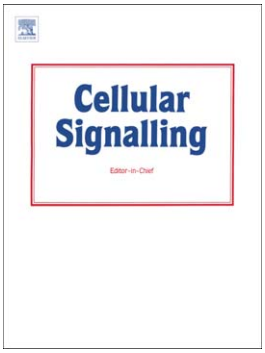


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# Novel Signaling of Dynorphin at κ-Opioid Receptor/Bradykinin B2 Receptor Heterodimers

**Bingyuan Ji[a,b], Haiqing Liu[c], Rumin Zhang[b], Yunlu Jiang[b], Chunmei Wang[b], Sheng Li[b], Jing Chen[b,d,*], Bo Bai[b,*]**

[a] *School of Life Science, Shandong Agricultural University, Taian, 271018, P.R. of China*

[b] *Neurobiology Institute, Jining Medical University, Jining, 272067, P.R. of China*

[c] *Department of Physiology, Taishan Medical College, Taian, 271000, P.R. of China*

[d] *Division of Translational and Systems Medicine, Warwick Medical School, University of Warwick, Coventry, UK*

[*] *Corresponding authors:*
*E-mail: bobai@mail.jnmc.edu.cn, Tel: +86 0537-3616002, Fax: +86 0537-3616777*
*E-mail: Jing.Chen@warwick.ac.uk, Tel: +44-24765968643, Fax: +44 2476968653*

**Abbreviations:** AC, adenylyl cyclase; B1R, bradykinin B1 receptor; B2R, bradykinin B2 receptor; BK, bradykinin; BRET, bioluminescence resonance energy transfer; CFP, cyan fluorescent protein; CNS, central nervous system; CRE, cAMP-response element; CREB, cAMP-response element-binding protein; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DOR, δ-opioid receptor; Dyn A(1–13), dynorphin A (1–13); DYN, dynorphin; FRET, fluorescence resonance energy transfer; FSK, forskolin; GPCR, G-protein-coupled receptor; HEK293, human embryonic kidney 293; IBMX, 3-isobutyl-1-methylxanthine; KOR, κ-opioid receptor; PBS, phosphate-buffered saline; PLA, proximity ligation assay; PLC, phospholipase C; RT-PCR, reverse transcription-polymerase chain reaction; SRE, serum response element; β2AR, β2 adrenergic receptor

**Abstract**

The κ-opioid receptor (KOR) and bradykinin B2 receptor (B2R) are involved in a variety of important physiological processes and share many similar characteristics in terms of their distribution and functions in the nervous system. We first demonstrated the endogenous expression of KOR and B2R in human SH-SY5Y cells and their co-localization on the membrane of human embryonic kidney 293 (HEK293) cells. Bioluminescence and fluorescence resonance energy transfer and the proximity ligation assay were exploited to demonstrate the formation of functional KOR and B2R heteromers in transfected cells. KOR/B2R heteromers triggered dynorphin A (1–13)-induced Gαs/protein kinase A signaling pathway activity, including upregulation of intracellular cAMP levels and cAMP-response element luciferase reporter activity, resulting in increased cAMP-response element-binding protein (CREB) phosphorylation, which could be dampened by the protein kinase A (PKA) inhibitor H89. This indicated that the co-existence of KOR and B2R is critical for CREB phosphorylation. In addition, dynorphin A (1–13) induced a significantly higher rate of

proliferation in HEK293-KOR/B2R and human SH-SY5Y cells than in the control group. These results indicate that KOR can form a heterodimer with B2R and this leads to increased protein kinase A activity by the CREB signaling pathway, leading to a significant increase in cell proliferation. The nature of this signaling pathway has significant implications for the role of dynorphin in the regulation of neuroprotective effects.

**Keywords**

G-protein-coupled receptor (GPCR); κ-Opioid receptor (KOR); Bradykinin B2 receptor (B2R); Resonance energy transfer (RET); cAMP-response element-binding protein (CREB); Heterodimerization

## 1. Introduction

G-protein-coupled receptors (GPCRs), which consist of seven membrane-spanning α-helical structures, are the largest family of cell surface receptors capable of binding to a wide array of molecules (i.e., ions, nucleotides, amino acids, photons, amines, and peptides) [1]. Once activated, a GPCR changes its conformation to activate specific heterotrimeric G proteins, followed by transduction of stimulatory or inhibitory signals from the extracellular milieu to intracellular signaling molecules [2-4]. Because they are involved in the regulation of nearly every physiological process, GPCRs have been considered as important targets for human therapeutics [5, 6]. Although GPCRs were initially thought to be, and function exclusively as, monomeric entities [7, 8], increasing evidence indicates that they can form homomeric or heteromeric GPCR complexes in intact cells[9-13].

Endogenous opioid peptides include three major types of neuropeptides (enkephalins, endorphins, and dynorphins (DYNs)), which exert their actions through three types of opioid receptors: the μ-opioid (MOR), δ-opioid (DOR), and κ-opioid (KOR) receptors. All three opioid receptors belong to the large family of GPCRs and are distributed in the central nervous system (CNS) and peripheral nervous system [14]. Among the opioid receptors, the KOR, which is specifically activated by the endogenous opioids DYNs [15], plays an important role in a variety of physiological processes including analgesia, anti-pruritic activity [16], fluid homeostasis, dieresis [14, 17], cardiovascular regulation [18], and modulation of the hypothalamic-pituitary axis [19]. KOR signals mainly through activation of the Gαi subunit to inhibit cAMP production [20]. The DYN/KOR system has neuroprotective effects *in vivo* and *in vitro*, such as improving the survival of cats with cerebral ischemia [21, 22] or ameliorating L-DOPA-induced dyskinesia symptoms in a rat model of Parkinson's disease [23].

The bradykinin B2 receptor (B2R) and bradykinin B1 receptor (B1R), which are both GPCRs, play prominent roles in a wide variety of physiological and pathological responses after being activated by bradykinin (BK) and des-Arg9-BK, respectively [24-26]. Typically, B2R is constitutively expressed in a variety of cell types, while B1R expression is induced by injury or inflammation [26]. Most of the physiological functions of kinins are mediated by B2R, which is present in most tissues including the CNS [27]. B2R participates in many signaling cascades, but generally couples with the Gαq protein, which activates phospholipase

C (PLC), leading to a transient increase in intracellular $Ca^{2+}$ [28]. Increasing data indicate that the Gαs-cAMP-PKA signaling pathway also functions downstream of B2R, leading to various biological effects [26, 29]. Like KORs, numerous studies have revealed that B2R exerts neuroprotective effects in the CNS, such as increasing memory preservation in Alzheimer's disease and promoting survival through suppressing apoptosis and inflammation in ischemic stroke [30-32].

Numerous GPCRs dimerize, and KORs and B2Rs are no exception [33-35]. KOR and B2R share many congenerous characteristics. They share similar distributions in the CNS and cardiovascular system and exhibit similar functions in cardiovascular protection and neuroprotection. In addition, both receptors mediate neuroprotection through the $PI_3K$/Akt signaling pathway [36, 37]. Interestingly, human neuroblastoma cells (SH-SY5Y cells) co-express KOR and B2R [38, 39], and these receptors have been implicated in the regulation of neurogenesis and neuroprotection [40]. Despite these similarities in distribution and functions, possible direct physical and functional interactions between KOR and B2R have not been examined.

In the present study, we aimed to investigate whether KOR and B2R can form heterodimers using bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfer (FRET), and the proximity ligation assay (PLA) in transfected human embryonic kidney 293 (HEK293) cells. Meanwhile, we examined whether signal transduction of the heterodimer was altered by dynorphin A (1–13) (Dyn A(1–13)) or BK, with particular focus on the effects of the heterodimers on the transcription factor cAMP-response element-binding protein (CREB), which plays a critical role in the modulation of neuroprotection [41-43]. This work further reveals the molecular mechanism underlying neuroprotection mediated by heterodimers in the CNS and provides an experimental basis for exploring new breakthroughs in brain injury and neurodegeneration.

