Dual-agonist occupancy of orexin receptor 1 and cholecystokinin A receptor heterodimers decreases G-protein-dependent signaling and migration in the human colon cancer cell line HT-29

Bo Bai[1a]

Xiaoyu Chen[2b]

Rumin Zhang[1a]

Xin Wang[1a]

Yunlu Jiang[1a]

Dandan Li[1a]

Zhengwen Wang[1a]

Jing Chen[1a, 3c, *]

jing.chen@warwick.ac.uk

[1a]Neurobiology Institute, Jining Medical University, Jining, Shandong 272067, People's Republic of China

[2b]Department of physiology, Taishan Medical University, Taian, Shandong 271000, People's Republic of China

[3c]Division of Translational and Systems Medicine, Warwick Medical School, University of Warwick, Coventry CV4 7AL, United Kingdom

*Corresponding author at: Division of Translational and Systems Medicine, Warwick Medical School, University of Warwick, Coventry CV4 7AL, United Kingdom.

Abstract

The orexin (OX1R) and cholecystokinin A (CCK1R) receptors play opposing roles in the migration of the human colon cancer cell line HT-29, and may be involved in the pathogenesis and pathophysiology of cancer cell invasion and metastasis. OX1R and CCK1R belong to family A of the G-protein–coupled receptors (GPCRs), but the detailed mechanisms underlying their functions in solid tumor development remain unclear. In this study, we investigated whether these two receptors heterodimerize, and the results revealed novel signal transduction mechanisms. Bioluminescence and Förster resonance energy transfer, as well as proximity ligation assays, demonstrated that OX1R and CCK1R heterodimerize in HEK293 and HT-29 cells, and that peptides corresponding to transmembrane domain 5 of OX1R impaired heterodimer formation. Stimulation of OX1R and CCK1R heterodimers with both orexin-A and CCK decreased the activation of Gαq, Gαi2, Gα12, and Gα13 and the migration of HT-29 cells in comparison with stimulation with orexin-A or CCK alone, but did not alter GPCR interactions with β-arrestins. These results suggest that OX1R and CCK1R heterodimerization plays an anti-migratory role in human colon cancer cells.

Keywords: G-protein–coupled receptor (GPCR); hHeterodimerization; oOrexin (hypocretin) receptor; cCholecystokinin A receptor; bBioluminescence resonance energy transfer (BRET); Förster resonance energy transfer (FRET); mMigration

1.1 Introduction

G-protein–coupled receptors (GPCRs) respond to a variety of external stimuli and endogenous ligands and activate G-proteins. In response to their ligands, GPCRs undergo ligand-dependent conformation changes and dimerization. Dimerization is a critical aspect of GPCR structure and function, and has implications for receptor trafficking and signaling.

GPCRs form heterodimers as well as homodimers. For example, GPR103 and OX1R heterodimerize, as do apelin receptor and bradykinin 1 receptor [1,2]. GPCR heterodimers can exhibit elevated activity or

novel function. For example, dopamine receptor D2R and somatostatin receptor SSTR5 interact physically through hetero-oligomerization, resulting in elevated functional activity in response to dopamine and somatostatin [3].

Orexin-A and -B, neuropeptides secreted by orexin neurons, bind to the GPCRs orexin receptor 1 (OX1R) and 2 (OX2R). OX1R primarily couples with Gαq, whereas OX2R couples with Gαq, Gαi, and Gαs subunits [4]. Orexin receptors are expressed in human colon cancer cell lines, and orexin stimulation plays important roles in regulating various behavioral and physiological responses of colon cancer cells, including migration, proliferation, and invasion [5].

Cholecystokinin receptors are upregulated in many human digestive malignancies. Clinicopathological analysis revealed that elevated expression of the cytoplasmic cholecystokinin receptors CCK1R and CCK2R correlated significantly with indicators of colon cancer severity, including depth of tumor invasion, venous invasion, and stage of progression [6]. In addition, immunohistochemical staining showed that nuclear CCK1R expression is significantly associated with lymphatic invasion, stage of progression, and poorer survival. Together, these findings indicate that nuclear CCK1R represents a potential biomarker for poor prognosis in patients with colon cancer, and suggest that CCK1R plays an important role in colon carcinogenesis. Furthermore, stimulation of CCK2R promotes human colon cancer cell growth via cyclooxygenase-2 induction and stimulation of prostaglandin E2 production, providing further evidence that cholecystokinin receptors possess tumor-promoting activities in colon cancer [7].

Collectively, these findings suggest that the functional activities of OX1R and CCK1R in colon cancer cell lines are due to dimerization. To test this hypothesis, we investigated whether these two receptors heterodimerize, and our results revealed novel signal transduction mechanisms. In addition, we examined whether a novel heterodimerization-mediated signal transduction is involved in the migration of human cancer cells.

~~2.~~2 Materials and methods

~~2.1.~~2.1 Reagents, plasmids, agonists, and antibodies

Human orexin-A and CCK (26–33) were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). Plasmids pcDNA3.1-OX1R and pcDNA3.1-CCK1R were obtained from the UMR cDNA Resource Center (University of Missouri-Rolla, USA). Lipofectamine 2000 was obtained from Invitrogen (Grand Island, NY, USA), and Duolink II in situ proximity ligation assay (PLA) detection kits were obtained from Sigma-Aldrich (St. Louis, MO, USA). Goat polyclonal anti-CCKAR antibody (NB100-2805) was obtained from Novus Biologicals (Littleton, CO, USA), Goat polyclonal anti-α1B-AR antibody (sc-27136) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA), anti-OX1R antibody (ab68718) was obtained from Abcam (Cambridge, UK), and anti-HA and anti-Myc antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

OX1R-eGFP, OX1R-Rluc, CCK1R-Rluc, CCK1R-eGFP, α1B-AR-Rluc, and α1B-AR-eGFP were constructed as described; all of these constructs encode a C-terminal *Renilla* luciferase or eGFP tag [8]. Similarly, enhanced cyan fluorescent protein (eCFP) was attached to the C-terminus of CCK1R or α1B-AR, and enhanced yellow fluorescent protein (eYFP) to the C-terminus of OX1R, by inserting the ORF of CCK1R or α1B-AR into eCFP-N1 and the ORF of OX1R into eYFP-N1 (Clontech, Mountain View, CA, USA), yielding CCK1R-eCFP or α1B-AR-eCFP and OX1R-eYFP [4], respectively.

To construct hemagglutinin (HA)-tagged OX1R and Myc-tagged CCK1R, sequences encoding their epitope tags (YPYDVPDYA and EQKLISEEDL, respectively) were inserted into the N-termini of pcDNA3.1-OX1R and pc3.1-CCK1R, respectively, by PCR. The sequences of the resultant constructs, HA-OX1R and Myc-CCK1R, respectively, were confirmed by commercial sequencing.

To construct Gαi2-Rluc, Gαq-Rluc, Gαs-Rluc, Gα12, and Gα13 fusion proteins, the coding sequence of humanized Rluc8 (kindly provided by Dr. Sanjiv Gambhir of the Department of Nuclear Medicine of Stanford University) was PCR-amplified and inserted into the coding sequence of each Gα subunit. Rluc was inserted between residues 91 and 92 of Gαi2, residues 97 and 98 of Gαq, residues 113 and 114 of Gαs, residues 115 and 116 of Gα12, and residues 106 and 107 of Gα13 [9]. All constructs were confirmed by DNA sequencing.

~~2.2.~~2.2 HIV transactivator of transcription-linked peptides

HIV TAT (YGRKKRRQRRR) was fused to the C-termini of the OX1R TM5 and TM7 peptides, yielding the amino acid sequences SCFFFVTYLAPLGLMGMAYFQIFYGRKKRRQRRR and YACFTFSHWLVYANSAANPIIYNFYGRKKRRQRRR, respectively. The inserted peptides were confirmed to have the correct orientation, because HIV TAT binds to phosphatidylinositol-(4,5)-bisphosphate on the inner surface of the membrane [10]. After custom synthesis, the identity of each TM peptide sequence was determined using a LC-MS system (Shimadzu 2020 and Waters 1010). The molecular weights of TM 5 and 7 were 4209.11 and 4355.05 Da, respectively. As shown in Fig. 3A and B, all TMs were synthesized correctly.

~~2.3.~~2.3 Cell culture and transfection

HEK293 and HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Paisley, UK) containing 10% inactivated fetal bovine serum (FBS) at 37 °C with 5% CO_2. The cells were transiently transfected using Lipofectamine 2000. In all transfection experiments, the total amount of transfected DNA was kept constant by addition of the appropriate amount of empty vector.

