Orthogonal Peptide-Templated Labeling Elucidates Lateral $\text{ET}_A\text{R}/\text{ET}_B\text{R}$ Proximity and Reveals Altered Downstream Signaling

Philipp Wolf, Alexander Mohr, Georgina Gavins, Victoria Behr, Karin Mörl, Oliver Seitz, and Annette G. Beck-Sickinger*
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Experimental section

Polymerase chain reaction primer

Primer sequences used for N-terminal fusion of (SP)-Cys-P1- and (SP)-Cys-P3-tag to GPCRs and the generation of the Nluc-tagged arrestin 3 constructs are summarized in Supplementary Table S1.

Table S1: Primer sequences.

| Code | Name | Sequence |
|------|------|----------|
| 1    | Cys-P1-AT1R fw | 5'-3' AAACCGTATCGCGCACCATGTGCGAGATCCAGGCCCTGGAGGAGGAGAACGCCAGCTGGAGCAGGAGAACGCCGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
| 2    | Cys-P3-AT1R fw | 5'-3' AAACCGTGCAGCCACCATGTGCGAGATCCAGCCTGGAGGAGGAGAACGCCGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
| 3    | Cys-P1-APJ fw | 5'-3' AAACCGTGCAGCCACCATGTGCGAGATCCAGGCCCTGGAGGAGGAGAACGCCGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
| 4    | Cys-P3-APJ fw | 5'-3' AAACCGTGCAGCCACCATGTGCGAGATCCAGGCCCTGGAGGAGGAGAACGCCGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
| 5    | pV2-eYFP-XbaI rev | GTGGAGCCAAACGCCAGTACAAAG |
| 6    | pVitro2-Sall fw | ACACAAAACGTGCAACTTTGAAACTC |
| 7    | pVitro2-Sall rev | TATTTGCACACCGTTGCTTGAATTAG |
| 8    | Sall-Cys-P1-AT1R-Stop fw | AAACCGTCGACACCATGTGCGAGATCCAGGCCCTGGAGGAGGAGAACGCCGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
| 9    | Sall-Cys-P1/P3-GPCR-Stop fw | AAACCGTCGACACCATGTGCGAGATCCAGGCCCTGGAGGAGGAGAACGCCGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
| 10   | AT1R-Stop rev | TTTGTCTAGATTACTCAACCTCAACATGTTGCGAG |
| 11   | APJ-Stop rev | TTTGTCTAGATTACTCAACCTCAACATGTTGCGAG |
| 12   | SP-Cys-P1-AT1R/APJ-MP fw | AAACCGGTTCGACAGCCACCATGTGCGAGATCCAGGCCCTGGAGGAGGAGAACGCCGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
| 13   | SP-Cys-P1-AT1R/APJ-MP rev | AAACCGGTTCGACAGCCACCATGTGCGAGATCCAGGCCCTGGAGGAGGAGAACGCCGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
| 14   | SP-Cys-P3-AT1R/APJ-MP fw | AAACCGGTTCGACAGCCACCATGTGCGAGATCCAGGCCCTGGAGGAGGAGAACGCCGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
| 15   | SP-Cys-P3-AT1R/APJ-MP rev | AAACCGGTTCGACAGCCACCATGTGCGAGATCCAGGCCCTGGAGGAGGAGAACGCCGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
| 16   | SP-Cys-P1-ET3R fw | TGGGACTGTTTGGATGTGTAATCAAGTGGGTGAGATCCAGGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
| 17   | SP-Cys-P1-ET3R rev | TGGGACTGTTTGGATGTGTAATCAAGTGGGTGAGATCCAGGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
| 18   | SP-Cys-P3-ET3R fw | TGGGACTGTTTGGATGTGTAATCAAGTGGGTGAGATCCAGGCCCTGGAGGAGGAGATCGCCCAGCTGGAGTACGGCGGCTCAATGATTCTCAACTCTTTCTACTCTG |
Solid phase synthesis of the TAMRA-MPAA-GSGSG conjugate

Materials

N-α-Fmoc-protected amino acids, ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma), and N,N'-diisopropylcarbodiimide (DIC) were purchased from Iris Biotech (Marktredwitz, Germany). G-Wang resin and O-(7-azabenzotriazolyl)-tetramethyloxonium hexafluorophosphate (HATU) were supplied from Novabiochem (Darmstadt, Germany). 6-Carboxytetramethylrhodamine (TAMRA) was purchased from ChemPep, Inc. (Wellington, Florida). Acetonitrile (ACN) was obtained from VWR (Darmstadt, Germany). Dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Biosolve (Valkenswaard, The Netherlands). N,N-Diisopropylethylamine (DIPEA), 1,2-ethanediethiol (EDT), 4-methoxytriphenylmethyl chloride (Mmt-Cl), mercapto phenyl acetic acid (MPAA), piperidine, thioanisole (TA), trifluoroacetic acid (TFA), and triisopropylsilane (TIS) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Diethyl ether was obtained from Merck (Darmstadt, Germany).
Synthesis of the control conjugate