## 2. Materials and methods

### *2.1. Peptides, reagents, and antibodies*

Human Dyn A(1–13) and BK were obtained from Phoenix Pharmaceuticals (Belmont, USA). The mammalian expression vector pcDNA3.1(+) and Lipofectamine™ 2000 were obtained from Invitrogen (Paisley, UK). Forskolin (FSK), 3-isobutyl-1-methylxanthine (IBMX), the Duolink PLA and protein kinase C (PKC) inhibitor Bisindolylmaleimide II (BisII) were all purchased from Sigma-Aldrich Shanghai Trading Co Ltd (Shanghai, China). PKA inhibitor H89 was purchased from Beyotime (Shanghai,China). Anti-KOR and anti-B2R antibodies were purchased from Novus (Littleton, USA). Anti-phospho-CREB and anti-total CREB antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

### *2.2. Plasmid constructs*

The pcDNA3.1-KOR and pcDNA3.1-B2R plasmids were obtained from the UMR cDNA Resource Center (University of Missouri-Rolla, USA). Cyan fluorescent protein (CFP) or mVenus was attached to the C-terminus of KOR, B2R, and mouse orexin type 2α receptor (mOX2αR) by inserting the open reading frame of KOR, B2R, or mOX2αR into the CFP-N1 or mVenus-N1 vector to create KOR-CFP, B2R-Venus, or mOX2αR-Venus. For the

KOR-Rluc plasmid, the coding sequence of human KOR lacking a stop codon was subcloned into pRluc-N1 (BioSignal Packard, Inc., Montreal, Canada). To construct pEGFP-B2R, cDNA encoding human B2R was cloned into pEGFP-C1 (Clontech, USA). The plasmids pCRE-Luc (which was used to detect the cAMP-response element (CRE)) and pSRE-Luc (which were used to detect the serum response element (SRE)) were purchased from Stratagene (La Jolla, CA, USA). The plasmid pRL-TK was purchased from Promega (Madison, WI, USA). To construct the Gαi2-Rluc8, Gαq-Rluc8, and Gαs-Rluc8 fusion proteins, Rluc8 (humanized regular Renilla Luciferase 8) was PCR-amplified and inserted at position 91 of Gαi2, 97 of Gαq, and 113 of Gαs, respectively [44]. All the resulting constructs were confirmed by commercial sequencing.

*2.3. Cell culture and transfection*

HEK293, SH-SY5Y, and CHO cells obtained from the American Tissue Culture Collection were cultured in Complete Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% $CO_2$ incubator. To establish stable cell lines, HEK293 cells were transfected with the plasmids pcDNA3.1-KOR, pcDNA3.1-B2R, and pcDNA3.1-KOR/pcDNA3.1-B2R, respectively. Cells were selected with G418 (Gibco, Invitrogen) for 8 weeks. Receptor expression in the stable cell lines was assessed by reverse transcription-polymerase chain reaction (RT-PCR).

*2.4. RT-PCR*

To detect the endogenous expression of KOR and B2R, total RNA was extracted and purified from human SH-SY5Y cells for RT-PCR. The primers were as follows: KOR, forward 5′-CTGGTGGGACCTCTTC-3′ and reverse 5′-ATGGGATTCAGGCTACT-3′ (product length: 320 bp); and B2R, forward 5′-GCTGCGGAACAACGAG-3′ and reverse 5′-TGGTACACCTCCCAAGAC-3′ (product length: 300 bp). Ten microliters of the reaction mixtures were subsequently electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

*2.5. Immunostaining and confocal microscopy*

HEK293 cells grown on coverslips were transiently transfected with pEGFP-B2R and pcDNA3.1-KOR and fixed after 24 h as previously described [33]. Non-specific binding was blocked by incubation with 3% bovine serum albumin prepared in phosphate-buffered saline (PBS) containing 0.01% Triton X-100 for 1 h at room temperature. Cells were washed three times with PBS and then incubated overnight with an anti-KOR monoclonal antibody (1:200) in PBS at 4°C. Cells were washed three times with PBS, followed by incubation with Alexa-Fluor®647 (red)-conjugated secondary antibodies (1:400) (Molecular Probes, Invitrogen) for 1 h at room temperature. After a final wash with PBS, cells were examined using a Leica DMRE laser scanning confocal microscope (Leica, Milton Keynes, UK).

*2.6. BRET measurements*

HEK293 cells were transiently co-transfected with vectors encoding Rluc fusion or Venus fusion proteins. Twenty-four hours after transfection, cells were washed twice, detached, and

transferred ($10^5$ cells/well) to a 96-well microplate (Corning 3600, white opaque plates) for 24 h in HEPES-buffered phenol red-free medium (Invitrogen, Life Technologies). Cells were then washed twice and resuspended in D-PBS (PBS containing 0.5 mM $MgCl_2$ and 0.1% (w/v) glucose). Coelenterazine H substrate was added to a final concentration of 5 μM. When appropriate, cells were pretreated with Dyn A(1–13) or BK at a final concentration of 100 nM for 10 min prior to addition of the substrate. Analysis was carried out immediately at 37°C using a Tristar LB941 plate reader (Berthold Technologies, Bad Wildbad, Germany). The BRET ratio was calculated by subtracting the ratio of the emission at 535 ± 30 nm to the emission at 485 ± 20 nm in cells expressing the Rluc fusion protein alone from the same ratio in cells co-expressing the Rluc and Venus fusion proteins. The results are expressed as the "BRET ratio" or alternatively as "mBRET" (mBRET = 1000 × BRET ratio). As a negative control, cells co-transfected with KOR-Rluc and mOX2αR-Venus were used.

BRET saturation curves were obtained by transfecting increasing amounts of plasmid encoding the Venus-tagged construct against a constant amount of the Rluc-tagged donor constructs. The total amount of plasmid DNA was kept constant by adding empty plasmid. Data were expressed as the mBRET, which was calculated as described above.

*2.7. FRET experiments*

To further explore the heterodimerization of KOR and B2R, FRET was performed in living cells. The donor plasmid KOR-CFP and the receptor plasmid B2R-Venus were co-transfected into HEK293 cells. As a negative control, KOR-CFP and mOX2αR-Venus were co-transfected into HEK293 cells. FRET signals were detected using an OLYMPUS I ×71 fluorescence inverted microscope and MetaFluor 7.0 software, which allowed the sequential integration of light signals detected with two filter settings (CFP: excitation at 436 ± 10 nm and emission at 480 ± 20 nm; mVenus: excitation at 480 ± 20 nm and emission at 535 ± 15 nm; FRET: excitation at 436 ± 10 nm and emission at 535 ± 15 nm). Corrected FRET was calculated using the equation: FRETc = FRET − (coefficient B × CFP) − (coefficient A × Venus), where CFP, Venus, and FRET values correspond to background-corrected images obtained through the CFP, Venus, and FRET channels. Coefficient B and coefficient A correspond to the values obtained for the CFP (donor) and Venus (acceptor) bleed-through co-efficients, respectively, which were calculated using cells transfected with CFP and mVenus individually [45, 46]. To correct the FRET levels for the various amounts of donor (eCFP) and acceptor (eYFP), normalized FRET was calculated using the following equation: $FRET_N$ = FRETc / (CFP × Venus), where FRETc, CFP, and Venus are equal to the fluorescence values obtained from single-transfected cells.

*2.8. PLA*

HEK293-KOR/B2R cells were grown and fixed with 4% paraformaldehyde prepared in PBS on 6-well tissue culture slides. To visualize receptor interactions, slides were blocked with Blocking Solution and incubated with rabbit anti-B2R and goat anti-KOR antibodies at 4°C overnight in a humidifying chamber. Slides were then washed and incubated with secondary anti-rabbit/goat antibodies conjugated with plus and minus Duolink II PLA probes (1:5) at 37°C for 1 h. Slides were washed again and then incubated with ligation-ligase solution (30 min at 37°C), followed by incubation with amplification-polymerase solution (3

h at 37°C). Slides were then mounted with a minimal volume of Duolink II mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) for 30 min. PLA signals [Duolink In Situ Detection Reagents Red (λ excitation/emission, 598/634 nm)] were identified as fluorescent spots under a fluorescence microscope (Leika TCS SP5) at room temperature.

*2.9. BRET assay for cAMP*

Intracellular cAMP was assayed using an Epac cAMP BRET biosensor [47]. Briefly, YFP-Epac-RLuc plasmids and receptors of interest were co-transfected into HEK293 cells. After 24 h, cells were collected, distributed in a 96-well white opaque microplate, and cultured for another 24 h in HEPES-buffered phenol red-free medium. Cells were then washed once with PBS and resuspended in D-PBS. BRET between RLuc and YFP was measured at 20°C in the presence of Coelenterazine H (5 μM) and the nonspecific phosphodiesterase inhibitor IBMX (40 μM). Cells were stimulated with agonists in the absence of FSK (10 μM) 5 min later. After incubation for 5 min, BRET readings were collected using a Tristar LB941 plate reader. The BRET signal was determined by calculating the ratio of emission of YFP (520–570 nm) to RLuc (370–480 nm).

*2.10. Luciferase gene reporter assays*

To conduct SRE and CRE luciferase assay, cells stably expressing KOR, B2R, or KOR/B2R were co-transfected with the pSRE-Luc (SRE tagged with luciferase) or pCRE-Luc (CRE tagged with luciferase) plasmid, which contain firefly luciferase, along with pRL-TK (Promega) expressing Renilla luciferase. About 24 h after transfection, cells were treated with Dyn A(1–13) (100 nM final concentration) as indicated for 6 h in serum-free DMEM at 37°C, washed with PBS, and lysed with the reagent containing the luciferase substrate. For SRE luciferase assay, cells were pretreated with 1 μM PKC inhibitor BisII for 2 h prior to ligand stimulation. As a control, the basal cells were also treated with BisII. The activities of firefly luciferase and Renilla luciferase were measured by the Dual-Luciferase Reporter Assay System using the Tristar LB941 instrument. The results were expressed as mean ± SEM of six independent experiments performed in duplicate. The ratio of the firefly luciferase luminescence signal to that of Renilla luciferase was determined.