~~2.4.~~2.4 Enzyme-linked immunosorbent assay (ELISA)

One day before transfection, HEK293 cells were plated in 96-well plates at a density of 1×10^4 cells per well. Transient transfection to express HA-OX1R and/or Myc-CCK1R was performed using Lipofectamine 2000. After an additional 24 h, cells were fixed for 30 min at room temperature with 4% paraformaldehyde in phosphate-buffered saline (PBS). The fixed cells were washed three times with PBS, and nonspecific binding sites were blocked with blocking buffer (3% dry milk in PBS). The cells were incubated overnight at 4 °C with rabbit polyclonal anti-HA or anti-Myc primary antibodies (1:500 each in blocking buffer), washed three times with PBS, and incubated with peroxidase-conjugated goat or rabbit secondary antibody in DMEM (1:2000 in blocking buffer) for 1 h at 37 °C. The cells were then incubated with 200 µl of 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich) for 30 min at 37 °C, the enzymatic reaction was stopped by adding 50 µl of 2 N H_2SO_4, and the optical density of each well at 450 nm was measured using an iMark Microplate reader (Bio-Rad, Hercules, California, USA). Optical densities were kept between 0.1 and 1.0 to avoid saturation.

~~2.5.~~2.5 Immunofluorescence assay

HEK293 cells were transfected with OX1R-eGFP and Myc-CCK1R plasmids in 12-well plates. After 24 h, the cells were distributed into 6-well plates at a density of 5×10^5 cells/well and cultured for an additional 24 h on glass coverslips pre-treated with 0.1 mg/ml poly-D-lysine. The cells were washed three times for 5 min each with PBS, fixed with 4% paraformaldehyde for 10 min, and then blocked with 1% bovine serum albumin for 1 h at room temperature. The coverslips were incubated with rabbit polyclonal anti-Myc primary antibody and secondary antibody labeled with Alexa Fluor 594 (Red), washed, and incubated with 4′,6-diamidino-2-phenylindole (DAPI) to stain nuclei. After additional washing, the coverslips were mounted onto glass microscope slides and examined using a Leica DMRE laser scanning confocal microscope (Leica, Milton Keynes, UK).

~~2.6.~~2.6 In situ PLA

Endogenous OX1R and CCK1R expression in HT-29 cells was assayed using Duolink II in situ PLA detection kits. Briefly, HT-29 cells were fixed in 4% paraformaldehyde for 15 min, washed with PBS, and incubated in blocking buffer for 1 h at 37 °C. The cells were subsequently incubated overnight at 4 °C with goat polyclonal anti-CCK1R antibody (Novus Biologicals) and rabbit polyclonal anti-OX1R antibody (Abcam). After washing with Duolink II buffer A at room temperature, the cells were incubated at 37 °C with PLA probes detecting rabbit or goat antibodies (Duolink II PLA probe anti-rabbit plus and Duolink II PLA probe anti-goat minus). After three washes with buffer A, the cells were incubated for 1 h at 37 °C with ligation solution, washed with buffer A, and then incubated for 100 min at 37 °C with amplification solution. The cells were washed twice with buffer B, immersed in 0.01 × Duolink II buffer B, and mounted using Duolink II Mounting Medium with DAPI. Punctate fluorescent signals indicative of close proximity (< 16 nm) [11] between OX1R and CCK1R and suggesting the formation of heterodimers were assessed by laser scanning confocal microscopy (Leica model DMRE; Leica, Milton Keynes, UK). Similar methods were used to detect the presence of OX1R and CCK1R heterodimers in HEK293 cells transfected with OX1R- and CCK1R-expressing vectors. HEK293 cells expressing OX1R alone or OX1R and α_{1b}-adrenoceptor were used as negative controls.

To explore the effects of TM peptides on OX1R and CCK1R dimerization, HEK293 cells were transfected with the plasmids pcDNA3.1-OX1R and pcDNA3.1-CCK1R in 12-well plates. After 24 h, the cells were distributed into 6-well plates and treated for 6 h with HIV TAT–fused TM peptides (10 µM) corresponding to TM5 or TM7 of OX1R, and then assayed for dimerization as described above.

~~2.7.~~2.7 BRET assays

HEK293 cells were transiently transfected with Rluc-tagged and eGFP- or GFP^2-tagged constructs. After 24 h, the cells were harvested in HEPES-buffered Phenol red–free complete medium containing 5% fetal calf serum (FCS), seeded in poly-D-lysine–coated 96-well plates, and incubated with acceptor pairs (Table ~~S~~1). Results were assessed using a Mithras LB940 plate reader (Berthold Technologies, Bad Wildbad, Germany) and the MicroWin 2000 software, as described [12,13].

Table 1 Substrates and filter settings used in BRET assays.

alt-text: Table 1

Method	Donor	Substrate	Donor ~~E~~emission [nm]	Acceptor	Acceptor ~~E~~emission [nm]
$BRET^1$	Rluc	Coelenterazine h	460	eGFP	535
$BRET^2$	Rluc	Deep Blue C™	400	GFP^2	515

eBRET	Rluc	EnduRen™	460	eGFP	535

~~2.8.~~2.8 FRET assays

FRET assays were performed as described [14–16]. Briefly, the donor plasmid CCK1R-eCFP and receptor plasmid OX1R-eYFP were co-transfected into HEK293 cells. The donor and acceptor channels were used to eliminate crosstalk in the FRET channel. To determine calibration coefficients corrected for excitation and emission crosstalk, CCK1R-eCFP and OX1R-eYFP were transfected individually into HEK293 cells as donor and acceptor channels, respectively. After 12–24 h, FRET signals were detected using the FRET Kit for the Leica AM TIRF MC system (Leica Microsystems). The efficiency of FRET (EA(i)) was calculated using the following equation:

$$EA(i) = \frac{B - A \times \beta - C \times (\gamma - \alpha \times \beta)}{C \times (1 - \beta \times \delta)}$$

where A, B, and C correspond to the intensities of the three signals (donor, FRET, and acceptor, respectively), and α, β, γ, and δ are the calibration factors generated by the acceptor- and donor-only references.

~~2.9.~~2.9 SRF-RE and NFAT-RE luciferase reporter assays

In luciferase reporter assays for serum response factor-response element (SRF-RE) and nuclear factor of activated T-cells-response element (NFAT-RE), cells expressing OX1R, CCK1R, or both were transfected with pSRF-RE-Luc or pNFAT-Luc PathDetect plasmid (Stratagene) containing the indicated response elements (REs) upstream of firefly luciferase, along with pRL-Tk (Promega), which encodes *Renilla* luciferase. After 24 h, the cells were starved and stimulated with orexin-A and/or CCK (26–33) for 6 h prior to harvesting. Luciferase activity was measured as described [4]. Firefly and *Renilla* luciferase activities were measured with a Dual-Luciferase Reporter (DLR) Assay System on a FLUOstar OPTIMA microplate reader (Promega).

~~2.10.~~2.10 Real-time analysis using the xCELLigence system

Prior to measurements, the xCELLigence real-time cell analysis (RTCA) dual-purpose (DP) system (ACEA) was initialized according to the manufacturer's instructions. HT-29 cells were plated at 5×10^4 cells/well in 100 µl of serum-free medium (upper chamber) and allowed to migrate toward the lower chamber (containing 5% FBS) for 24 h. Cells were treated with agonists as indicated. The xCELLigence system monitors the electrical impedance of the gold-sensing electrode underneath the cultured cell layer and calculates the arbitrary cell index (CI) as CI = (Zi − Z0)/15, where Zi represents the electrical impedance at a given time point and Z0 represents the background electrical impedance.

~~2.11.~~2.11 Statistical analysis

All data are shown as means ± SEM. Data were presented and analyzed using Prism 5.0 graphing software (GraphPad). Sigmoidal curves were fitted to the dose-response data using nonlinear regression. Statistical analysis was performed using one-way analysis of variance followed by Tukey's multiple comparison post-test.

~~3.~~3 Results

~~3.1.~~3.1 Co-expression and co-localization of OX1R and CCK1R in HEK293 cells

To rule out the possibility that co-expression of OX1R and CCK1R affects the expression levels and membrane distribution of both receptors, we assessed cell surface expression of OX1R and CCK1R by enzyme-linked immunosorbent assay (ELISA) using anti-HA and -Myc antibodies, respectively. Cell surface expression was unaffected by co-expression of both proteins in HEK293 cells (Fig. 1A).