S-Mmt-protected MPAA was prepared as described in the main manuscript. The control peptide was synthesized on a G-Wang resin (15 μmol scale). Automated synthesis was performed with a SYRO I peptide synthesizer (MultiSynTech; Witten, Germany), using 8 equiv. of N-α-Fmoc-protected amino acids, 8 equiv. Oxyma, and 8 equiv. DIC dissolved in DMF. Automatic coupling steps were carried out twice with a reaction time of 40 min each. For Fmoc deprotection, 40 % (v/v) piperidine in DMF was applied for 3 min and 20 % (v/v) piperidine in DMF for 10 min. The S-Mmt-MPAA-OH (4.5 equiv.) was coupled twice for 45 min each in DMF using 4.5 equiv. HATU, and 8 equiv. DIC dissolved in DMF. After Mmt deprotection, using DCM/TFA/TIS (96:2:2, 2x1 min), TAMRA was coupled with 1.9 equiv HATU and 2 equiv DIPEA for approximately 18 h at RT. Peptides were cleaved from the resin with TFA/H2O/TIS (96:2:2) and precipitated from diethyl ether. Purification peptide was carried out on a RP-HPLC (Shimadzu) equipped with a Phenomenex Kinetex C18 100 Å column by applying a linear binary gradient of eluent A (0.1 % TFA in water, v/v) and B (0.08 % TFA in ACN). Peptide purity was confirmed by analytical RP-HPLC, and peptide identity was verified by mass spectrometry: MALDI-ToF mass spectrometry (UltraflexIII, Bruker Daltonics), and ESI-ion trap mass spectrometry (HCT, Bruker). Peptides were kept as a 10⁻⁴ M stock aqueous solution, containing 0.1 % TFA.

Generation of nanoluciferase-arrestin-3

The cDNA of bovine arrestin-3 (arr-3) was amplified from the previously described pcDNA3.1-Rluc3-Arr3 plasmid[1] introducing a 5’ AflII restriction site (primer: 28 and 29). The Nluc cDNA, containing a 5’ secretion sequence, was amplified from the pNL1.3-secLuc plasmid (kindly provided by A. Kaiser, Leipzig University) introducing a 5’ AsiSI restriction site (primer: 30 and 31). The PCR products were fused by overlap extension (OE) PCR (primer: 29 and 30) and digested with AsiSI/HindIII prior to ligation into a pcDNA3.1 vector.

Inositol phosphate accumulation assay

COS-7 cells were grown in 25 cm² culture flasks until 70-80 % confluency was reached. Transient transfection was performed using Metafectene® Pro (according to the manufacturer’s protocol) with a total of 4000 ng plasmid DNA per 25 cm² flask. The apelin receptor was co-transfected with the chimeric GoΔGq4myr protein (3/1 ratio) (kindly provided by E. Kostenis (Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany)).[2] After 24 h, transfected cells were seeded into 384-well plates (15,000 cells in 20 μL/well) and cultured until the experiment. Cells were stimulated with different peptide concentrations (10⁻⁷-10⁻¹² M) in HBSS, containing 20 mM LiCl, for 60 min at standard conditions. Cell lysis and inositol phosphate detection was performed as described by the manufacturer. Assay readout was
performed on a Tecan Spark plate reader (Tecan Group, Männedorf, Switzerland). Fluorophore emission was measured at (620/10 nm; 665/8 nm) after donor excitation (320/25 nm) and the HTRF ratio \((10,000 \times 665 \text{ nm}_{\text{emission}}/620 \text{ nm}_{\text{emission}})\) was calculated. Concentration-response curves were generated by normalization to the respective wild type/control (bottom value = 0 %; top value = 100 %) and determination of \(EC_{50}/pEC_{50}\) and \(E_{\text{max}}\) values was performed with the software GraphPad PRISM 5.0 (San Diego, USA).

**Arrestin 3 recruitment assay**

COS-7 cells were transiently transfected using Metafectene® Pro (according to the manufacturer’s protocol) in 25 cm² cell culture flask after reaching 70 % confluency. Transfection of was performed at a 1/20 ratio of Nluc-arr3 (BRET donor) to ET₅R-GFP (BRET acceptor) plasmid DNA, balancing the total amount of plasmid DNA (4000 ng) per cell culture flask with mock DNA. One day post transfection, cells were seeded into 96-well µclear plates (100,000 cells/well) and cultured at standard conditions. All BRET experiments were performed at 37 °C. The medium was replaced with BRET buffer (25 mM HEPES in HBSS, pH 7.3) and furimazine (Promega (Madison, Wisconsin)) was added (final concentration: 2.1 μM). For kinetic experiments, the baseline was monitored for 5 min before peptide addition (final concentration: 100 nM). For concentration-dependent analyses, peptides were applied in a concentration range \((10^{-12} \text{ to } 10^{-7} \text{ M})\) and the BRET signal was measured after 7.5 min. BRET studies were carried out on a Tecan Spark plate reader (Tecan Group, Männedorf, Switzerland). The luminescence was monitored from 400-440 nm and fluorophore emission was detected between 505-590 nm. The fluorescence to luminescence ratio was calculated (BRET ratio) and netBRET values were determined by subtraction of BRET signals derived from unstimulated cells. Concentration-response curves were generated by normalization to the respective wild type/control (bottom value = 0 %; top value = 100 %) and determination of \(EC_{50}/pEC_{50}\), and \(E_{\text{max}}\) values was performed with the software GraphPad PRISM 5.0 (San Diego, USA).