*2.11. Western blotting*

HEK293 and SH-SY5Y cells were lysed and the proteins concentration was quantified with a BCA Analysis Kit (KeyGEN, Nanjing, China). Proteins were separated on 10% SDS-PAGE gels and subsequently transferred to PVDF membranes. The membranes were blocked with 5% nonfat milk at room temperature for 1 h and then incubated with a primary antibody (1:1000) against KOR, B2R, or GAPDH in TBST at 4°C overnight. After washing three times with TBST for 10 min, the membrane was incubated with a HRP-conjugated secondary antibody (1:5000) at room temperature for 1 h and washed three times for 10 min with TBST. The immunoreactive proteins were visualized by ECL chemiluminescent reagent (Pierce Biotechnology). An anti-GAPDH antibody was used as a loading control.

To assay CREB activation, HEK293 cells expressing KOR, B2R, or KOR/B2R cells were pre-treated with or without protein kinase A (PKA) inhibitor H89 (10 μM, 30 min) or phospholipase C (PLC) inhibitor U73122 (10 μM, 30 min), followed by stimulation with Dyn

A(1–13) (100 nM) for 5 min, respectively. Following treatment, cells were washed, harvested, and lysed in RIPA lysis buffer. Thereafter, 10 μg of cell extracts was separated by 10% SDS-PAGE. Gel transfer was performed at 150 mA for ~90 min. Phosphorylation of CREB was detected by immunoblotting with an antibody against phospho-CREB (1:1000). As a loading control, the same membranes were re-probed with an anti-total CREB antibody (1:1000). The band intensities were measured by densitometric analysis, and the change in CREB phosphorylation was calculated as the phospho-CREB/CREB ratio.

*2.12. Cell proliferation assays*

HEK293-KOR/B2R and human SH-SY5Y cells were seeded into a 96-well plate at a density of $10^4$ cells per well. After 24 h, the cells were treated with 1, 10, and 100 nM Dyn A(1–13) for another 24 h. Cell proliferation was measured using a CCK-8 (Dojindo, Japan) viability assay. HEK293, HEK293-KOR, HEK293-B2R, and HEK293-KOR/B2R cells were seeded in a 96-well plate at a density of $10^4$ cells per well in quadruplicate. After 24 h, all cells were stimulated with 100 nM Dyn A(1–13) for 24 h. The CCK-8 assay was performed according to the manufacturer's instructions. Following incubation, the absorbance of the suspension was measured at 450 nm. Each sample was run in quadruplicate. Wells containing media alone were included as a control.

*2.13. Statistical analysis*

All the data are shown as the means ± SEM. The data were presented and analyzed using GraphPad Prism v6.0. Sigmoidal curves were fitted to the dose-response data using non-linear regression. A one-way ANOVA was used for multiple group comparisons, followed by Tukey's test. All experiments were repeated at least three times, and representative experiments are shown in the figures.

**3. Results**

*3.1. Endogenous expression of KOR and B2R in SH-SY5Y cells*

Bands of 320 and 300 bp were amplified using RT-PCR, suggesting that SH-SY5Y cells expressed KOR and B2R (Fig. 1A and B). SH-SY5Y cells also expressed KOR (43 kDa) and B2R (45 kDa) proteins (Fig. 1C and D). However, neither of the receptors was detected in HEK293 cells.

*3.2. Co-localization of KOR and B2R in HEK293-KOR/B2R cells*

To identify the co-localization of KOR and B2R in HEK293 cells co-transfected with pcDNA3.1-KOR and N-terminal EGFP-tagged B2R, immunofluorescence image analysis was carried out using confocal microscopy. KOR and B2R were predominantly expressed at the cell membrane (Fig. 1E), implying that they may interact. Nuclei were visualized by DAPI staining.

*3.3. Heterodimerization of KOR and B2R determined by BRET and FRET analyses*

To determine whether KOR and B2R can form a heterodimer, BRET analysis was performed in HEK293 cells co-transfected with the plasmids KOR-Rluc and B2R-Venus. The

BRET ratio was significantly higher in cells co-expressing KOR-Rluc and B2R-Venus than in cells transfected with KOR-Rluc alone or co-transfected with Rluc and B2R-Venus, Venus and KOR-Rluc, or KOR-Rluc and mOX2αR-Venus (Fig. 2A). These results indicated the constitutive association of KOR and B2R. To evaluate whether KOR/B2R heteromerization is influenced by agonists of the two receptors, BRET analyses were performed in the presence of Dyn A(1–13) or/and BK. Neither agonist affected BRET signals (data not shown).

To investigate whether the interaction of KOR and B2R is specific, BRET saturation assays were performed. BRET signals representing the transfer of energy between KOR-Rluc and B2R-Venus increased as a hyperbolic function of the concentration of the Venus fusion construct added until a plateau was reached, whereas Rluc and B2R-Venus as well as Venus and KOR-Rluc exhibited a nonspecific linear relationship (Fig. 2B). These results indicated that the interaction between KOR and B2R is specific and is not due to receptor overexpression and random collisions.

For competitive BRET analysis, fixed amounts of KOR-Rluc/B2R-Venus and increasing amounts of unlabeled KOR or B2R were co-expressed in HEK293 cells. BRET signals decreased as the concentration of unlabeled KOR or B2R increased (Fig. 2C and D). These data further confirmed the specific formation of KOR and B2R heterodimers.

FRET was performed to further explore the interaction between KOR and B2R. HEK293 cells were transfected with KOR-CFP (a), B2R-Venus (b), KOR-CFP and mOX2αR-Venus (c), or KOR-CFP and B2R-Venus (d) (Fig. 3A). In images of FRET, the site of interaction will be marked as yellow or red. Moreover, a more intense color means a stronger interaction. There was no significant FRET in all single-transfected cells (Fig. 3A and B). As a negative control, no significant FRET was visualized in cells co-transfected with KOR-CFP and mOX2αR-Venus. However, a notable FRET signal was clearly detected in HEK293 cells co-transfected with KOR-CFP and B2R-Venus. FRET signals could also be detected in CHO cells co-transfected with KOR-CFP and B2R-Venus (Fig. 3C), suggesting that FRET occurred in distinct cell lines.

### *3.4. Heterodimerization of KOR and B2R determined by the PLA*

To further confirm the formation of KOR-B2R heteromeric complexes, the PLA was performed to specifically detect the close proximity of these two receptors in HEK293 cells stably expressing both receptors (Fig. 4A). There were strong PLA signals in HEK293-KOR/B2R cells, but no signals in the control group incubated with only one secondary antibody (Fig. 4B). These results from stable cells suggest that KOR and B2R can interact and that the observed proximity between the receptors corresponds to direct receptor–receptor interactions.

### *3.5. Heterodimerization of KOR and B2R increases the intracellular cAMP concentration upon Dyn A(1–13) stimulation*

We used the cAMP BRET biosensor to determine whether the heterodimer affects cAMP accumulation. The sensor consists of an N-terminal truncated variant of EPAC tagged with Rluc and YFP at the N and C termini, respectively (Fig. 5A). Upon binding of cAMP, the signal of the biosensor decreases due to a conformational change that presumably increases the distance between the donor and acceptor. The reduction of the BRET signal with FSK is

consistent with its role as a stimulator that promotes cAMP production (Fig. 5B). After treatment with Dyn A(1–13), BRET signals were significantly lower in HEK293-KOR/B2R cells than in HEK293-KOR and HEK293-B2R cells (Fig. 5C). However, when treated with BK, BRET signals in HEK293-KOR/B2R cells did not differ from those in HEK293-B2R cells (Fig. 5D), suggesting that BK cannot enhance the cAMP level through the KOR/B2R heterodimer.

*3.6. Detection of interactions between Gα subunits and KOR/B2R heterodimers using BRET*

To investigate potential receptor-G protein interactions, BRET assays were performed in living HEK293 cells co-expressing Gαs-Rluc8, Gαi2-Rluc8, or Gαq-Rluc8 and either KOR-Venus, B2R-Venus, or B2R, in combination with the complementary untagged G protein subunits Gβ1/Gγ2. The BRET ratio between Venus-tagged KOR and Rluc8-tagged Gαs significantly increased after Dyn A(1–13) stimulation compared with that in cells expressing KOR or B2R alone (Fig. 6A), suggesting that heterodimerization of KOR and B2R leads to Gαs protein binding and activation upon agonist stimulation. However, when KOR and B2R were co-expressed, the changes of the BRET ratio between Venus-tagged KOR and Rluc8-tagged Gαi2 after agonist stimulation were smaller than in cells expressing KOR alone (Fig. 6B). No BRET signal changes were detected between Gαq and receptors in any of the three cell types (Fig. 6C). Moreover, basal BRET signals were also observed in all cases, reflecting constitutive interactions between the receptor and G proteins.