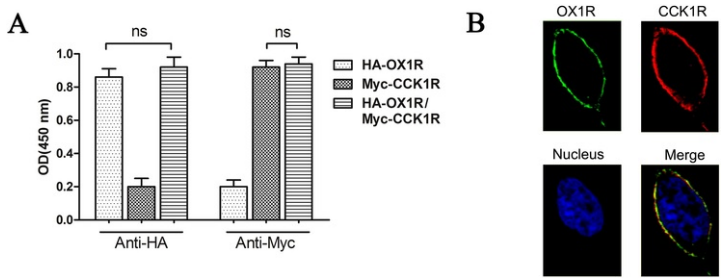

Fig. 1 Co-expression and co-localization of OX1R and CCK1R in HEK293 cells.

(A) ELISA assays of cell surface expression of HA-OX1R and Myc-CCK1R. Cells were transfected with HA-tagged OX1R and/or Myc-tagged CCK1R, and ELISA assays were performed on intact cells using anti-HA and anti-Myc antibodies. Data represen means ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison post hoc test (ns: not significant, $p > 0.05$).

(B) Confocal analysis of co-localization of OX1R and CCK1R in transfected HEK293 cells. C-terminally eGFP-tagged OX1R and N-terminally Myc-tagged CCK1R were expressed in HEK293 cells and visualized using laser confocal microscopy. Confocal images of OX1R and CCK1R were merged to show regions of co-localization. Nuclei were stained with the DNA-specific dye DAPI (blue).

Immunofluorescence image analysis of HEK293 cells co-expressing C-terminal eGFP-tagged OX1R and N-terminal Myc-tagged CCK1R confirmed that OX1R and CCK1R co-localized on cell membranes (Fig. 1B). The cell membrane expression of both proteins suggested that the two receptors might interact.

~~3.2.~~3.2 Heterodimerization of OX1R and CCK1R measured by BRET assays

We investigated the heterodimerization of OX1R and CCK1R using BRET saturation assays, which detect protein–protein interactions in living cells. We also observed the homodimerization of OX1R and CCK1R in the same manner. HEK293 cells were co-transfected with a constant amount of the luminescent donor (OX1R-Rluc) and increasing amounts of the fluorescent acceptor (CCK1R-eGFP, OX1R-eGFP, or α_{1b}-adrenoceptor-eGFP). The BRET ratio between OX1R-Rluc and CCK1R-eGFP increased with the expression of CCK1R-eGFP or OX1R-eGFP until a plateau was reached (Fig. 2A). Moreover, the BRET ratio of homodimerization of OX1R or CCK1R was significantly reduced. In the negative control group, however, increasing expression of α_{1b}-adrenoceptor-eGFP resulted in a low BRET ratio, suggesting a nonspecific linear relationship. This is consistent with a previous report that BRET signals are low when OX1R-Rluc8 and α_{1b}-adrenoceptor-eYFP are co-transfected [17]. These results indicate that OX1R-Rluc and CCK1R-eGFP can form heterodimers. Receptor constructs in the “opposite” configuration (CCK1R-Rluc and OX1R-eGFP) were also tested to confirm the dimerization of OX1R and CCK1R (Fig. 2B). As reported [18], increasing expression of secretin receptor (SecR-eGFP) also resulted in a low BRET ratio.

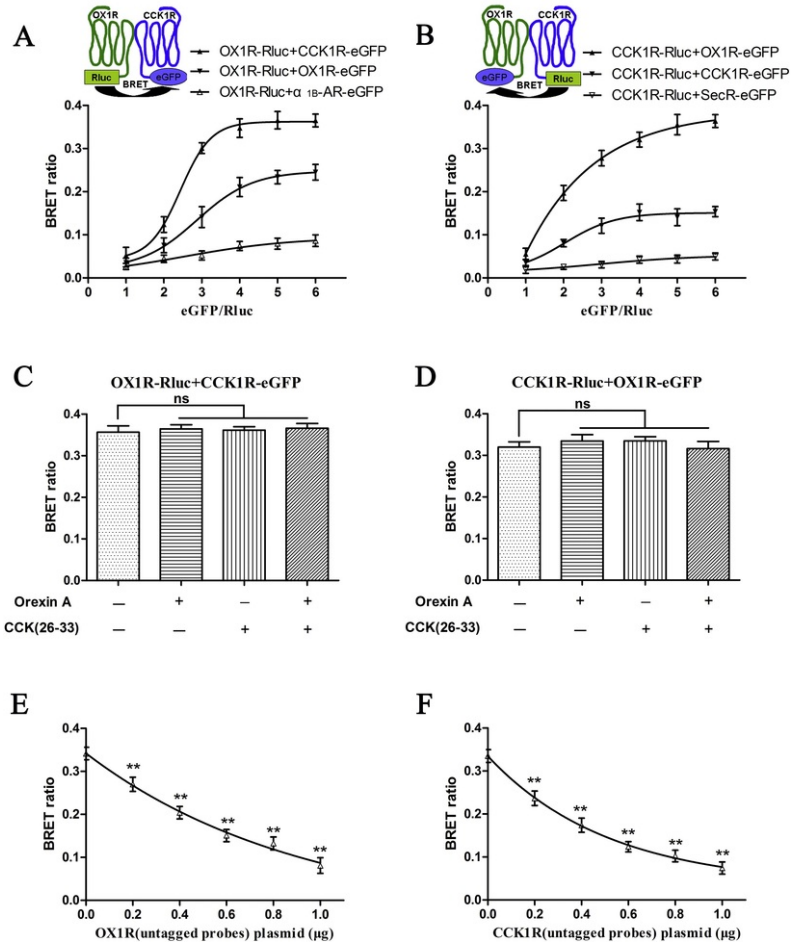

Fig. 2 Heterodimerization of OX1R and CCK1R, demonstrated by BRET assays in live cells.

(A and B) HEK293 cells were co-transfected with a constant amount of the OX1R-Rluc (A) or CCK1R-Rluc (B) construct, each at 0.15 μg/well, and increasing amounts of the eGFP construct (0.15–0.9 μg/well). Calculated BRET ratios were plotted relativ to total fluorescence/luminescence ratios, and data were analyzed by nonlinear regression curve fitting (one site–specific binding) using GraphPad Prism. BRET ratios were analyzed and expressed as means ± SEM of four experiments.

(C and D) Effects of orexin-A and/or CCK (26–33) on BRET ratios. Plasmids expressing OX1R-Rluc and CCK1R-eGFP (C) or CCK1R-Rluc and OX1R-eGFP (D) were co-transfected (1:3) into HEK293 cells. Twenty-four hours later, the Rluc substrate coelenterazine-h was added for 5 min, and the cells were treated with orexin-A (100 nM), CCK (26–33) (1000 nM), or vehicle for 10 min. BRET ratios were analyzed and expressed as means ± SEM of four experiments (one-way analysis of variance; ns: not significant, $p > 0.05$).

(E and F) Competition binding assay. HEK293 cells were co-transfected with a fixed amount of CCK1R-Rluc and OX1R-eGFP plasmids and increasing amounts of OX1R (C) or CCK1R (D) not fused to a donor or acceptor BRET probe. Data represent means ± SEM of three independent experiments. **, $p < 0.01$ vs. control groups.

alt-text: Fig. 2

Additional BRET assays were performed to determine whether ligands induce heterodimerization. BRET ratios were observed in HEK293 cells co-transfected with OX1R-Rluc and CCK1R-eGFP at a 1:3 ratio and treated with orexin-A, in cells treated with CCK (26–33) alone, and in cells treated for 10 min with orexin-A and CCK (26–33) (Fig. 2C). The BRET ratios of these cells were 0.364 ± 0.010, 0.361 ± 0.009, and 0.366 ± 0.012, respectively. When the experiment was repeated following energy donor–acceptor exchange between the two receptors, orexin-A or/and CCK (26–33)-induced BRET ratios remained unchanged (Fig. 2D). These results implied that ligand binding might not be

necessary for heterodimerization of OX1R and CCK1R. Moreover, sequential addition of orexin-A and CCK (26–33) did not similarly alter the BRET ratio between OX1R and CCK1R (Fig. S1).

To perform competitive BRET analysis, we co-expressed fixed amounts of CCK1R-Rluc/OX1R-eGFP and increasing amounts of unlabeled OX1R or CCK1R in HEK293 cells. BRET signals decreased as the concentration of unlabeled OX1R or CCK1R increased (Fig. 2E and F). These data further confirmed the specific formation of OX1R and CCK1R heterodimers.

Furthermore, BRET ratios were significantly reduced after incubation with HIV TAT–fused TM5, but not TM7 peptide, for each energy donor–acceptor combination, suggesting that TM5 impairs the heterodimerization of OX1R and CCK1R (Fig. 3C and D).

