/or efficacy of CXCL12 to induce coupling of CXCR4 to Gαi or β_arrestin (β_AR).

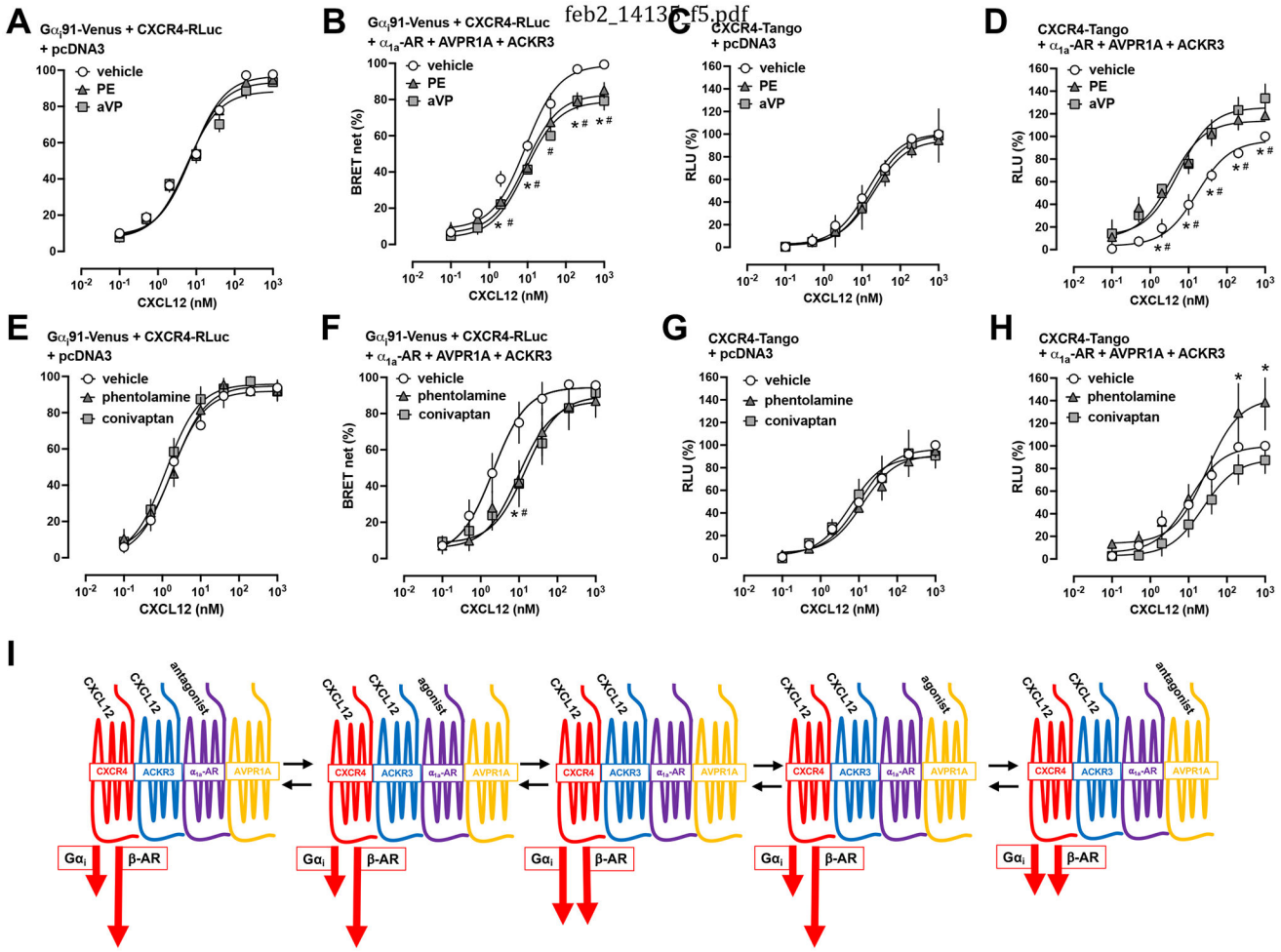

Figure 5.

Ligand-binding to heteromerization partners regulates agonist-induced coupling of CXCR4 to signaling transducers. BRET of CXCL12-induced engagement of CXCR4 with Gαi was measured in cells co-transfected with CXCR4-RlucII, Gαi-91Venus plus pcDNA3 (control) (**a/e**) or all heteromerization partners (**b/f**). PRESTO-Tango assays to measure CXCL12-induced recruitment of β-arrestin to CXCR4 were performed in cells transfected with CXCR4-Tango plus pcDNA3 (control) (**c/g**) or all heteromerization partners (**d/h**). Cells were co-stimulated with CXCL12 plus vehicle, phenylephrine (PE, 1 μM) or arginine vasopressin (aVP, 0.1 μM) (**a-d**) or pre-treated with vehicle, phentolamine (1 μM) or conivaptan (0.1 μM) for 15 min prior to CXCL12 stimulation (**e-h**). RLU (%): Relative luminescence units in % of the maximal RLU of cells treated with vehicle and CXCL12 (=100%). N=4 per condition. *: $p<0.05$ for control vs. phenylephrine or phentolamine. #: $p<0.05$ for control vs. aVP or conivaptan. (**i**) Simplified schematic summarizing the effects of ligand-binding to heteromerization partners on agonist-induced coupling of CXCR4 to signaling transducers. The lengths of the red arrows represent the general tendency of the changes in potency and/or efficacy of CXCL12 to induce coupling of CXCR4 to Gαi or β_arrestin (β_AR), as compared with cells stimulated with CXCL12 alone (center).