*3.7. Dyn A(1–13) increases CRE-luc activity in HEK293-KOR/B2R cells*

To determine the effect of KOR and B2R heterodimers on downstream signaling, we detected the activity of CRE and SRE in HEK293-KOR, HEK293-B2R, and HEK293-KOR/B2R cells. CRE-luc activity was significantly higher in HEK293-KOR/B2R cells than in HEK293-KOR and HEK293-B2R cells ($P<0.01$, Fig. 7A), whereas SRE-luc activity was decreased compared to that in HEK293-KOR cells ($P<0.01$, Fig. 7B). Furthermore, the SRE-luc activity was completely blocked by PKC inhibitor BisII in HEK293-KOR cells and the same treatment has no effects on SRE-luc activities in other groups (Fig. 7B), suggesting that Dyn A(1-13) induced enhanced SRE activation depends upon PKC activity in HEK293-KOR cells. All the results showed that heterodimerization of KOR and B2R increased CRE activity depending on PKA activities when Dyn A(1–13) was added to HEK293-KOR/B2R cells, which further confirmed that the KOR/B2R heterodimer leads to an increase in Gαs binding.

*3.8. Dyn A(1–13) increases CREB phosphorylation in HEK293-KOR/B2R cells*

GPCR coupling to the stimulatory G protein (Gαs) stimulates adenylyl cyclase (AC) to increase CREB phosphorylation, while coupling to the inhibitory G protein (Gαi) inhibits AC and decreases CREB activation. To determine whether KOR/B2R heterodimers affect CREB phosphorylation, the time- and dose-dependent effects of Dyn A(1–13) on CREB activation were investigated. The ability of KOR and/or B2R to stimulate phosphorylation of CREB was assessed by treating cells with Dyn A(1–13) ($10^{-7}$ M) for 0–60 min. Activation of either receptor did not affect the total CREB protein expression, and treatment with Dyn A(1–13) inhibited the activation of CREB in HEK293-KOR cells, with maximal inhibition at 5 min

and a return to near basal conditions after 60 min. However, a robust increase (maximal at 5 min) in CREB phosphorylation was observed in HEK293-KOR/B2R cells, with a subsequent decline (Fig. 8A and C). In dose-dependent experiments, CREB activation decreased when concentrations of Dyn A(1–13) increased in HEK293-KOR cells. By contrast, CREB activation increased with increasing concentrations of Dyn A(1–13) in HEK293-KOR/B2R cells (Fig. 8B and D). B2R expression alone led to very little activation of CREB, whereas co-expression of KOR and B2R led to significantly higher CREB phosphorylation than that in HEK293-B2R cells (Fig. 8B and D). Similarly, a dose-dependent effect on CREB activation was also observed in human SH-SY5Y cells (Fig. 8E), suggesting that Dyn A(1–13) can induce CREB phosphorylation.

### *3.9. CREB phosphorylation mediated by KOR/B2R heterodimers is PKA-dependent and PLC-independent*

To examine whether CREB phosphorylation is PKA-dependent in HEK293-KOR/B2R cells, the cells were treated with the PKA inhibitor H89 prior to Dyn A(1–13). The level of CREB phosphorylation in HEK293-KOR/B2R cells was significantly lower with H89 treatment than without H89 treatment (Fig. 9A and B). To determine whether the Gq/PLC/PKC pathway mediates CREB activation, a PLC-selective inhibitor, U73122, was applied. Pretreatment with 10 μM U73122 for 30 min did not significantly change the level of CREB phosphorylation in HEK293-KOR/B2R cells (Fig. 9C and D). As a positive control, however, U73122 remarkably reduce the level of CREB phosphorylation in HEK293-OX1R cells ($P<0.01$) (Fig. S1). These results further indicate that the KOR/B2R heterodimer signals through the Gαs/cAMP/PKA/CREB pathway after stimulation by Dyn A(1–13) in HEK293-KOR/B2R cells.

### *3.10. Dyn A(1–13) enhances proliferation of HEK293-KOR/B2R and SH-SY5Y cells*

The above results indicate that KOR and B2R form a heterodimer, thereby synergistically increasing cell signal transduction. To further investigate the role of the dimer in cell function, we performed a cell viability assay. Dyn A(1–13) had a significant effect on the proliferation of HEK293-KOR/B2R cells, and this functional outcome of KOR/B2R co-expression followed a dose-dependent pattern, reaching significance at 10 nM ($P<0.01$) (Fig. 10A). Treatment with $10^{-7}$ M Dyn A(1–13) for 24 h significantly increased the proliferation of HEK293-KOR/B2R cells, while the proliferation of HEK293-KOR and HEK293-B2R cells was not affected (Fig. 10B). In HEK293 cells, there were no significant proliferative responses to Dyn A(1–13). Thus, KOR and B2R heteromerization is important for cell proliferation. Corresponding to its effects on CREB phosphorylation, Dyn A(1–13) promoted the proliferation of human SH-SY5Y cells (Fig. 10C).

## 4. Discussion

The ability of GPCRs to interact with each other as dimers or higher-order oligomers has received much attention. These complexes induce diverse signaling pathways, leading to effects on nearly every aspect of GPCR functions. For example, receptor heteromerization alters the cell surface delivery and retention of certain GPCRs [48], modulates G

protein-coupling [49], and causes cross-activation or cross-inhibition of signaling. KOR and B2R are becoming an increasingly important subject for investigation in a variety of neurological processes such as neuroprotection [37, 50].

In the present study, we confirmed the co-expression of KOR and B2R in SH-SY5Y cells by RT-PCR and Western blotting. Furthermore, immunofluorescence staining showed the co-localization of KOR and B2R on the surface of HEK293 cells. We further identified the heterodimerization of KOR and B2R in co-transfected HEK293 cells using both BRET and FRET technology. The results showed that KOR constitutively heteromerizes with B2R, but not with the unrelated receptor mOX2αR (Fig. 2 and Fig. 3). BRET saturation and competitive analyses confirmed that the interaction was specific. A series of studies suggest that ligands may modulate the extent of dimerization [10, 33, 51], whereas Dyn A(1–13) or BK treatment had no effect on the interaction between KOR and B2R.

The PLA was performed to further confirm the interaction between KOR and B2R. This assay is a powerful tool for studying protein-protein interactions *in vitro* and *in vivo* [52, 53]. The current study demonstrates that KOR can form heterodimers with B2R in stably transfected cells (Fig. 4). Because the PLA assay is conducted on fixed tissue, transient interactions can be easily detected, in contrast with BRET and FRET assays. Furthermore, signal amplification by rolling-circle amplification increases the number of fluorophores, meaning the PLA has high sensitivity with less background and the unique ability to quantify dimerization signals.

Taken together, we provide sufficient evidence of KOR-B2R heterodimers. In general, GPCR heterodimerization may influence signaling pathways, and heteromer-specific signaling is a marker of heteromer formation [12, 13, 54, 55]. For example, heteromers of the CB1 and D2 receptors can induce an apparent shift in CB1 signaling from Gαi to Gαs [56]. Lee et al. demonstrated that D1-D2 heteromers switch D1 from Gαs to Gαq signaling [57]. Generally, the mGlu2 and 5-HT2A receptors couple with Gi and Gq proteins, respectively. Several studies report that hallucinogenic 5-HT2A receptor agonists activate both Gq and Gi proteins when the 5-HT2A receptor forms heteromers with the mGlu2 receptor [58, 59]. Our previous study also found that orexin receptor 1 and KOR can form a heterodimer, which couples with Gαs, leading to increased PKA activity and upregulation of intracellular cAMP levels [60]. KORs are coupled with heterotrimeric Gi/o proteins [61], inhibiting AC and cAMP levels and decreasing calcium levels via the Gβγ subunit. By contrast, B2R is generally reported to signal through the Gαq-PLC pathway, stimulating a transient increase in intracellular $Ca^{2+}$ [26]. Here, we explored whether KOR/B2R heterodimers affect signal transduction and levels of signaling molecules. Using an EPAC cAMP BRET biosensor, we studied ligand-induced changes of cAMP, a second messenger that directly affects the functions of many regulatory proteins. The cAMP BRET biosensor is a powerful tool to measure cAMP levels in real time. When the cAMP level increases, BRET ratios decrease due to a conformational change that leads to a larger distance between Rluc and YFP [47]. GPCRs that couple to Gαs or Gαi are thus suitable targets for investigations with this sensor. Moreover, the BRET signal with this sensor is reversible after the agonist is removed. cAMP accumulation was significantly higher in co-transfected cells than in single-transfected cells. Interestingly, Dyn A(1–13), but not BK, activated cAMP signaling through KOR/B2R heteromers (Fig. 5), suggesting that heteromerization leads to different affinities for ligands,

resulting in a novel signaling pathway. Similar results were reported in other studies. For example, treatment with isoproterenol increases the level of phosphorylated MAPK in cells co-expressing DOR and β2 adrenergic receptor (β2AR), whereas opioid-mediated phosphorylation of MAPK is not significantly altered, indicating that the DOR/β2AR heterodimer has different affinities for both ligands [62].