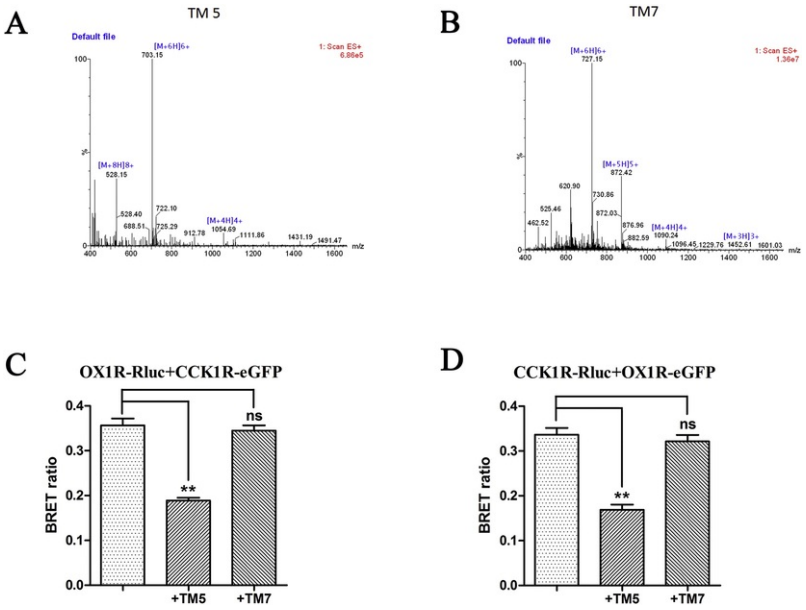

Fig. 3 Effects of interfering peptides on OX1R and CCK1R heterodimers.

(A and B) Identification of transmembrane domain (TM) peptide sequences. Peptide sequences of (A) TM5 and (B) TM7 of OX1R, measured by liquid chromatography–mass spectrometry (LC-MS).

(C and D) HEK293 cells were co-transfected with OX1R-Rluc and CCK1R-eGFP (C) or CCK1R-Rluc and OX1R-eGFP (D) and incubated at 37 °C for 2 h with HIV TAT–fused TM peptides (10 μM) corresponding to TM5 or TM7 of OX1R. BRET ratios were analyzed and expressed as means ± SEM of four experiments (one-way analysis of variance; ns: not significant, $p > 0.05$; **, $p < 0.01$ vs. control group).

alt-text: Fig. 3

~~3.3.~~3.3 Heterodimerization of OX1R and CCK1R determined by FRET assays

We also used FRET to explore the interaction between OX1R and CCK1R. For these FRET assays, HEK293 cells were transfected with OX1R-eYFP and/or CCK1R-eCFP. Sites of interaction on the acquired FRET images are marked yellow or red, with more intense color indicating a stronger interaction (Fig. 4A). Lower FRET signals were observed in all singly transfected cells and co-transfected α_{1b}-adrenoceptor-eCFP and OX1R-eYFP cells, whereas FRET signals were significantly higher in cells transfected with both CCK1R-eCFP and OX1R-eYFP (Fig. 4A and B), further confirming that OX1R and CCK1R can form heterodimers.

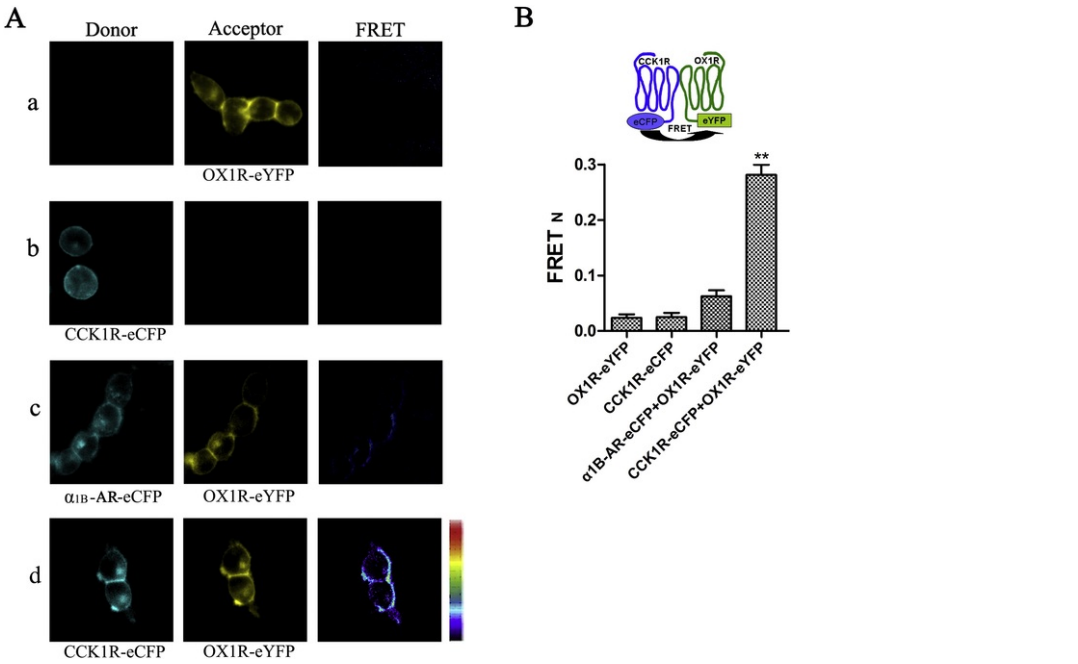

Fig. 4 FRET assays of heterodimerization of OX1R and CCK1R.

(A) FRET imaging of constitutive OX1R–CCK1R heteromeric interactions in living cells. HEK293 cells were transiently transfected with the plasmids encoding (a) OX1R-eYFP, (b) CCK1R-eCFP, (c) α_{1b}-adrenoceptor-eCFP and OX1R-eYFP, or (d) CCK1R-eCFP and OX1R-eYFP. Left-hand panels, CFP; center panels, YFP; right-hand panels, corrected FRET.

(B) Normalized FRET values, calculated as described in Experimental Procedures. Data represent means ± SEM of four independent experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison post ho test. **, $p < 0.01$ vs. other groups.

alt-text: Fig. 4

~~3.4.~~3.4 In situ PLA confirms formation of endogenous OX1R and CCK1R heterodimers in HT-29 cells

In situ PLA is sufficiently sensitive to detect molecular interactions between two endogenous proteins. In this regard, it is similar to immunoprecipitation, but it has the advantage that it does not require membrane solubilization. Labeling heterodimers by PLA requires both receptors to be sufficiently close (< 16 nm) to allow two antibody–DNA probes to form double-stranded segments. The signal is further amplified in the presence of fluorescent nucleotides [19]. First, PLA was used to determine the expression of endogenous OX1R and CCK1R heterodimers in HT-29 cells. OX1R–CCK1R heterodimers in cultured HT-29 cells were detected as red punctate staining (Fig. 5A, b). As expected, red dots were also detected in HEK293 cells stably co-expressing both OX1R and CCK1R (Fig. 5B, c), as well as in cells expressing only OX1R or OX1R and α_{1b}-adrenoceptor, used as controls (Fig. 5B, a and b), suggesting that endogenous OX1R–CCK1R heterodimers were present in both cell lines. Pre-incubation with TM5 peptides, but not TM7 peptides, dramatically decreased the amount of positive PLA products in HEK293 cells co-expressing OX1R and CCK1R (Fig. 5B, d and e, C), confirming the BRET results showing that TM5 peptides disrupted the heterodimerization of OX1R and CCK1R.

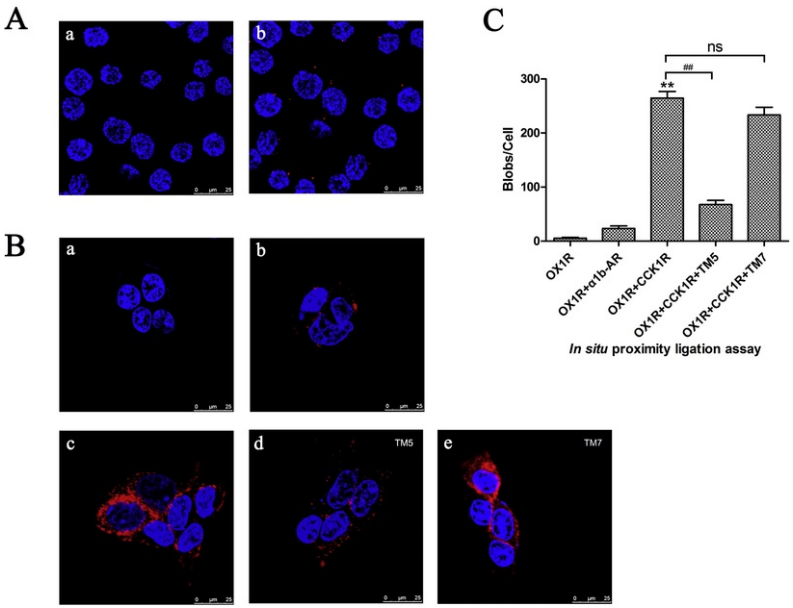

Fig. 5 Confirmation of OX1R and CCK1R heterodimers by in situ proximity ligation assay (PLA).