**Statistical analysis**

Statistical analyses were performed with the software GraphPad PRISM 5.0 (San Diego, USA). Significances were calculated by one-way ANOVA and Tukey’s t-test.
Results

Activation profile of Cys-P1/P3-ET\(_{A}\)R/ET\(_{B}\)R-GFP and proof of principle labeling

Figure S1: Activation profile of Cys-P1/P3-ET\(_{A}\)R/ET\(_{B}\)R-GFP and proof of principle labeling. GPCR activation (A: ET\(_{A}\)R; B: ET\(_{B}\)R) was investigated by inositol phosphate accumulation assay (transiently transfected COS-7 cells; n≥3 performed in triplicates). Concentration-response curves represent the mean over all assay repetitions. Membrane localization was determined in HEK293 cells either expressing wt, Cys-P1- or Cys-P3-tagged receptors (lower panels). Peptide-templated acyl transfer was carried out on Cys-P1/P3-ET\(_{A}\)R-GFP (C) or Cys-P1/P3-ET\(_{B}\)R-GFP (D) using 200 nM TAMRA-P2/-P4 in transiently transfected HEK293 cells (n≥3). Scale bar: 10 µm. Figure B is partially adapted from Gavins et al. (2021).

Table S2: Receptor activation after N-terminal modification. Activation of either wild type (wt) ET\(_{A}\)R/ET\(_{B}\)R-GFP-spark, or Cys-P1/P3-ET\(_{A}\)R/ET\(_{B}\)R-GFP was assessed in inositol phosphate accumulation assays transiently transfected COS-7 cells; n≥3 performed in triplicates).

| Receptor Tag | ET\(_{A}\)R-GFP EC\(_{50}\) [nM] | ET\(_{B}\)R-GFP EC\(_{50}\) [nM] | E\(_{\text{max}}\) [%] ± SEM | ET\(_{A}\)R-GFP EC\(_{50}\) [nM] | ET\(_{B}\)R-GFP EC\(_{50}\) [nM] | E\(_{\text{max}}\) [%] ± SEM |
|--------------|-------------------------------|-------------------------------|----------------------|-------------------------------|-------------------------------|----------------------|
| wt           | 0.6                           | 9.2 ± 0.03                    | 100 ± 1              | 2.4                           | 8.6 ± 0.03                    | 99 ± 2               |
| Cys-P1       | 0.6                           | 9.2 ± 0.07                    | 102 ± 3              | 1.4                           | 8.8 ± 0.07                    | 98 ± 3               |
| Cys-P3       | 0.5                           | 9.3 ± 0.07                    | 99 ± 2               | 2.0                           | 8.7 ± 0.09                    | 104 ± 4              |
Figure S2: Determination of orthogonal peptide-templated acyl transfer specificity. (A) Structure of control peptide lacking the coiled-coil motif. (B) Peptide-templated labeling using either 200 nM 6-carboxytetramethylrhodamine (TAMRA)-control peptide, TAMRA-P2 or TAMRA-P4 on HEK293 transiently expressing either Cys-P1- or Cys-P3-ETAR-GFP (n=3). Scale bar: 10 µm. (C) Fluorescence quantification after peptide-templated labeling. Untransfected HEK293 cells or HEK293 expressing either Cys-P3-ETAR or Cys-P1-ETBR were labeled with either Atto488-P2 or Atto565-P4. After labeling, the fluorescence was measured for each condition. Successful and efficient labeling was only observed for Cys-P1/P2 and Cys-P3/P4.
Figure S3: Receptor activation after peptide-templated labeling. (A) ET\(_\text{A}\)R-GFP, Cys-P1-, and Cys-P3-ET\(_\text{A}\)R-GFP or (B) ET\(_\text{B}\)R-GFP, Cys-P1-, and Cys-P3-ET\(_\text{B}\)R-GFP were labeled with 6-carboxytetramethylrhodamine (TAMRA)-P2 or -P4, using 0.1 mM TCEP. Agonist-triggered calcium flux was recorded after labeling (\(n\geq3\)). Black: wt GPCR; green: Cys-P1-GPCR; red: Cys-P3-GPCR. Dashed lines indicate TCEP-treated cells. Signal transduction data was normalized to the wt receptors (untreated) and represent the mean over all assay repetitions.
Figure S4: Determination of labeling probe selectivity. (A) Orthogonal coiled-coil peptide sequences (single-letter amino acid code) are depicted and interaction pattern are highlighted (hydrogen bonding: red; hydrophobic interactions: blue). Interaction specificity of the P1/P2 and P3/P4 coiled coil motifs was assessed by fluorescence microscopy by applying either 6-carboxytetramethylrhodamine (TAMRA)-P2 or TAMRA-P4 probe peptides to P3-tagged ET\(_{\beta}\)R-GFP (B) and P1-tagged ET\(_{\alpha}\)R-GFP (C) expressing HEK293 cells (n=3). Scale bar: 10 µm.
**Figure S5:** Determination of labeling efficiency on live cells. HEK293 cells, expressing either Cys-P1- or Cys-P3-tagged ET_{a}-GFP or ET_{b}-GFP were labeled with TAMRA-P2 (TMR-P2) or TAMRA-P4 (TMR-P4), respectively. Total GFP and TAMRA fluorescence were measured separately (A) and the GFP/TAMRA (B) ratio was determined (n=4 in quadruplicates).
### GPCR interaction characteristics derived from proximity-dependent FRET