To further examine whether the KOR-B2R heterodimer is coupled to Gαs proteins to increase cAMP levels, the BRET assay was performed to detect the effects of the heteromer on G-protein coupling. Interestingly, although it is reported that KOR and B2R mainly couple with Gαi and Gαq, respectively [26, 61], significant agonist-promoted BRET was observed between receptors and Gαs proteins in co-transfected HEK293 cells when stimulated with Dyn A(1–13). The results indicate that Gαs protein is activated by KOR/B2R heterodimers. By contrast, activation of Gαs was not detected in HEK293 cells expressing KOR or B2R alone, suggesting that KOR/B2R heterodimers enhance Gαs-protein coupling and lead to a novel signaling pathway.

The Gα subunit dissociates from Gβγ dimers after the GPCR is activated and subsequently interacts with downstream effectors and initiates a cascade of downstream second messenger pathways, eventually inducing gene transcription by various response elements including CRE, SRE, NFAT-RE, and serum response factor response element (a mutant form of the SRE). To explore the influence of KOR/B2R heterodimers on the Gαs/Gαq/Gαi signaling pathways, we detected downstream signals of CRE and SRE in stably transfected cells using a dual reporter gene assay, which is a powerful tool to decipher downstream G-protein signaling [63]. SRE activity was lower in co-transfected cells than in HEK293-KOR cells, while there was no significant difference between HEK293-KOR/B2R cells and HEK293 cells (Fig. 7B). By contrast, CRE activity was significantly higher in co-transfected cells than in single-transfected cells and HEK293 cells (Fig. 7A), which is consistent with the evaluation of cAMP accumulation, confirming that the major downstream signaling of KOR/B2R heteromers is the cAMP pathway via Gαs protein. Taken together, our data suggest that dimerization of KOR and B2R up-regulates the Gαs-mediated cAMP signaling pathway. Previous studies demonstrate that a GPCR heteromer can “choose” a completely new G protein. For example, the dopamine receptors D1R and D5R usually couple with Gαs/olf proteins, D2R and D4R couple with Gαi/o proteins, while the D1R-D2R and D5R-D2R heteromers both couple preferentially with Gαq proteins [64]. These results indicate that the ligand-binding site of receptors is altered by dimer formation and that the receptor heterodimer can form distinct conformations to couple with distinct G proteins, leading to a specific signaling pathway. Similarly, our findings also support the idea that allosteric interactions between KOR and B2R protomers can elicit changes of signaling cascades.

In general, the main role of cAMP is to activate cAMP-dependent PKA, which phosphorylates cytoplasmic and nuclear proteins and activates the transcription of target genes [65]. As a nuclear transcriptional factor, CREB mediates cAMP-induced gene expression via binding to the CRE in gene promoter regions, which plays an important role in diverse biological processes such as development and differentiation of the nervous system [66], learning, memory, addiction, and modulation of cancer pain [67].

In the present study, Dyn A(1–13) led to a time-dependent and dose-dependent increase in phosphorylation of CREB in HEK293-KOR/B2R cells, whereas total CREB levels remained

unaltered (Fig. 8). However, the level of CREB phosphorylation was lower in HEK293 cells transfected with KOR or B2R alone than in co-transfected cells. The increased phosphorylation of CREB might be mediated by the increase in the intracellular cAMP concentration, which was consistent with our previous data (Fig. 5). Interestingly, Dyn A(1–13) also induced a dose-dependent increase in phosphorylation of CREB in human SH-SY5Y cells expressing both receptors (Fig. 8E). Furthermore, we tested CREB activation in HEK293-KOR/B2R cells in the presence of the PKA inhibitor H89 or the PLC inhibitor U73122. The results indicate that H89, but not U73122, blocked CREB activation in HEK293-KOR/B2R cells (Fig. 9), suggesting that CREB is phosphorylated via the Gαs/cAMP/PKA/CREB signaling pathway upon co-expression of the two receptors. Thus, the underlying mechanism may be conformational changes in the receptors affecting dimer-G protein binding, although, apart from heterodimers, monomers of KOR and B2R would still activate Gαi and Gαq, respectively.

In addition to its roles in other physiological processes, increasing studies strongly support a role for CREB as a neuroprotectant. Mäkelä et al. reported that the peroxisome proliferator-activated receptor-γ agonist GW1929 influences CREB signaling in human dopaminergic neurons, contributing to increased cell viability [41]. Liu et al. demonstrated that activation of CREB pathways contributes to neurite extension of PC12 cells [42]. Several studies report that DYN/KOR and BK/B2R systems both participate in the process of neuroprotection [37, 68-70]. To further explore whether the heterodimer of KOR and B2R affects cell viability, cell proliferation was investigated. After treatment with Dyn A(1–13), the proliferation of HEK293-KOR/B2R cells was significantly higher than that of HEK293-KOR and HEK293-B2R cells (Fig. 10). Furthermore, Dyn A(1–13) induced the proliferation of human SH-SY5Y cells in dose-dependent manner (Fig. 10C). These results indicate that Dyn A(1–13) plays a neuroprotective role via increasing the proliferation of SH-SY5Y cells.

Dyn A(2–17), a des-tyrosyl fragment of DYN A, has a very low affinity for KOR. Liu et al. found that Dyn A(1–17) and Dyn A (2–17) protect neurons from damage with an equal potency [22], suggesting that the neuroprotective effect of DYN is not mediated through binding to the KOR, but rather through a novel mechanism. As we know, Dyn A mediated neuroinhibitory effects through the opioid receptors. Interestingly, Lai et al. found that non-opioid fragment Dyn A(2-13) can promote hyperalgesia through agonist action at bradykinin receptors, especially B2R, in a neuropathic pain model [71]. Conversely, non-opioid [des-Arg$^{7}$]-Dyn A(4-11) and its analogues, which also directly interact with BK receptors, can inhibit Dyn A-induced neuro-excitatory effects and hypersensitivities[72, 73], but this ligand was quickly degraded. Compared with [des-Arg$^{7}$]-Dyn A(4-11), its analogue [D-Leu$^{5}$, des-Arg$^{7}$]-DynA-(4−11) modified at Leu$^{5}$ is very stable, and the ligand can also binds to the BK receptors with high affinity and possesses the same potent antihyperalgesic effects[74]. These studies suggested that modified Dyn A has a promising treatment for chronic neuropathic pain and play potential neuroprotection via a novel mechanism. Here, we demonstrated that KOR and B2R could form a heterodimer, which induced the Gαs/cAMP/PKA/CREB signaling pathway after treatment with Dyn A(1–13), finally resulting in a significant increase in cell proliferation.

In summary, we provide clear evidence that the KOR and B2R can form heterodimers in co-transfected HEK293 cells, leading to new Gαs coupling after stimulation with Dyn A(1–13). Activation of the Gαs/cAMP/PKA signaling pathway causes an increase in CREB phosphorylation to enhance cell proliferation. Our study therefore defines the underlying mechanisms of neuroprotection modulated by heterodimerization and helps devise novel strategies to combat neurodegenerative diseases. Knockout KOR and/or B2R in SH-SY5Y cells are necessary for our present and further study. However, *in vivo* experiments are required to further investigate the roles of KOR/B2R heterodimers.

## Disclosure statement

The authors declare that they have no conflicts of interest with the contents of this article.

## Author contributions

Bingyuan Ji, Rumin Zhang, Yunlu Jiang and Sheng Li performed the experiments; Haiqing Liu and Chunmei Wang analyzed the data; Bingyuan Ji wrote the manuscript; Bo Bai and Jing Chen designed the experiments and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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# Figure Legends

**Fig. 1 Expression and co-localization of KOR and B2R in SH-SY5Y and HEK293 cells.** RT-PCR revealed that SH-SY5Y cells, but not HEK293 cells, express KOR and B2R **(A, B)**. Similar expression was noted for both receptors at the protein level by western blotting **(C, D)**. Lane M: DNA ladder, Lane HEK: Untransfected HEK293 cells. **(E)** Confocal microscopy analysis of the co-localization of KOR and B2R was performed in HEK293 cells co-transfected with pEGFP-B2R (green) and pcDNA3.1-KOR. pcDNA3.1-KOR was immunostained with Alexa-Fluor 647 (red). Confocal microscopy images of KOR and B2R were merged to show regions of co-localization. DAPI was used to stain nuclei. Immunofluorescence was measured with a Leica fluorescence microscope.