(A) In situ PLA was performed in HT-29 cells to detect heterodimers between endogenously expressed OX1R and CCK1R. HT-29 cells were incubated with (a) anti-OX1R antibody (control) or (b) anti-CCK1R antibody and anti-OX1R antibody. Punctate fluorescent signals (red dots) indicating close proximity of OX1R and CCK1R, implying heterodimer formation, were revealed by confocal microscopy.

(B) Detection of OX1R and CCK1R heterodimers in HEK293 cells. Confocal microscopy images from PLA experiments performed in HEK293 cells expressing (a) OX1R alone, (b) α_{1b}-adrenoceptor and OX1R, or (c) both OX1R and CCK1R. HEK293 cells expressing OX1R and CCK1R were treated with HIV TAT–fused TM peptides (10 μM) corresponding to (d) TM5 or (e) TM7 of OX1R. Heteromeric complexes appear as red spots and cell nuclei in blue (DAPI). Scale bars, 25 μm.

(C) In situ PLA signals, signifying the OX1R–CCK1R interaction, were quantified as blobs/cell. Data represent means ± SEM of three independent experiments. Statistical significance was assessed by a paired Student's *t*-test. **, $p < 0.01$ vs. OX1R alone or α_{1b}-adrenoceptor and OX1R group; ##, $p < 0.01$ for the indicated pairs; ns, not significant.

alt-text: Fig. 5

~~3.5.~~3.5 G-protein activation patterns associated with OX1R and CCK1R heterodimers

BRET^2 assays were performed to determine the activation patterns of Gαi2, Gαq, Gα12, Gα13, and Gαs in association with heterodimers. The BRET^2 probe measured the distance between the helical domain of Gα (Gα-Rluc8) and the N-terminus of $G\gamma_2$ (GFP^2-$G\gamma_2$) during GDP–GTP exchange. The BRET^2 signal was reduced following receptor activation [20,21].

We next measured BRET^2 signals in HEK293 cells co-transfected with plasmids expressing GFP^2-tagged $G\gamma_2$ and Gβ1- and Rluc8-tagged Gα [Gαq-Rluc8 (A); Gαi2-Rluc8 (B); Gα12-Rluc8 (C); Gα13-Rluc8 (D); and Gαs-Rluc8 (E)], as well as pcDNA3.1-OX1R and/or pcDNA3.1-CCK1R (Fig. 6). The cells were stimulated with 100 nM orexin-A and/or 1000 nM CCK (26–33).

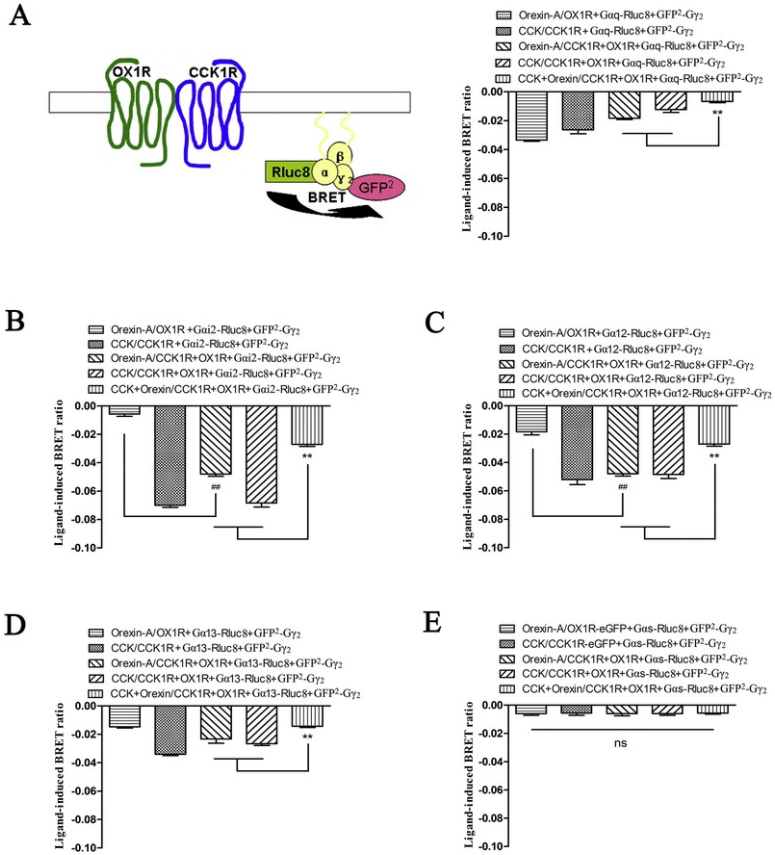

Fig. 6 BRET analysis of the effects of OX1R-CCK1R heterodimerization on Gα protein subunit activation.

HEK293 cells expressing OX1R and/or CCK1R were stimulated with 100 nM orexin-A and/or 1000 nM CCK (26–33) for 1 min, and the levels of expression of GFP^2-Gγ2, Gβ1, and (A) Gαq-Rluc8, (B) Gαi2-Rluc8, (C) Gα12-Rluc8, (D) Gα13-Rluc8, or (E) Gαs Rluc8 were measured. Data represent means ± SEM of at least four independent experiments. $^{\#\#}$, $p < 0.01$, cells co-expressing both OX1R and CCK1R treated with 100 nM orexin-A vs. cells expressing OX1R alone; **, $p < 0.01$, cells co-expressing both OX1R and CCK1R treated with 100 nM orexin-A and 1000 nM CCK (26–33) vs. orexin-A or CCK (26–33) alone; ns: not significant, as determined by a paired Student's *t*-test.

alt-text: Fig. 6

We observed substantial reductions in $BRET^2$ signals for Gαq, Gα12, Gα13-Rluc, and GFP^2-$G\gamma_2$ when HEK293 cells expressing OX1R alone were stimulated with orexin-A, and for Gαq, Gαi2, Gα12, Gα13-Rluc, and GFP^2-$G\gamma_2$ when HEK293 cells expressing CCK1R alone were stimulated with CCK (26–33) (Fig. 6A, B, C, and D). Taken together, these findings indicate that OX1R couples to Gαq, Gα12, and Gα13, whereas CCK1R couples to Gαq, Gαi2, Gα12, and Gα13. Interestingly, when compared with HEK293 cells expressing OX1R alone, $BRET^2$ signals for Gαi2, Gα12-Rluc8, and GFP^2-$G\gamma_2$ subunits were further reduced when HEK293 cells co-expressing both OX1R and CCK1R were stimulated with orexin-A, indicating that OX1R and CKK1R heterodimers induce unique patterns of G-protein activation upon stimulation with a single agonist (Fig. 6B and C).

Furthermore, in comparison with cells treated with a single agonist, HEK293 cells co-expressing OX1R and CCK1R exhibited significantly lower $BRET^2$ signals for Gαq, Gαi2, Gα12, Gα13-Rluc8, and GFP^2-$G\gamma_2$ following co-stimulation with orexin-A and CCK (26–33) (Fig. 6A, B, C, and D). These findings suggest that dual-agonist occupancy of OX1R and CCK1R heterodimers induces unique conformational changes in these receptors, reducing the dissociation of G-protein subunits. Notably, however, $BRET^2$ signals for Gαs-Rluc8 and GFP^2-$G\gamma_2$ were unaltered, suggesting that heterodimerization between OX1R and CCK1R has little effect on Gαs activation after stimulation with orexin-A and/or CCK (26–33) (Fig. 6E).