**Table S3**: GPCR interaction characteristics derived from proximity-dependent FRET assays for GPCRs from different species derived from N-terminal proximity-dependent FRET assays after peptide-templated GPCR labeling in live cells. Titration experiments were performed, using constant amount of donor DNA (P1-GPCR) and adding increasing amounts of acceptor DNA (P3-GPCR) for transient transfection, which were subsequently and simultaneously labeled with Atto488-P2 (FRET donor) and Atto565-P4 (FRET acceptor). Formation of specific interaction is indicated by hyperbolic fitting (HEK293 cells, n≥2 each performed in quadruplicates).

| FRET pair | FRET<sub>max</sub> ± SEM | FRET<sub>50</sub> ± SEM | Quality of fit [R²] |
|-----------|-------------------------|------------------------|---------------------|
| Acceptor* | Donor*                  | observed<sup>#</sup>    | regression<sup>§</sup> |                     |
| ETAR      | ETAR                    | 0.76 ± 0.09            | 0.96 ± 0.08         | 0.15 ± 0.06         | 0.94                |
| ETBR      | ETBR                    | 0.57 ± 0.08            | 0.57 ± 0.05         | 0.25 ± 0.07         | 0.96                |
| AT1R      | AT1R                    | 0.72 ± 0.13            | 0.98 ± 0.09         | 0.85 ± 0.17         | 0.97                |
| APJ       | APJ                     | 0.58 ± 0.13            | 0.75 ± 0.06         | 0.63 ± 0.12         | 0.98                |
| ETAR      | ETAR                    | 0.58 ± 0.09            | 0.65 ± 0.05         | 0.18 ± 0.04         | 0.95<sup>a</sup>    |
| AT1R      | ETAR                    | n.d.                   | n.d.                | n.d.                | 0.92<sup>b</sup>    |
| APJ       | ETAR                    | n.d.                   | n.d.                | n.d.                | 0.99<sup>b</sup>    |
| AT1R      | ETAR                    | n.d.                   | n.d.                | n.d.                | 0.90<sup>b</sup>    |
| APJ       | ET1R                    | n.d.                   | n.d.                | n.d.                | 0.91<sup>b</sup>    |
| APJ       | AT1R                    | n.d.                   | n.d.                | n.d.                | 0.93<sup>b</sup>    |

<sup>a</sup> hyperbolic fit  
<sup>b</sup> linear fit  
*acceptor construct carried the N-terminal Cys-P3-tag, whereas donor constructs were equipped with the Cys-P1-tag  
<sup>#</sup> observed FRET<sub>max</sub> correlates to the experimentally determined netFRET derived from the highest A/D ratio  
<sup>§</sup> refers to the FRET<sub>max</sub> value derived from the hyperbolic fit in signal saturation
**Determination of ligand selectivity and GPCR activation profiles**

**Figure S6:** Determination of ligand selectivity and GPCR activation profiles. Peptide ligands AngII, Ap13 or ET-1 were administered to determine activation of ET₄R (A), ET₃R (B), AT₃R (C), and APJ (D) in inositol phosphate accumulation assays by Gᵢ signaling (in transiently transfected COS-7 cells; n≥2 each performed in triplicates). Concentration-response curves represent the average of all assay repetitions.