**Fig. 2 Heterodimerization of KOR and B2R determined by BRET assays in living cells. (A)** Heterodimerization of KOR and B2R was measured in the four indicated conditions by BRET. Twenty-four hours after transfection, the fluorescence and luminescence of each sample were measured prior to every experiment to confirm equal expression of Rluc while monitoring the increase in Venus expression. BRET ratios were analyzed and expressed as the means ± SEM of four experiments. $^{\$\$}P<0.01$ *vs.* KOR-Rluc+Venus group and KOR-Rluc+mOX2αR-Venus group; $^{***}P<0.001$ *vs.* Rluc+B2R-Venus group. **(B)** BRET saturation assay. HEK293 cells were transfected with a constant amount of the Rluc construct and increasing amounts of the Venus construct. The calculated BRET signals were plotted as a function of the total fluorescence/luminescence ratios, and data were analyzed using linear and non-linear regression curve fitting in GraphPad Prism (GraphPad Software, CA, USA). **(C–D)** Competition binding assay. HEK293 cells were co-transfected with a fixed amount of the KOR-Rluc and B2R-Venus plasmids and increasing amounts of B2R **(C)** or KOR **(D)** not fused to a donor or acceptor BRET probe. Data were expressed as means ± SEM of three experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* control group.

**Fig. 3 Assessment of dimerization of KOR and B2R using FRET assays. (A)** FRET imaging of constitutive KOR-B2R heteromeric interactions in living cells. HEK293 cells were transiently transfected (co-expressed or individually expressed) with the following plasmids: (a) KOR-CFP, (b) B2R-Venus, (c) KOR-CFP and mOX2αR-Venus, and (d) KOR-CFP and B2R-Venus. Left-hand panels, CFP; center panels, Venus; right-hand panels, corrected FRET. **(B)** Calculated normalized FRET values were assessed and shown as described in the *Materials and Methods*. $^{***}$ P<0.001 *vs.* other groups. Results are the means ± SEM of at least three independent experiments. **(C)** FRET imaging of constitutive KOR-B2R heteromeric interactions in living CHO cells.

**Fig. 4 KOR and B2R are in close proximity on the surface of HEK293 cells. (A)** Detection of interactions between KOR and B2R in co-transfected HEK293 cells by the PLA. **(B)** Omission of one secondary antibody after incubation with anti-KOR and anti-B2R antibodies in co-transfected HEK293 cells. PLA signals were detected by Detection Reagents Red (red). Nuclei were stained with DAPI (blue). Each red dot represents the detection of a protein-protein interaction. All images are representative of three independent experiments.

PLA: PLA fluorescence signal. PLA/DAPI: merged PLA/DAPI signals.

**Fig. 5 Evaluation of the cAMP response in HEK293 cells using an EPAC BRET biosensor. (A)** Schematic diagram of the presumptive molecular rearrangement of the EPAC protein with and without cAMP. **(B)** FSK decreased the BRET signal upon binding to CAMYEL (cAMP sensor using YFP-Epac-RLuc)), as measured by the decrease in 535/465 nm fluorescence. $^{****}$P<0.001. **(C–D)** Measurement of BRET signals in HEK293 cells co-expressing the cAMP biosensor and KOR, B2R, or KOR and B2R and stimulated with Dyn A(1–13) **(C)** or BK **(D)**. $^{*}$P<0.05, $^{***}$P<0.001, NS: not significantly different. All the results are expressed as means ± SEM of four experiments.

**Fig. 6 BRET analysis of the effects of KOR/B2R heterodimerization on Gα protein subunit activation.** HEK293 cells transiently expressing KOR, B2R, or KOR and B2R, together with Gαs-Rluc8, Gαi2-Rluc8, or Gαq-Rluc8 and unlabeled β1 and γ2, were prepared and analyzed as described in the figure. **(A)** Real-time analysis of the interaction between KOR/B2R heterodimers and Gαs using BRET in living cells. **(B)** Real-time analysis of the interaction between KOR/B2R heterodimers and Gαi2 using BRET in living cells. **(C)** Real-time analysis of the interaction between KOR/B2R heterodimers and Gαq using BRET in living cells. $^{**}$P<0.01, HEK293 cells co-expressing KOR and B2R *vs.* HEK293 cells expressing B2R only; $^{\#\#}$P<0.01, HEK293 cells expressing KOR only *vs.* HEK293 cells co-expressing KOR and B2R, as determined by the Student's t-test. Results are the means ± SEM of at least four independent experiments.

**Fig. 7 Effect of KOR and B2R heterodimerization on CRE and SRE activities.** Twenty-four hours after transfection with pCRE-luc **(A)** or pSRE-luc **(B)** together with pRL-TK, HEK293-KOR, HEK293-B2R, and HEK293-KOR/B2R cells were treated with Dyn A(1–13) (100 nM final concentration) as indicated for 6 h prior to harvest. When indicated, cells were pretreated with PKC inhibitor BisII (1 μM for 2 h). The firefly and Renilla luciferase activities were assayed using the Dual-Luciferase® Reporter Assay System according to the manufacturer's instructions. The ratio of the firefly luciferase luminescence signal to that of Renilla luciferase was determined. The results are expressed as the means ± SEM of six independent experiments performed with duplicate samples. (A) $^{\#\#}$P<0.01, KOR group not treated with agonist *vs.* KOR group treated with Dyn A(1-13); $^{**}$ P<0.01 *vs.* other groups. (B) $^{**}$P<0.01.

**Fig. 8 KOR/B2R dimer activity mediates CREB phosphorylation following Dyn A(1–13) treatment. (A)** HEK293 cells expressing KOR, B2R, or KOR and B2R were treated with a constant concentration of Dyn A(1–13) ($10^{-7}$ M) for 0, 5, 10, 15, 30, and 60 min. **(B)** HEK293 cells expressing KOR, B2R, or KOR and B2R were treated with Dyn A(1–13) ($10^{-11}$–$10^{-7}$ M) for 5 min. The time-dependent and dose-dependent effects of the treatments were quantified **(C–D)**. $^{*}$P<0.05, $^{**}$P<0.01 compared with single-transfected groups. **(E–F)** Human SH-SY5Y cells were treated with Dyn A(1–13) ($10^{-9}$–$10^{-6}$ M) for 5 min. $^{*}$P<0.05, $^{**}$P<0.01 *vs.* control group. All the data represent the means ± SEM of three measurements from three independent experiments.

**Fig. 9 KOR/B2R dimer activity mediates CREB phosphorylation in a PKA-dependent and PLC-independent manner. (A–B)** HEK293 cells stably expressing KOR, B2R, or KOR/B2R were treated with a constant concentration of Dyn A(1–13) ($10^{-7}$ M) for 5 min. When indicated, cells were pretreated with PKA inhibitor H89 (10 μM for 30 min). $^{**}P<0.01$ *vs.* other groups. **(C–D)** HEK293 cells stably expressing KOR and B2R were stimulated with Dyn A(1–13) ($10^{-7}$ M) for 5 min. When indicated, cells were pretreated with PLC inhibitor U73122 (10 μM for 30 min). $^{**}P<0.01$ *vs.* control group. All the data represent the means ± SEM of three measurements from three independent experiments.

**Fig. 10 Proliferation of transfected cell lines in response to Dyn A(1–13). (A)** Dose-dependent effects of Dyn A(1–13) on HEK293-KOR/B2R cell proliferation. Co-transfected cells not treated with Dyn A(1–13) were the control. **(B)** HEK293, HEK293-KOR, HEK293-B2R, and HEK293-KOR/B2R cells were cultured for 24 h in a 96-well plate prior to treatment with 100 nM Dyn A(1–13) and starved overnight in serum-free media. Cell proliferation was measured. Untransfected HEK293 cells were the control. **(C)** Dose-dependent effects of Dyn A(1–13) on human SH-SY5Y cell proliferation. Cells not treated with Dyn A(1–13) were the control. Data points were determined from three independent experiments, each with quadruplicate samples. $^{***}P<0.001$, $^{**}P<0.01$.