To clarify the effect of OX1R and CCK1R heterodimerization on G-protein activation, we incubated HEK293 cells co-expressing OX1R and CCK1R with HIV TAT–fused TM peptides corresponding to TM5 or TM7 of OX1R, and

then observed the activation patterns of Gαq or Gαi2 in association with heterodimers. BRET^2 signals for Gαq and GFP^2-Gγ_2 subunits were significantly reduced after incubation with HIV TAT–fused TM5, but not TM7, following treatment of orexin-A or/and CCK (26–33) (Fig. 7A). Orexin-A–induced Gαi2 activation was unexpected after incubation with HIV TAT–fused TM5, because OX1R is thought to couple to Gαq, Gα12, and Gα13, and orexin-A is unable to bind CCK1R (Fig. 7B). This observation suggests that the orexin-A–induced Gαi2 activation is triggered by heterodimerization of OX1R and CCK1R. CCK (26–33) did not modify the Gαi2 activation profile in the presence of HIV TAT–fused TM5 or TM7 peptide, indicating that OX1R–CCK1R heterodimers or CCK1R alone can couple to Gαi2 protein (Fig. 7B). Our earlier results showed that OX1R–CCK1R heterodimers decreased Gαi2 activation by co-stimulation with orexin-A and CCK (26–33) (Fig. 6B). By contrast, Gαi2 activation was elevated after incubation with HIV TAT–fused TM5, but not TM7 (Fig. 7B). These observations are consistent with the conformational changes in OX1R–CCK1R heterodimers induced by TM5 peptides.

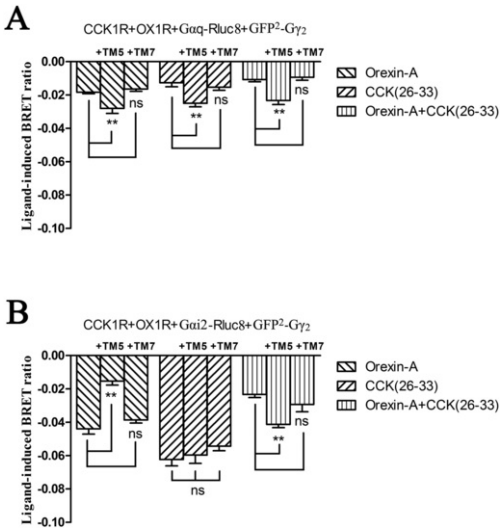

Fig. 7 Effects of HIV TAT–TM peptides on Gα protein subunit activation.

HEK293 cells co-expressing OX1R and CCK1R were incubated at 37 °C for 2 h with HIV TAT–fused TM peptides (10 μM) corresponding to TM5 or TM7 of OX1R. BRET was used to analyze the levels of expression of GFP^2-Gγ2, Gβ1, and (A) Gαq-Rluc8 o (B) Gαi2-Rluc8, stimulated with 100 nM orexin-A and/or 1000 nM CCK (26–33) for 1 min. Data represent means ± SEM of four independent experiments. Statistical significance was assessed by Student's *t*-test, comparing cells treated with TM5 or TM7 with untreated cells (**, $p < 0.01$; ns: not significant, $p > 0.05$).

alt-text: Fig. 7

~~3.6.~~3.6 OX1R and CCK1R heterodimerization does not alter recruitment of β-arrestins

Stimulation of HEK293 cells expressing OX1R-Rluc and β-arrestin1-eGFP with 100 nM orexin-A resulted in a robust and continually increasing ligand-induced BRET signal, indicating the recruitment of β-arrestin1 to activated OX1R (Fig. 8A). Incubation of these cells with 1000 nM CCK (26–33) alone did not increase BRET signals, whereas co-treatment of these cells with orexin-A and CCK (26–33) resulted in a kinetic profile similar to that observed upon treatment with orexin-A alone (Fig. 8A). Orexin-A treatment of cells transfected with untagged CCK1R, OX1R-Rluc, and β-arrestin1-EGFP increased BRET signals, with a similar kinetic profile but reduced magnitude (Fig. 8B). However, incubation of cells co-expressing CCK1R and OX1R with CCK (26–33) increased BRET signals, indicating the proximity of CCK1R and OX1R in heteromeric complexes that recruit β-arrestin1 (Fig. 8B). Similarly, β-arrestin2 was recruited to these heteromeric complexes in a ligand-dependent manner (Fig. 8C and D).

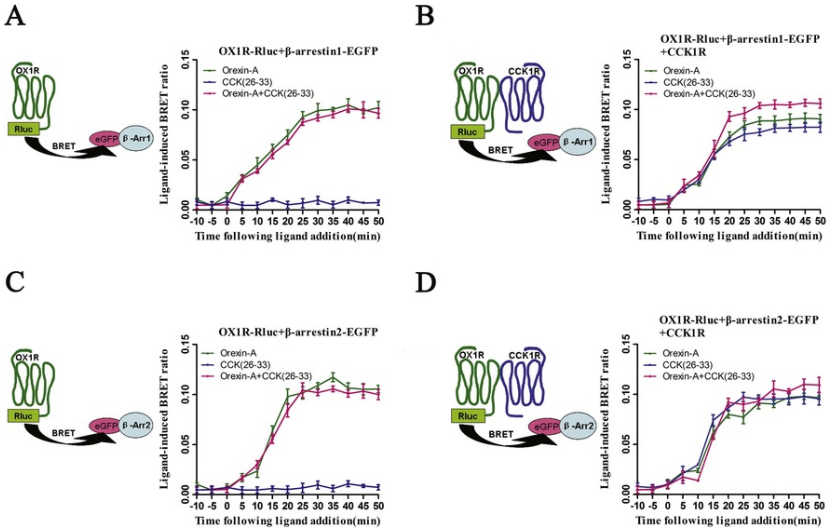

Fig. 8 Characterization of β-arrestin recruitment to OX1R–CCK1R heterodimers.

HEK293 cells transiently co-expressing OX1R-Rluc and (A, B) β-arrestin1-eGFP or (C, D) β-arrestin2-eGFP in the absence (A, C) or presence (B, D) of CCK1R were monitored by extended BRET for 10 min at 37 °C to generate kinetic curves. Following the addition of orexin-A (100 nM) and/or CCK (26–33) (1000 nM) or vehicle, monitoring was continued for an additional 50 min. Data represent means ± SEM of four independent experiments.

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When the experiment was repeated following exchange of energy donor, stimulation of HEK293 cells expressing CCK1R-Rluc, β-arrestin1-eGFP, and untagged OX1R with 100 nM orexin-A resulted in a robust and continually increasing ligand-induced BRET signal, indicating the recruitment of β-arrestin1 and β-arrestin2 to CCK1R by orexin-A, dependent on OX1R expression (Fig. S2).

~~3.7.~~3.7 Heterodimerization of OX1R and CCK1R decreases NFAT-RE and SRF-RE activity

OX1R and CCK1R couple to Gαq or Gα12 and induce transcriptional activation via NFAT-RE and SRF-RE. To determine the effects of OX1R and CCK1R heterodimers on downstream signaling, we measured the activities of NFAT-RE and SRF-RE in HEK293-OX1R, HEK293-CCK1R, and HEK293-OX1R/CCK1R cells. Treatment of HEK293 cells co-expressing OX1R and CCK1R with orexin-A or CCK (26–33) resulted in significantly lower NFAT-RE activity than similar treatment of cells expressing OX1R or CCK1R alone (Fig. 9A). In addition, dual-agonist treatment of HEK293 cells co-expressing both receptors further decreased NFAT-RE activity (Fig. 9A). Similarly, although CCK (26–33) treatment increased SRF-RE activity in HEK293-CCK1R cells, SRF-RE activity was significantly lower in HEK293-OX1R/CCK1R cells stimulated with CCK (26–33), and dual-agonist treatment further decreased SRF-RE activity (Fig. 9C). These findings indicate that dual-agonist occupancy of OX1R and CCK1R heterodimers decreases Gαq- and Gα12-dependent downstream signaling.

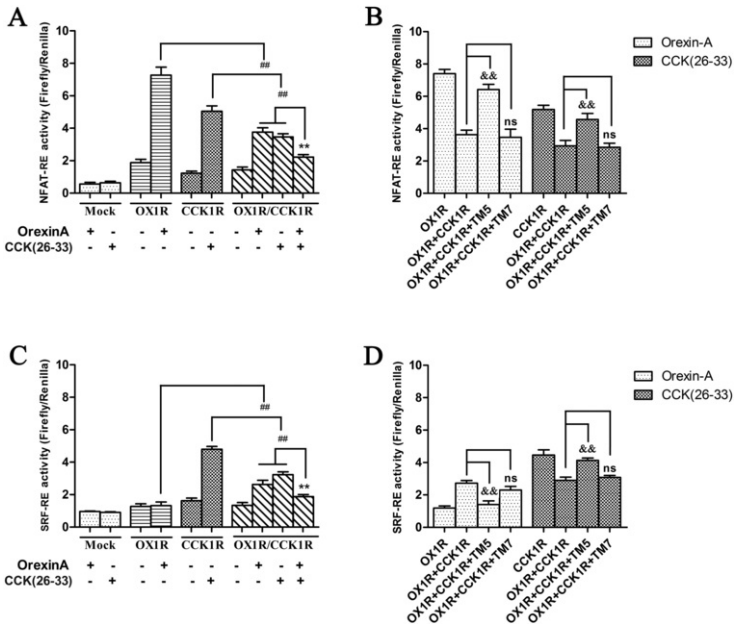

Fig. 9 Effect of OX1R and CCK1R heterodimerization on NFAT-RE and SRF-RE activities.