**Table S4:** Gᵢ activation profiles of GPCR depending on cardiovascular-active peptide ligands. Receptor activation was assessed in concentration-dependent inositol phosphate accumulation assays (in transiently transfected COS-7 cells; n≥2 each performed in triplicates). Transfected cells were stimulated either with AngII, Ap13 or ET-1. Concentration-response curves represent the average of all assay repetitions.

| Peptide ligand | ET₄R | ET₃R | AT₃R | APJ |
|----------------|------|------|------|-----|
| GPCR           | EC₅₀ [nM] | pEC₅₀ ± SEM | Eₘₐₓ [%] ± SEM | EC₅₀ [nM] | pEC₅₀ ± SEM | Eₘₐₓ [%] ± SEM | EC₅₀ [nM] | pEC₅₀ ± SEM | Eₘₐₓ [%] ± SEM |
| AngII          | n.d. | n.d. | n.d. | n.d. | n.d. | 0.6 | 9.2 ± 0.03 | 100 ± 1 |
| Ap13           | n.d. | n.d. | n.d. | n.d. | n.d. | 2.4 | 8.6 ± 0.03 | 99 ± 2 |
| AT₃R           | 0.4 | 9.3 ± 0.02 | 100 ± 1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| APJ            | n.d. | n.d. | 1.7 | 8.8 ± 0.02 | 100 ± 1 | n.d. | n.d. | n.d. | n.d. |

n.d. = not detectable, Ang II - angiotensin II; AT₃R - angiotensin II receptor 1; Ap13 - apelin-13; APJ - apelin receptor; ET₄R - endothelin A receptor; ET₃R - endothelin B receptor; ET-1 - endothelin 1
Figure S7: Agonist-internalization of GPCRs labeled by peptide-templated acyl transfer reaction in live cells. HEK293 were either transfected with Cys-P3-ETAR (A), Cys-P1-ETBR (B), SP-Cys-P3-AT1R (C), or SP-Cys-P3-APJ (D). Membrane embedded GPCRs were labeled with Atto488-P2 or Atto565-P4, respectively, prior to agonist application. ETAR- and ETBR-expressing HEK293 cells were stimulated with either 500 nM ET-1 (dual agonist) or [4Ala1,3,11,15,Nle7]-ET-1 (linear ET-1, ETBR-selective agonist) for 1 h after labeling prior to picture acquisition (n=3). For cells expressing AT1R or APJ, 500 nM AngII or Ap13 were applied, respectively, for 60 min after labeling prior to picture acquisition (n=3). Scale bar: 10 µm.
Figure S8: Impact of receptor activation on the agonist-induced internalization of the ET\(_{a}\)R, co-expressed with either ET\(_{a}\)R, AT\(_{1}\)R, or APJ. HEK293 were transfected with Cys-P1-ET\(_{a}\)R and either Cys-P3-ET\(_{a}\)R (A), SP-Cys-P3-AT\(_{1}\)R (B), or SP-Cys-P3-APJ (C). Membrane embedded GPCRs were labeled with Atto488-P2 (green) and Atto565-P4 (red), respectively. After peptide-templated labeling, cells were stimulated with either 500 nM ET-1 (for ET\(_{a}\)R or ET\(_{b}\)R activation), 500 nM [4Ala\(^{1,3,11,15}\),Nle\(^{7}\]\)-ET-1 (linear ET-1, for selective-ET\(_{a}\)R activation in the presence of ET\(_{a}\)R), 500 nM AngII (for AT\(_{1}\)R activation), or Ap-13 (for APJ activation) for 60 min under cell culture conditions prior to fluorescence microscopy (n≥2, representative images are shown). Scale bar: 10 µm.
Figure S9: Membrane residence time of N-terminally labeled GPCRs in the absence of agonist administration. (A) Membrane-embedded Cys-P3-ET_{a}R, Cys-P1-ET_{b}R, SP-Cys-P3-AT_{1}R, or SP-Cys-P3-APJ were stained using the Atto488-P2 or Atto565-P4 peptide (ET_{a}R: blue, ET_{b}R: light green, AT_{1}R: orange, APJ: purple). (B) Membrane residence time of Cys-P1-ET_{b}R co-expressed with Cys-P3-ET_{a}R, SP-Cys-P3-AT_{1}R or SP-Cys-P3-APJ were labeled using the Atto488-P2 and Atto565-P4 peptide probe (ET_{b}R/mock: light green, ET_{b}R/ET_{a}R: blue, ET_{b}R/AT_{1}R: orange, ET_{b}R/APJ: purple). Picture acquisition was performed at distinct time point (0, 15, 30, and 60 min) post-labeling without agonist application. Membrane fluorescence was quantified for each time point and normalized to 0 min (100 %) and background fluorescence (0 %). Quantitative data represent the average over all assay repetitions (n≥2 with 10–15 cells analyzed per time point and experiment). Significance was determined by one-way ANOVA and Tukey’s post test, n.s.: not significant, **: P<0.01; ***: P<0.001). Scale bar: 10 µm.
Selective ET₆R-mediated Ca²⁺ flux in the presence of co-transfected ET₆R