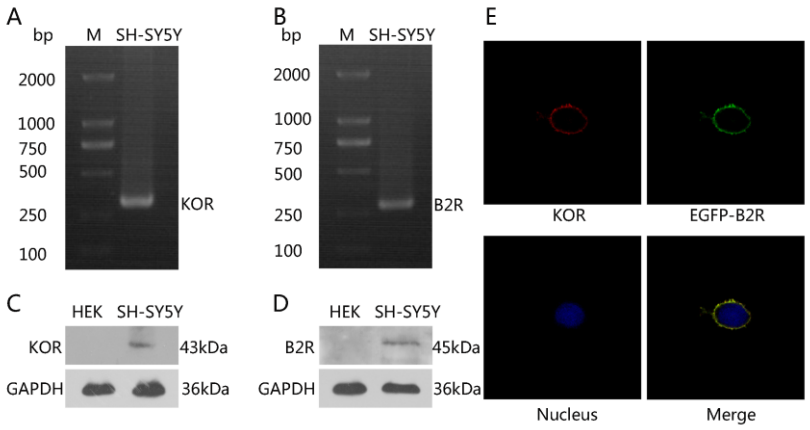



Figure 1

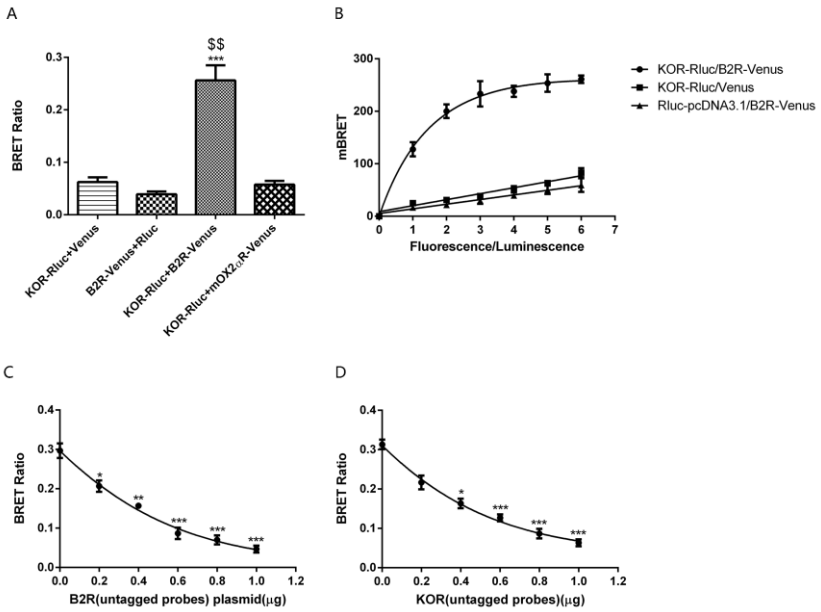
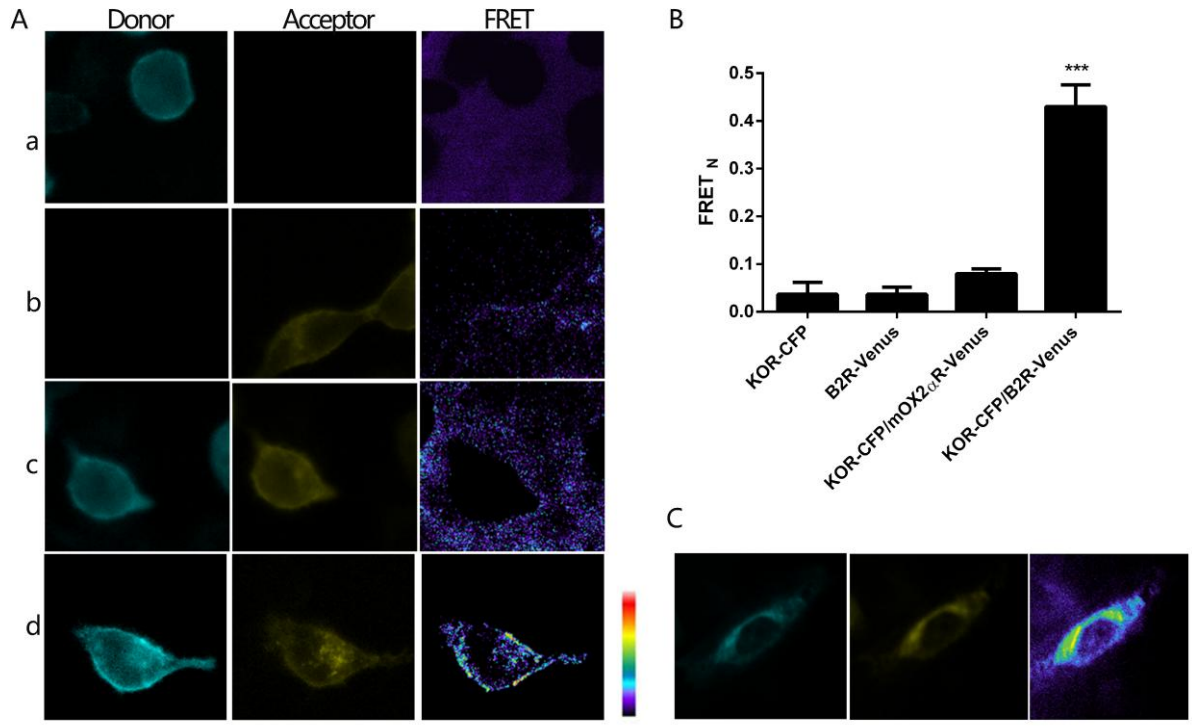



Figure 2

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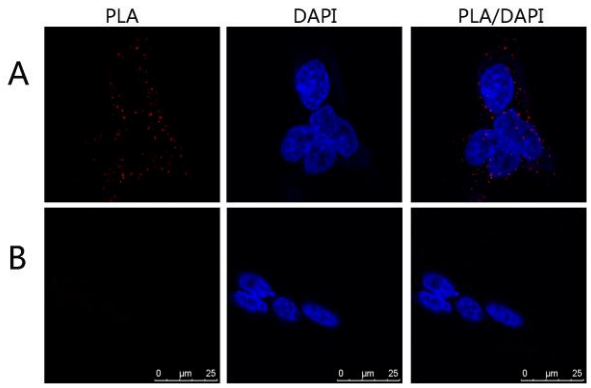