(A and C) Twenty-four hours after transfection with NFAT-RE (A) or SRF-RE (C), with RL-Tk, HEK293-OX1R, HEK293-CCK1R, and HEK293-OX1R/CCK1R cells were treated with 100 nM orexin-A and/or 1000 nM CCK (26–33) for 6 h prior to harvest.

(B and D) Twenty-four hours after transfection with NFAT-RE (B) or SRF-RE (D), with RL-Tk, HEK293 cells co-expressing OX1R and CCK1R were incubated at 37 °C for 2 h with HIV TAT–fused TM peptides (10 μM) corresponding to TM5 or TM7 of OX1R. Cells were treated with 100 nM orexin-A or 1000 nM CCK (26–33) for 6 h prior to harvesting. Firefly and *Renilla* luciferase activities were assayed using a Dual-Luciferase Reporter Assay System, and ratios of firefly to *Renilla* luciferase luminescence were calculated. Data represent means ± SEM of at least four independent experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison post hoc test. $^{\#\#}$, $p < 0.01$, cells co-expressing both OX1R and CCK1R treated with orexin-A or CCK (26–33) vs. cells expressing OX1R or CCK1R alone; **, $p < 0.01$, cells co-expressing both OX1R and CCK1R treated with orexin-A and CCK (26–33) vs. orexin-A or CCK (26–33) alone; $^{\&\&}$, $p < 0.01$, TM5 treated vs. untreated cells; ns: not significant, $p > 0.05$.

alt-text: Fig. 9

To further clarify the effect of OX1R and CCK1R heterodimers on NFAT-RE and SRF-RE, we incubated HEK293 cells co-expressing with OX1R and CCK1R with HIV TAT–fused TM peptides corresponding to TM5 or TM7 of OX1R. Incubation with HIV TAT–fused TM5, but not TM7, resulted in significantly higher NFAT-RE activity than treatment with orexin-A or CCK (26–33) alone (Fig. 9B). Finally, incubation with TM5 decreased induction of SRF-RE activity by orexin-A treatment, but increased SRF-RE activity in response to CCK (26–33) treatment (Fig. 9D). This observation is consistent with our earlier data that TM5 impaired the conformation of OX1R and CCK1R heterodimerization.

~~3.8.~~3.8 Heterodimerization of OX1R and CCK1R alters the cell migration using RTCA

The xCELLigence system records the electrical impedance of a gold-sensing electrode beneath a cultured cell layer in the form of arbitrary CI units, and can thus monitor cell migration in real time. The CI values of HT-29 cells treated with both orexin-A (100 nM) and CCK (26–33) (1000 nM) were significantly lower than those of cells treated with orexin-A or CCK (26–33) alone, suggesting that dual-agonist occupancy of OX1R and CCK1R heterodimers affects the migration of HT-29 human colon cancer cells (Fig. 10). The cell migration data provide further evidence in support of the hypothesis that heterodimerization of OX1R and CCK1R mediates a unique response of HT-29 cells to dual-agonist stimulation.

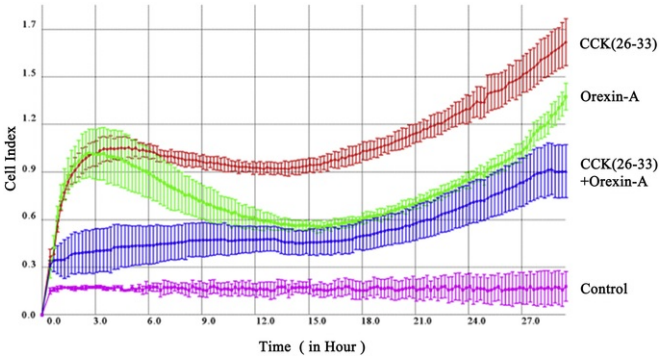

Fig. 10 Heterodimerization of OX1R and CCK1R alters cancer cell migration.

Migration CI curves of HT-29 cells during differentiation, measured by RTCA assay. HT-29 cells were treated with orexin-A (100 nM) and/or CCK (26–33) (1000 nM), and the resultant CI growth curves were compared. Data represent means ± SEM of four independent experiments.

alt-text: Fig. 10

4.4 Discussion

It is well established that GPCRs can exist as monomeric entities and form dimers and/or oligomers when expressed in a heterologous cell system, as well as in native tissues. For example, human muscarinic M_1 receptor is present on the basolateral surface of cells as a 75:25 mixture of receptor monomers and dimers/oligomers [22]. However, the proportion of these forms in a receptor population can be regulated by interaction with ligands or other modulators, and these effects have major implications for receptor function and behavior.

Accumulating evidence indicates that GPCRs are involved in cancer development, including proliferation, migration, and invasion. Furthermore, GPCR dimerization is regarded as important in fine-tuning the behavior of cancer cells. For example, a synthetic peptide that blocks the dimerization of CXCR4 inhibits malignant cell migration in response to CXCL12/SDF-1A [23,24].

Both orexin and cholecystokinin receptors are involved in migration of human colon cancer cells. In this study, we found that OX1R and CCK1R were co-expressed in the human colon cancer cell line HT-29. In addition, we analyzed OX1R and CCK1R dimerization by BRET, FRET, and in situ PLA assays in HEK293 cells. The PLA results confirmed that OX1R and CCK1R heterodimerize in HT-29 cells, indicating that these dimers are functional in vivo. Our investigation of the effects of interfering peptides on interactions between OX1R and CCK1R heterodimers revealed that peptides encoding TM5, but not TM7, could bind to OX1R, suggesting that TM5 is involved in interface formation. These findings suggest that these interfering peptides have therapeutic potential, as they can disrupt dimerization and influence receptor function. Indeed, a peptide derived from TM6 of β2AR disrupts dimer formation and decreases receptor function [25], whereas a synthetic peptide that disrupts the dimerization of CXCR4 inhibits malignant cell migration in response to CXCL12/SDF-1A [24].

GPCR dimerization is of physiological relevance and often acts as the first step in the induction of intracellular signals in response to ligand binding [26–28]. GPCR dimer–monomer interconversion may be important for precise control of signal transduction [29–31]. For example, heterodimerization of APJ and the κ-opioid receptor (KOR) affects cAMP accumulation after treatment with apelin-13 or DynA(1–13) [32]. In the ventral tegmental area, heterodimers of orexin receptor and corticotropin-releasing factor receptor serve as targets for cocaine and promote long-term disruption of negative crosstalk between orexin-A and CRF [33]. Heteromultimerization of cannabinoid (CB) 1 receptor and OX1R generates a unique complex, in which both protomers are regulated by orexin-A [34]. We also found that heterodimerization of OX1R and CCK1R led to alterations in signaling. Although OX1R and CCK1R were previously reported to couple with Gαq and Gα12/13, respectively, BRET revealed that co-stimulation of both OX1R and CCK1R in HT-29 cells decreased the dissociation of Gαq, Gαi, Gα12, and Gα13 subunits following stimulation with both orexin-A and CCK (26–33), indicating that activation of Gαq, Gαi, Gα12, and Gα13 may be reduced by dual-agonist occupancy of OX1R/CCK1R heterodimers. By contrast, activation of Gαs was not significantly changed. Indeed, occupancy by the dual agonists norepinephrine (NE) and angiotensin II (AngII) causes the conformation of the heterodimer of AngII AT1 receptor (AT1-R) and NE α2C-adrenergic receptor (α2C-AR) to differ from that induced by individual active protomers, and triggers atypical Gαs-cAMP–PKA signaling [35]. Thus, dual-agonist occupancy may result in novel functional activities of GPCR heterodimers, suggesting a promising new target for therapeutics. The findings reported in the present study are consistent with this, in that dual-agonist stimulation of OX1R and CCK1R induced significant changes in downstream signaling, suggesting that GPCR dimerization is an underlying mechanism of fine-tuning of receptor function, especially upon dual-agonist stimulation.

We used reporter gene assays to measure the activation of Gα subunits after dual-agonist stimulation. Upon activation of GPCRs, the Gα protein subunit dissociates from the βγ-dimeric subunit, initiating a cascade of downstream second-messenger pathways. This may eventually induce gene transcription by various REs, including cAMP response element (CRE), NFAT-RE, serum response element (SRE), and SRF-RE, corresponding to the Gαs, Gαi, Gαq, and Gα12 pathways, respectively. The results of this study also suggest that dual-agonist stimulation reduces NFAT-RE and SRF-RE activities, suggesting that decreased Gαq and Gα12 activation also affects downstream gene transcription.