Figure S10: Selective ET₆R-mediated Ca²⁺ flux in the presence of co-transfected ET₆R. COS-7 cells were transiently transfected with constant amounts of ET₆R-GFP increasing amounts of Cys-P3-ET₆R in a ratio range from 1:0 to 1:3. Cells, expressing both ET₆R and ET₆R were stimulated with the ET₆R/ET₆R dual agonist ET-1 (A) or the ET₆R-selective [4Ala₄,3,11,15, Nle₇]-ET-1 (linear ET-1). The ET₆R-selective antagonist sitaxentan (red symbol, sita) was applied for selective ET₆R activation by ET-1 in the presence of co-expressed ET₆R (C). ET₆R-GFP expression was monitored by GFP fluorescence. Ca²⁺ flux mediated by Gq protein (turquoise) activation was monitored in COS-7 cells in duplicates and concentration-response curve represented the average over all assay repetitions (n=3; significance was determined by one-way ANOVA and Tukey’s post test, n.s.: not significant, *: P<0.05, **: P<0.01).
**Table S5:** Selective ET<sub>B</sub>R-mediated Ca<sup>2+</sup> flux in the presence of co-transfected ET<sub>A</sub>R. COS-7 cells were transiently transfected with constant amounts of ET<sub>B</sub>R-GFP increasing amounts of Cys-P3-ET<sub>A</sub>R in a ratio range from 1:0 to 1:3. Cells, expressing both ET<sub>A</sub>R and ET<sub>B</sub>R were stimulated with the ET<sub>A</sub>R/ET<sub>B</sub>R dual agonist ET-1 or the ET<sub>A</sub>R-selective [4Ala<sup>1,3,11,15</sup>, Nle<sup>7</sup>]-ET-1. ET<sub>B</sub>R-GFP expression was monitored by GFP fluorescence. Ca<sup>2+</sup> flux was monitored in COS-7 cells in duplicates and concentration-response curved represent the average over all assay repetitions (n=3).

| Co-transfection ratio | ET<sub>B</sub>R expression [%] ± SEM | ET-1 | Linear ET-1 | EC<sub>50</sub> [nM] | pEC<sub>50</sub> ± SEM | E<sub>max</sub> [%] ± SEM | EC<sub>50</sub> [nM] | pEC<sub>50</sub> ± SEM | E<sub>max</sub> [%] ± SEM |
|-----------------------|----------------------------------|------|-------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| ET<sub>B</sub>R/ET<sub>A</sub>R | 1:0 | 100 ± 1 | 1.5 | 8.8 ± 0.09 | 100 ± 5 | 4.7 | 8.3 ± 0.09 | 103 ± 6 |
| | 1:0.5 | 105 ± 8 | 0.9 | 9.0 ± 0.15 | 96 ± 7 | 3.1 | 8.5 ± 0.10 | 90 ± 5 |
| | 1:1 | 113 ± 8 | 0.9 | 9.1 ± 0.12 | 99 ± 5 | 4.5 | 8.3 ± 0.08 | 81 ± 4 |
| | 1:2 | 116 ± 3 | 0.8 | 9.1 ± 0.10 | 93 ± 4 | 2.4 | 8.6 ± 0.18 | 61 ± 6 |
| | 1:3 | 150 ± 8 | 1.2 | 8.9 ± 0.14 | 74 ± 5 | 3.1 | 8.5 ± 0.19 | 39 ± 5 |

**Table S6:** Selective ET<sub>B</sub>R-mediated Ca<sup>2+</sup> flux in the presence of co-transfected ET<sub>A</sub>R. COS-7 cells were transiently transfected with constant amounts of ET<sub>B</sub>R-GFP and a 3-fold excess of Cys-P3-ET<sub>A</sub>R. Cells, expressing both ET<sub>A</sub>R and ET<sub>B</sub>R were stimulated with the ET<sub>A</sub>R/ET<sub>B</sub>R dual agonist ET-1 in the absence and presence of the ET<sub>A</sub>R-selective antagonist sitaxentan or DMSO (vehicle control). ET<sub>B</sub>R-GFP expression was monitored by GFP fluorescence. Ca<sup>2+</sup> flux was monitored in COS-7 cells in duplicates and concentration-response curved represent the average over all assay repetitions (n=3).

| Co-transfection ratio | ET<sub>B</sub>R expression [%] ± SEM | ET-1 + vehicle (DMSO) | Linear ET-1 + 500 nM Sitaxentan | EC<sub>50</sub> [nM] | pEC<sub>50</sub> ± SEM | E<sub>max</sub> [%] ± SEM | EC<sub>50</sub> [nM] | pEC<sub>50</sub> ± SEM | E<sub>max</sub> [%] ± SEM |
|-----------------------|----------------------------------|----------------------|-----------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| ET<sub>B</sub>R/ET<sub>A</sub>R | 1:0 | 100 ± 1 | 0.5 | 9.3 ± 0.07 | 100 ± 3 | 0.6 | 9.3 ± 0.09 | 102 ± 4 |
| | 1:3 | 206 ± 15 | 0.6 | 9.2 ± 0.13 | 88 ± 4 | 0.6 | 9.2 ± 0.18 | 51 ± 4 |