Figure 4

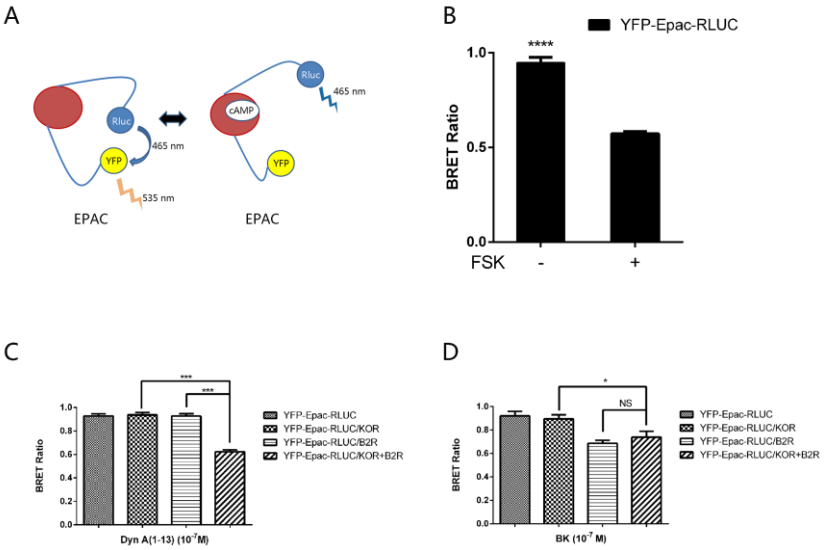


Figure 5

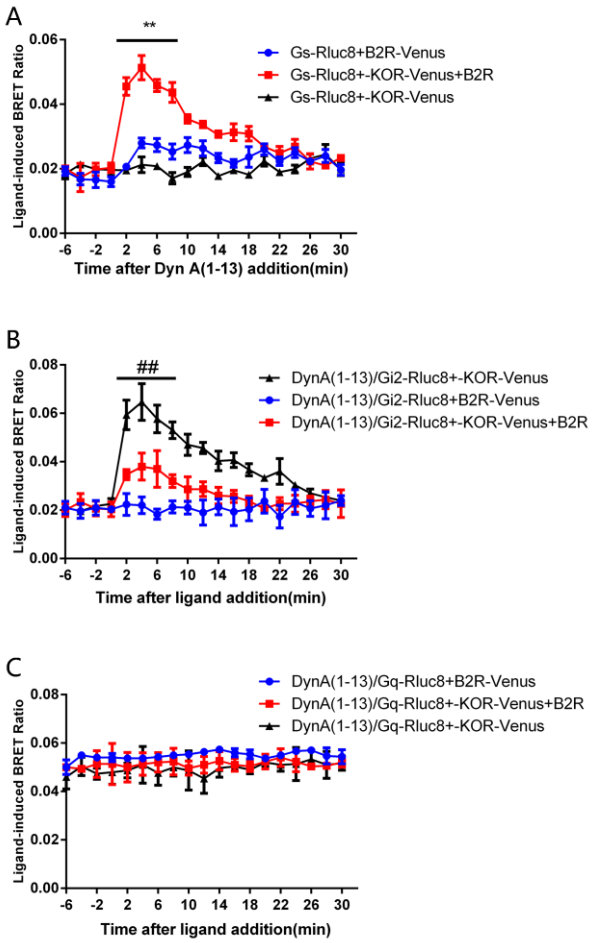


Figure 6

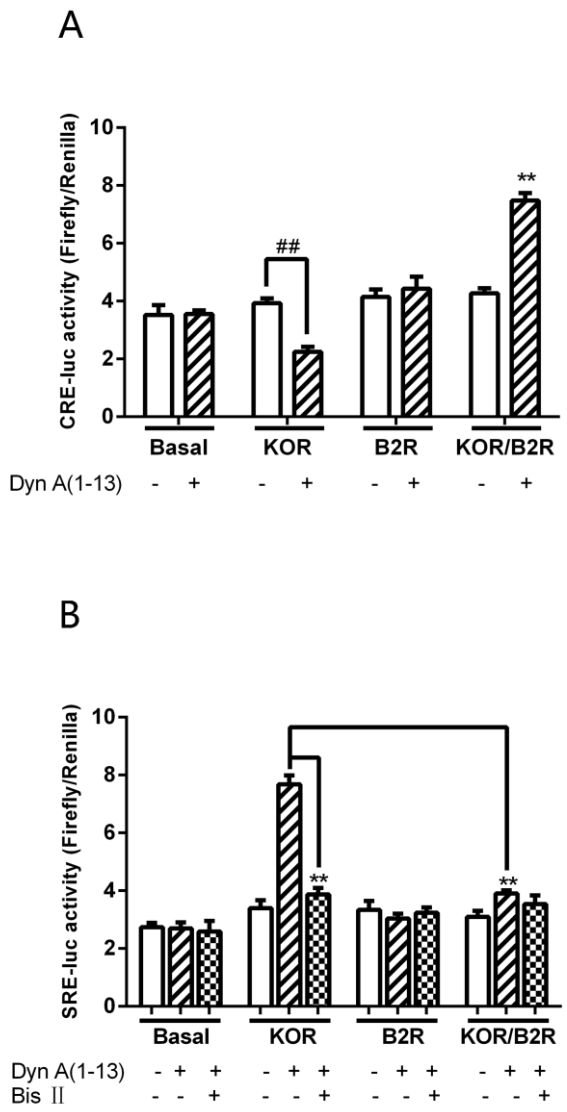


Figure 7

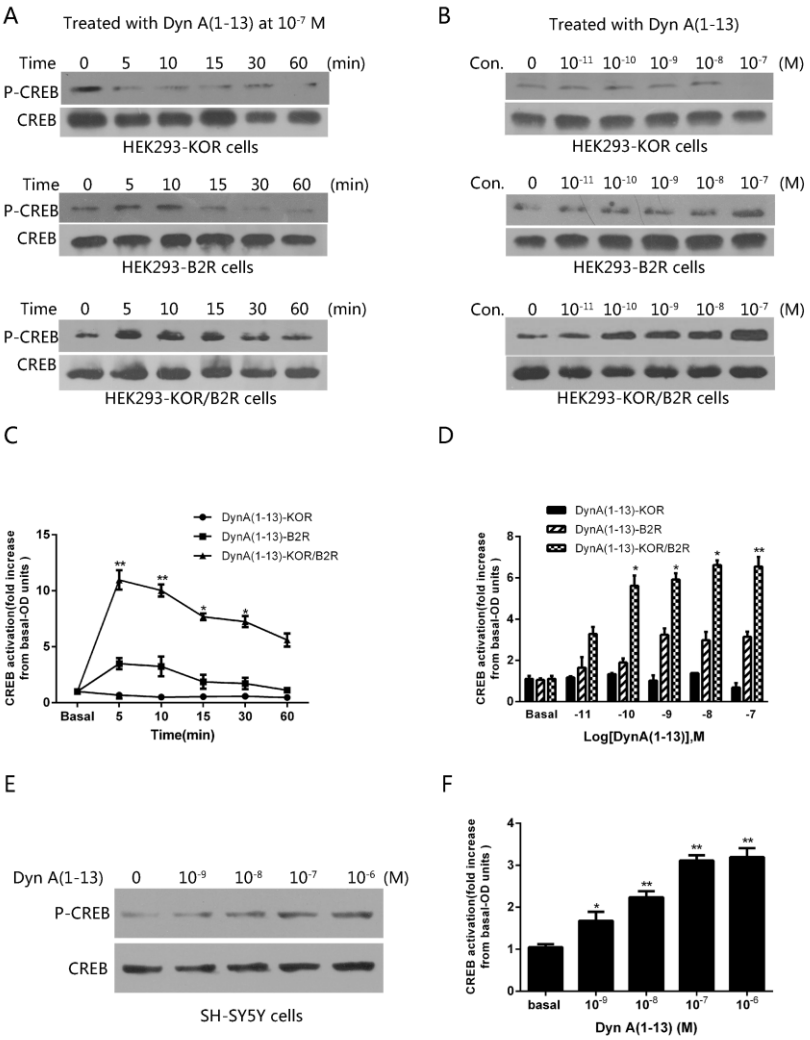


Figure 8

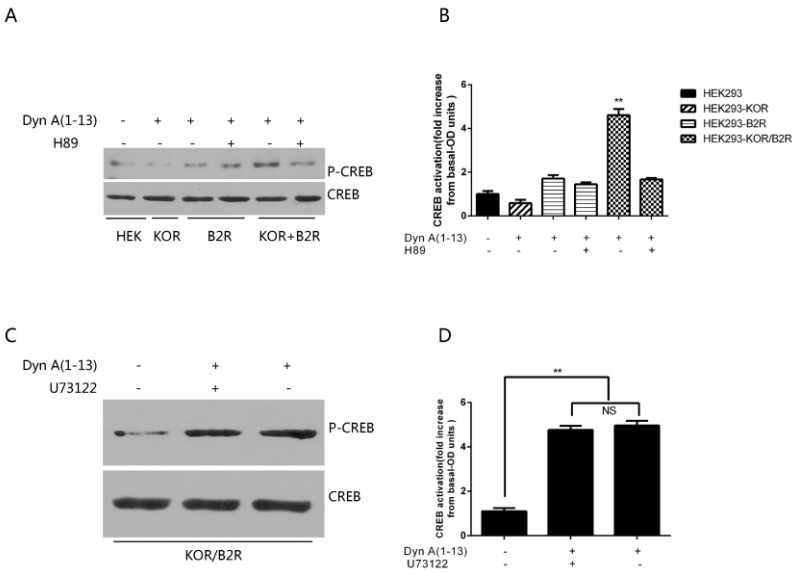


Figure 9

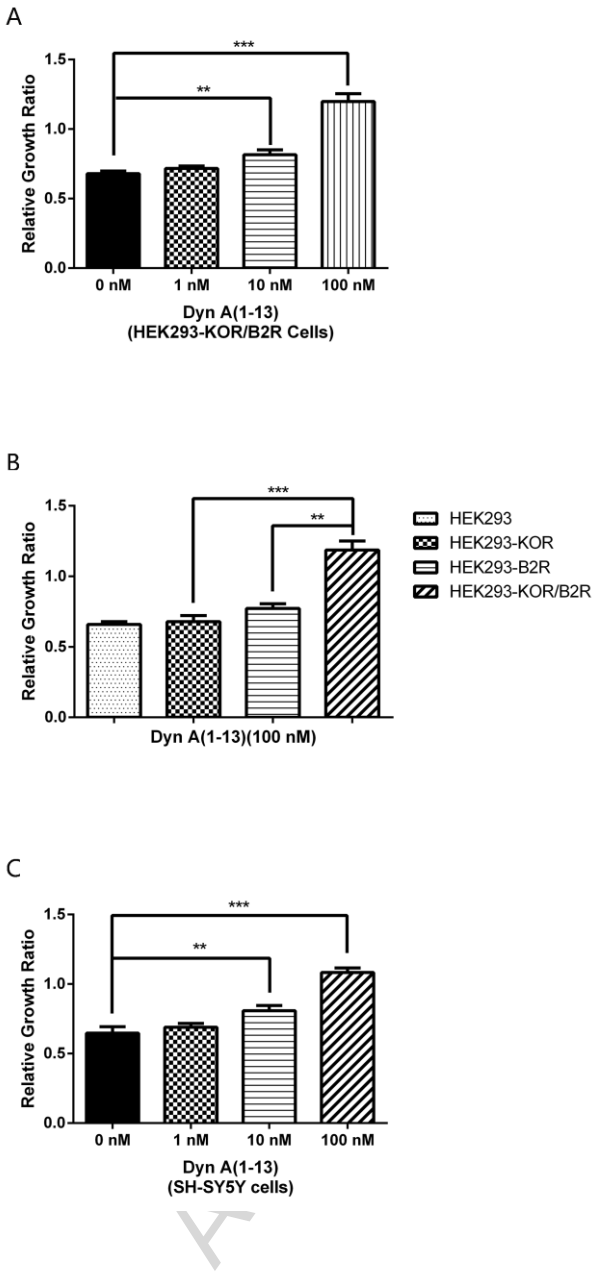


Figure 10

## Highlights

- KOR and B2R form heterodimers that function as a novel pharmacological entity.
- KOR/B2R heteromers mediate the Gαs/cAMP/CREB signaling pathway.
- Dyn A(1–13) enhances proliferation of HEK293-KOR/B2R and SH-SY5Y cells.
- We reveal the molecular mechanism underlying neuroprotection mediated by KOR/B2R heterodimers.