Furthermore, OX1R and CCK1R heterodimerization does not alter the recruitment of β-arrestins, which mediate the internalization of heterodimers, resulting in reduced G-protein activation and downstream gene transcription. Several studies suggest coordinated trafficking of GPCR heterodimers [34,36,37], but these reports are contradicted by others suggesting that monomeric receptors predominate following internalization [38]. Our findings did not resolve whether native OX1R and CCK1R monomeric or OX1R and CCK1R heterodimerization enter the clathrin-mediated endocytosis pathway, but it is possible that stabilized receptors undergo internalization. However, our results show that Gαq activation was reduced when OX1R–CCK1R heterodimerization was stimulated with orexin-A or/and CCK (26–33). Meanwhile, Gαi2 and Gα12 were activated by treatment with orexin-A or CCK (26–33) alone, but not by co-stimulation. Taken together, these findings indicate that orexin-A may acquire the capacity to activate Gαi2 and Gα12 when OX1R and CCK1R heterodimerize.

To further investigate whether OX1R–CCK1R heterodimers affect cell migration, we performed xCELLigence assays. Cells co-stimulated with orexin-A and CCK (26–33) migrated significantly less than cells treated with orexin-A or CCK (26–33) alone. Thus, OX1R–CCK1R dimerization may decrease the migration of human colon cancer cells. However, combining the evidence from the effects of co-stimulation with agonists of these two receptors on cancer cell migration, the results indicate that the dimerization may play important roles for these two receptors exerting their functional activities.

Recent studies show that monovalent drugs specific for GPCR heterodimers and bivalent ligands are powerful tools for evaluating the activation of signaling pathways and cellular responses induced by GPCR dimerization and oligomerization. Because heterodimerization of OX1R and CCK1R significantly decreases the migration of human colon cancer cells, future studies should investigate the effects of monovalent drugs or bivalent ligands specific for OX1R–CCK1R heterodimers on the growth and metastasis of solid tumors, including colon cancer.

In conclusion, this study is the first to report that OX1R forms heterodimers with CCK1R, and that G-protein–dependent signal transduction is reduced by orexin-A and CCK (26–33) co-stimulation. Dimerization of OX1R and CCK1R may inhibit the Gαq/Gαi/Gα12/Gα13 signaling pathways, as well as downstream NFAT-RE and SRF-RE activation, but activate the β-arrestin signaling pathway. Furthermore, OX1R–CCK1R heterodimers may mediate the migration of HT-29 cells in an agonist-dependent manner, indicating that the balance between orexin and CCK in vivo may fine-tune the in vivo metastasis of colon tumors. However, in vivo investigations are required to further evaluate the importance of OX1R–CCK1R dimerization in cancer.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2017.03.003.

Conflict of Interest

Disclosure Summary: The authors have nothing to declare.

Acknowledgements

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Additional BRET assays were performed to determine whether ligands induce heterodimerization. BRET ratios were observed in HEK293 cells co-transfected with OX1R-Rluc and CCK1R-eGFP at a 1:3 ratio and treated with orexin-A, in cells treated with CCK (26–33) alone, and in cells treated for 10 min with orexin-A and CCK (26–33) (Fig. 2C). The BRET ratios of these cells were 0.364 ± 0.010, 0.361 ± 0.009, and 0.366 ± 0.012, respectively. When the experiment was repeated following energy donor–acceptor exchange between the two receptors, orexin-A or/and CCK (26–33)-

induced BRET ratios remained unchanged (Fig. 2D). These results implied that ligand binding might not be necessary for heterodimerization of OX1R and CCK1R. Moreover, sequential addition of orexin-A and CCK (26–33) did not similarly alter the BRET ratio between OX1R and CCK1R (Fig. S1).

When the experiment was repeated following exchange of energy donor, stimulation of HEK293 cells expressing CCK1R-Rluc, β-arrestin1-eGFP, and untagged OX1R with 100 nM orexin-A resulted in a robust and continually increasing ligand-induced BRET signal, indicating the recruitment of β-arrestin1 and β-arrestin2 to CCK1R by orexin-A, dependent on OX1R expression (Fig. S2).

The following are the supplementary data related to this article.

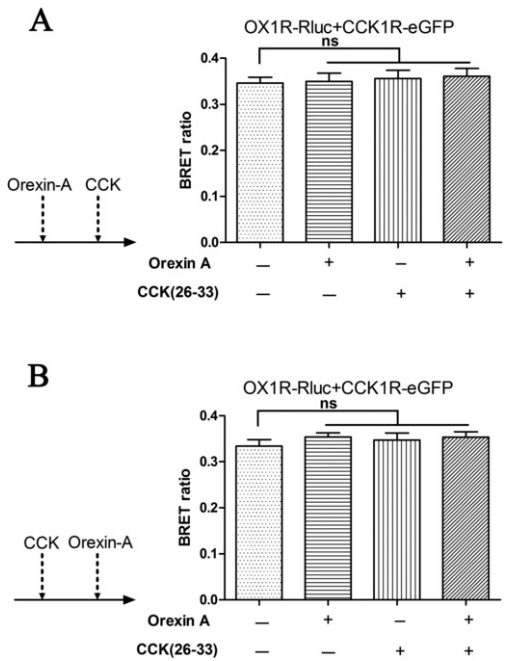

Fig. S1 Orexin-A and CCK (26-33) sequential stimulation effects on the OX1R and CCK1R heterodimer.

BRET was measured in HEK293 cells co-expressing OX1R-Rluc and CCK1R-eGFP and stimulated or not for 10 min with orexin-A (100 nM), CCK (26-33) (1000 nM) or both orexin-A + CCK (26-33), as indicated. For orexin-A + CCK (26-33) co-stimulation, experiments were performed by preincubating with orexin-A (5 min) before CCK (26–33) injection (A) or preincubating with CCK (26–33) before orexin-A injection (B). BRET ratios were analyzed and expressed as the mean ± SEM of four experiments (one-way analysis of variance; ns, not significant, $p > 0.05$).

alt-text: Fig. S1

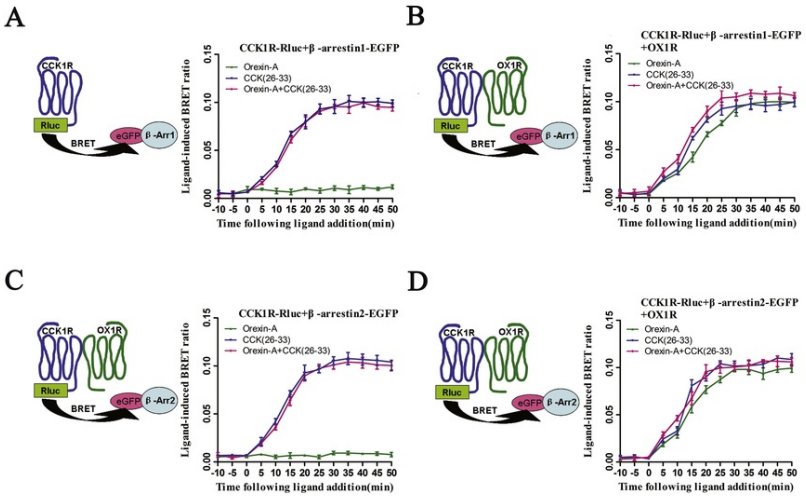

Fig. S2 Heterodimerization of OX1R and CCK1R does not alter their interaction with β-arrestins.

HEK293 cells transiently co-expressing CCK1R-Rluc and (A, B) β-arrestin1-eGFP or (C, D) β-arrestin2-eGFP in the absence (A, C) or presence (B, D) of OX1R were monitored by extended BRET for 10 min at 37 °C to generate kinetic curves. Following the addition of orexin-A (100 nM) and/or CCK (26-33) (1000 nM) or vehicle, monitoring was continued for an additional 50 min. Data represent the mean ± SEM of four independent experiments.

alt-text: Fig. S2

Highlights

- Identification of heterodimerization of OX1R and CCK1R, with peptides corresponding to transmembrane domain 5 of OX1R impairing heterodimer formation.
- OX1R forms heterodimers with CCK1R and that G-protein-dependent signal transduction is reduced by orexin-A and CCK (26-33) co-stimulation.
- Dimerization of OX1R and CCK1R may inhibit the Gαq/Gαi/Gα12/Gα13 signaling pathways
- ~~The heterodimer leads to decreased NFAT-RE activity and SRF-RE activity, but still activate the β-arrestin signaling pathway~~.
- The heterodimerization may play an anti-migratory role in human colon cancer cells.

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