Impact of GPCR co-expression on ET<sub>B</sub>R-mediated Ca<sup>2+</sup> flux in HEK293

**Figure S11:** Impact of GPCR co-expression on ET<sub>B</sub>R-mediated Ca<sup>2+</sup> flux in HEK293 cells. (A) Constant amounts of ET<sub>B</sub>R-GFP were co-transfected with increasing amounts of Cys-P3-ET<sub>A</sub>R in a ratio range from 1:0 to 1:1. Cells, expressing both ET<sub>A</sub>R and ET<sub>B</sub>R were stimulated with the ET<sub>A</sub>R/ET<sub>B</sub>R dual agonist ET-1 (upper row) or the ET<sub>B</sub>R-selective [4Ala<sup>1,3,11,15</sup>, Nle<sup>7</sup>]-ET-1 (lower row). ET<sub>B</sub>R-GFP expression was monitored by GFP fluorescence. (B) To validate the specific effect, constant amounts of AT<sub>1</sub>R-YFP were co-transfected with increasing amounts of Cys-P3-ET<sub>A</sub>R in a ratio range from 1:0 to 1:1 and cells AngII for AT<sub>1</sub>R activation. AT<sub>1</sub>R-YFP expression was monitored by YFP fluorescence. Ca<sup>2+</sup> flux mediated by Gq protein (turquoise) activation was monitored in HEK293 cells in duplicates and concentration-response curved represent the average over all assay repetitions (n=3; significance was determined by one-way ANOVA and Tukey’s post test, n.s.: not significant, ***: P < 0.001.
**Table S7:** Characterization of ET₃R signaling in transiently transfected HEK293 cells. Constant amounts of ET₃R-GFP were co-transfected with increasing amounts of Cys-P3-ET₃R. Cells, expressing both ET₃R and ET₆R were stimulated with the ET₃R/ET₆R dual agonist ET-1, the ET₆R-selective [4Ala¹³,¹¹,¹⁵, Nle⁷]-ET-1. ET₆R-GFP expression was monitored by GFP fluorescence. Ca²⁺ flux was monitored in HEK293 cells in duplicates and concentration-response curved represent the average over all assay repetitions (n=3).

| Co-transfection ratio | ET₆R expression [%] ± SEM | ET-1 | Linear ET-1 |
|-----------------------|--------------------------|------|-------------|
|                       |                          | EC₅₀ [nM] | pEC₅₀ ± SEM | Eₘₐₓ [%] ± SEM | EC₅₀ [nM] | pEC₅₀ ± SEM | Eₘₐₓ [%] ± SEM |
| 1:0                   | 100 ± 1                  | 2.0   | 8.7 ± 0.12 | 100 ± 6       | 1.7      | 8.8 ± 0.10 | 98 ± 5       |
| 1:0.25                | 112 ± 21                 | 1.8   | 8.8 ± 0.11 | 98 ± 5        | 1.5      | 8.8 ± 0.13 | 90 ± 6       |
| ET₆R/ET₃R            | 1:0.5                    | 139 ± 21 | 1.8  | 8.8 ± 0.18 | 109 ± 9   | 1.7      | 8.8 ± 0.17 | 80 ± 6       |
|                       | 1:0.75                   | 100 ± 20 | 1.1  | 9.0 ± 0.27 | 95 ± 11   | 1.4      | 8.8 ± 0.15 | 76 ± 5       |
|                       | 1:2                      | 116 ± 27 | 3.2  | 8.5 ± 0.21 | 95 ± 11   | 2.4      | 8.6 ± 0.24 | 49 ± 6       |

**Table S8:** Characterization of AT₃R signaling in transiently transfected HEK293 cells. Constant amounts of AT₃R-YFP were co-transfected with increasing amounts of Cys-P3-AT₃R. Cells, expressing both receptors were stimulated with the AngII for selective AT₃R activation. AT₃R-YFP expression was monitored by YFP fluorescence. Ca²⁺ flux was monitored in HEK293 cells in duplicates and concentration-response curved represent the average over all assay repetitions (n=3).