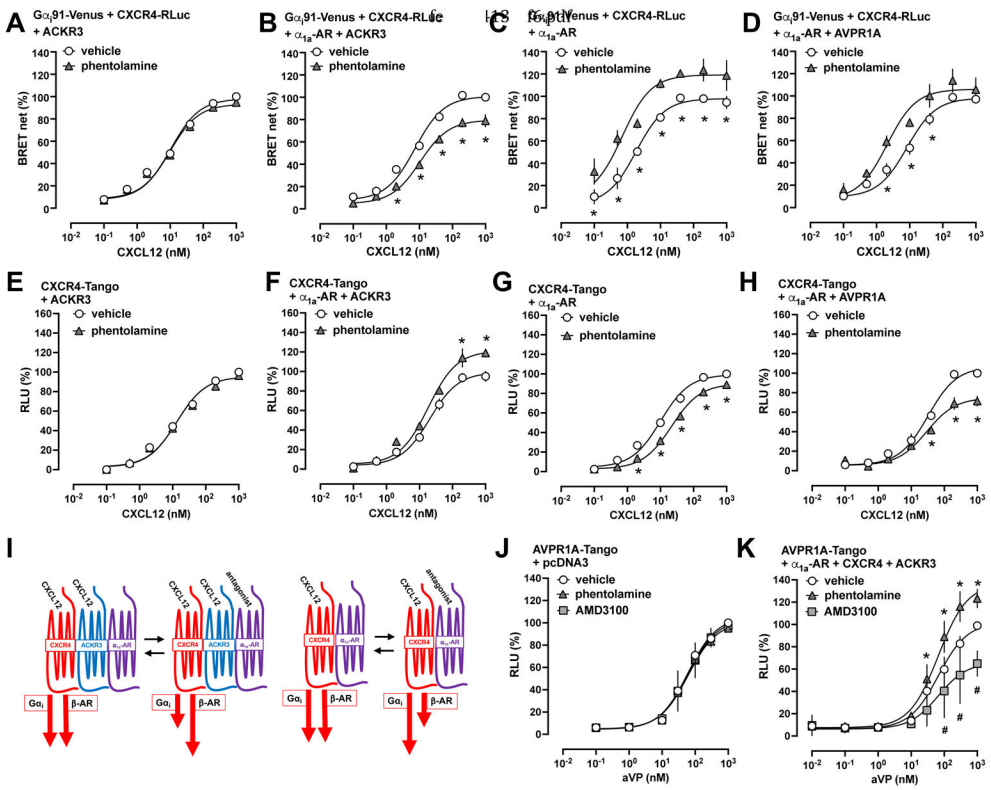

Figure 6.

The regulation of agonist-induced coupling of CXCR4 to signaling transducers by antagonist-binding to α_{1a}-AR depends on the composition of heteromerization partners and antagonist-binding to heteromerization partners regulates agonist-induced β-arrestin recruitment to AVPR1A. (**a-d**) BRET of CXCL12-induced engagement of CXCR4 with Gαi was measured in cells co-transfected with CXCR4-RlucII, G αi-91Venus plus receptors as indicated. PRESTO-Tango assays to measure CXCL12-induced recruitment of β-arrestin to CXCR4 were performed in cells transfected with CXCR4-Tango plus receptors as indicated (**e-h**). Cells were pre-treated with vehicle or phentolamine (1 μM) for 15 min prior to CXCL12 stimulation. N=4 per condition. *: $p<0.05$. (i) Simplified schematic summarizing the effects of of ACKR3 on CXCL12-induced CXCR4 activation in the presence of antagonist-bound α_{1a}-AR. The lengths of the red arrows represent the general tendency of the changes in potency and/or efficacy of CXCL12 to induce coupling of CXCR4 to Gαi or β_arrestin (β_AR), as compared with cells stimulated with CXCL12 alone (center). The presence and absence of ACKR3 resulted in the same effects when cells co-expressed CXCR4, α_{1a}-AR and AVPR1A (not depicted). (**j/k**) PRESTO-Tango assays to measure aVP-induced recruitment of β-arrestin to AVPR1A were performed in cells transfected with AVPR1A-Tango plus pcDNA3 (control, (**i**)) or all heteromerization partners (**j**). Cells were pre-treated with vehicle, phentolamine ((1 μM) or AMD3100 (1 μM) for 15 min prior to aVP stimulation. RLU (%): Relative luminescence units in % of the maximal RLU of cells treated with vehicle and CXCL12 or aVP (=100%). N=4 per condition. *: $p<0.05$ vehicle vs. phentolamine. #: $p<0.05$ vehicle vs. AMD3100.