| Co-transfection ratio | AT₃R expression [%] ± SEM | AngII |
|-----------------------|--------------------------|-------|
|                       |                          | EC₅₀ [nM] | pEC₅₀ ± SEM | Eₘₐₓ [%] ± SEM |
| 1:0                   | 100 ± 1                  | 0.4   | 9.4 ± 0.10 | 103 ± 4       |
| 1:0.25                | 81 ± 3                   | 0.5   | 9.3 ± 0.11 | 103 ± 5       |
| AT₃R/ET₆R            | 1:0.5                    | 95 ± 6 | 0.3   | 9.5 ± 0.10 | 116 ± 4       |
|                       | 1:0.75                   | 85 ± 8 | 0.4   | 9.4 ± 0.12 | 102 ± 5       |
|                       | 1:2                      | 81 ± 5 | 0.5   | 9.3 ± 0.12 | 105 ± 5       |
Figure S12: Investigation of arrestin recruitment to activated ET_{B}R in the presence of co-expressed GPCRs. (A) For kinetic studies of arr3 recruitment, COS-7 cells were transiently transfected with constant amounts of ET_{B}R-GFP and increasing amounts of Cys-P3-tagged ET_{A}R, AT_{1}R or APJ (left to right). The recruitment was monitored for 5 min (basal, light grey) before ligand addition and 15 min after addition of ET-1 (black) or linear ET-1 (dark grey). Kinetic analyses were performed in quadruplicates (n=3) and one representative kinetic trace is shown. Concentration-dependent investigation of arr3 recruitment to ET_{B}R-GFP in the presence of Cys-P3-ET_{A}R (B/C), SP-Cys-P3-AT_{1}R (E) or SP-Cys-P3-APJ (F). Transfected cells were stimulated with ET-1 (B, E, F) or the ET_{B}R-selective [4Ala^{1,3,11,15}, Nle^{7}]-ET-1 (C). ET_{B}R expression was monitored by total GFP fluorescence. Arr3 recruitment was investigated in triplicates and concentration-response curves represent the average of all assay repetitions (n=3). Significance was determined by one-way ANOVA and Tukey’s post test, n.s.: not significant, *: P<0.05). Scale bar: 10 µm.
Table S9: Characterization of arr3 recruitment to ET₆R-GFP was assessed by concentration-dependent analyses in the presence Cys-P3-ET₆R, SP-Cys-P3-AT₁R, or SP-Cys-P3-APJ. Transfected COS-7 cells were stimulated with ET-1 or the ET₆R-selective [4Ala¹,³,11,15, Nle⁹]-ET-1 (linear ET-1). ET₆R expression was monitored by total GFP fluorescence. Arr3 recruitment was investigated in triplicates and concentration-response curves represent the average of all assay repetitions (n=3).

| Co-transfection ratio | ET₆R expression [%] ± SEM | ET-1 EC₅₀ [nM] | ET-1 pEC₅₀ ± SEM | ET-1 E_max [%] ± SEM | Linear ET-1 EC₅₀ [nM] | Linear ET-1 pEC₅₀ ± SEM | Linear ET-1 E_max [%] ± SEM |
|-----------------------|--------------------------|----------------|-----------------|---------------------|-----------------------|--------------------------|---------------------------|
| 1:0                   | 100 ± 1                  | 19             | 7.7 ± 0.9       | 102 ± 5             | 23                    | 7.6 ± 0.06               | 100 ± 3                   |
| ET₆R/ET₆R 1:0.25      | 100 ± 1                  | 16             | 7.8 ± 0.10      | 100 ± 5             | 14                    | 7.8 ± 0.05               | 91 ± 2                    |
| ET₆R/ET₆R 1:0.5       | 105 ± 1                  | 13             | 7.9 ± 0.11      | 104 ± 5             | 14                    | 7.9 ± 0.05               | 94 ± 2                    |
| ET₆R/ET₆R 1:1         | 136 ± 6                  | 15             | 7.8 ± 0.09      | 99 ± 4              | 15                    | 7.8 ± 0.06               | 94 ± 6                    |
| 1:0                   | 100 ± 1                  | 13             | 7.9 ± 0.09      | 100 ± 4             | n.d.                  | n.d.                     | n.d.                      |
| ET₆R/AT₁R 1:0.25      | 107 ± 1                  | 16             | 7.8 ± 0.07      | 106 ± 4             | n.d.                  | n.d.                     | n.d.                      |
| ET₆R/AT₁R 1:0.5       | 115 ± 14                 | 11             | 8.0 ± 0.10      | 104 ± 5             | n.d.                  | n.d.                     | n.d.                      |
| ET₆R/AT₁R 1:1         | 144 ± 2                  | 13             | 7.9 ± 0.09      | 98 ± 0.09           | n.d.                  | n.d.                     | n.d.                      |
| 1:0                   | 95 ± 5                   | 13             | 7.9 ± 0.08      | 100 ± 4             | n.d.                  | n.d.                     | n.d.                      |
| ET₆R/APJ 1:0.25       | 105 ± 8                  | 14             | 7.9 ± 0.09      | 98 ± 4              | n.d.                  | n.d.                     | n.d.                      |
| ET₆R/APJ 1:0.5        | 104 ± 1                  | 12             | 7.9 ± 0.10      | 97 ± 5              | n.d.                  | n.d.                     | n.d.                      |
| ET₆R/APJ 1:1          | 118 ± 1                  | 14             | 7.9 ± 0.09      | 94 ± 4              | n.d.                  | n.d.                     | n.d.                      |

n.d. = not determined
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