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Supporting Information

Shuttling of Peptide-Drug Conjugates by G Protein-Coupled Receptors Is Significantly Improved by Pulsed Application

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Supporting Information

Table of Content:

I. Experimental Details:

i.) Primer

Table 1: designed primers and details of the RT-PCR analysis of murine mHypoE-N39 cells.

encoded Protein		Sequence [5´-3´]	T_a [°C]	Product length [bp]
mY_1R	s	CCTCTCCTTCTCAGACTTGC	60	775
	as	GTCACAAAAGTTGAAGAAGAACTGC	60	
mY_2R	s	AAGAGCATGCGCACAGTAACCAAC	60	236
	as	TGGGCCAGGTGGTAGACAATGGGGCAACGA	60	
mY_4R	s	GACTTGCTACCCATCCTCAT	54	492
	as	ATCACCACCGTCTCATCTA	54	
mY_5R	s	TCTGATTCATACAGAATTGC	54	653
	as	GGATTAAGACAACAGGACATC	54	
GAPDH	s	ACCACAGTCCATGCCATCAC	60	452
	as	TCCACCACCCTGTTGCTGTA	60	

T_a = annealing temperature; s = sense; as = antisense, m = murine; bp = base pairs

ii.) Semi-quantitative RT-PCR. mHypoE-N39 cells were cultured to confluence as previous described, washed twice with PBS, harvested by trypsination and suspended in 10 ml fresh medium. The cell suspension was centrifuged for 5 min at 800 rpm, the supernatant was discarded and the cell pellet was frozen in liquid nitrogen and stored at -70 °C until RNA isolation was performed.

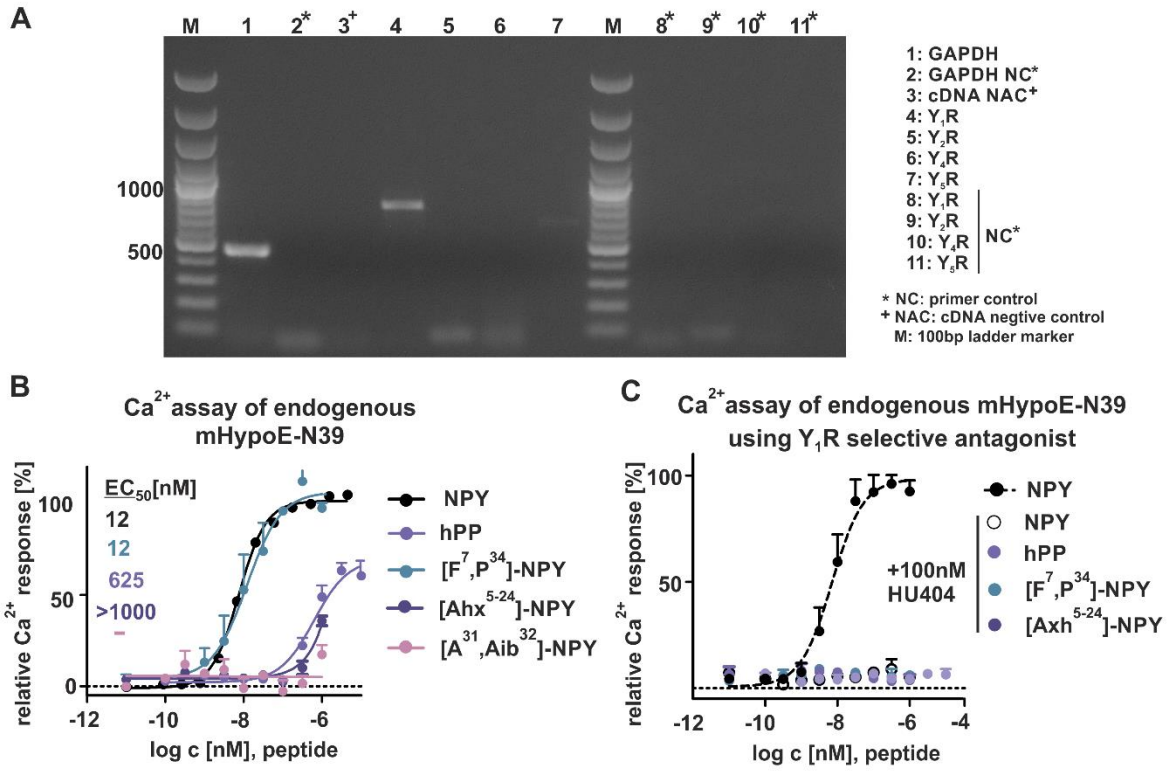
RNA extraction was accomplished using RNeasy Mini Kit (Qiagen) following an additional DNase I digestion step according to the manufacturer´s protocol. RNA amount was determined photometrically using a microplate reader (Infinite 200 PRO NanoQuant, Tecan). Reverse transcription by QuantiNova™ Reverse Transcription Kit was performed in a total volume of 20 µl composed of 1 µg RNA, 2 µg oligo$(dT)_{12\text{-}18}$ primers, 4 µl buffer, 2 µl 0.1 mol/L dithiothreitol, 0.5 µl dNTP-mix (10 µM), 0.5 µl RNaseOUT™ Recombinant Ribonuclease Inhibitor and 160 units of SuperScript®II Reverse Transcriptase. For cDNA negative control water was added instead of the enzyme.

RT-PCR reaction was performed in a total volume of 25 µl using 2 µl of generated cDNA; 100 pmol/µl of specific primer (forward and reverse, Table 1); 2.5 µl 10x Taq-buffer; 1.5 µl 25 mM $MgCl_2$; 0.5 µl dNTP mix; 1 µl DMSO and 0.25 units of Taq DNA-polymerase(0.25 µl). for primer control water was added instead of cDNA. PCR protocol was applied according to the following protocol: 5 min initial denaturation at 95 °C, 35 cycles of denaturation at 95 °C for 30 s, annealing step for 30 s at different temperatures and extension at 72 °C for 90 s; followed by final extension step for 10 min at 72 °C. Analysis of PCR products was performed by electrophoresis on 1 % agarose gel supplemented with ethidium bromide and images were taken using a transilluminator (DarkHood SH-40/50, Biostep).

iii.) Ca^{2+}assay. mHypoE-N39 cells were cultured in 25 cm^2 up to 70-80% confluence in a humidified atmosphere at 37 °C and 5% CO_2. Transfection of 4000 ng plasmid encoding the $G\alpha_{\Delta 6qi4myr}$ protein was performed by using Metafecten® Pro (Biontex Laboratories GmbH)

according to the manufacturer´s protocol. One-day post transfection, cells were re-seeded (10.000 cells/well) into a black poly-D-lysine covert 96-well plates (Greiner Bio-one) and incubated overnight at 37 °C. Two-days post transfection Ca^{2+} experiments were performed with the help of the FLIPR® Calcium Assay Kit (Molecular Devices). Cells were stained with 1.0 µM Fluor-2-AM staining dye. For measurement, a subset of respective stimulated cells was treated with NPY, [F^{7},P^{34}]-NPY, hPP, [AHX^{5-24}]-NPY and [A^{31},Aib^{32}]-NPY in a concentration range 10^{-11} M to 10^{-6} M and the Ca^{2+}-response was detected using the FlexStation (Molecular Devices). For antagonist experiments, cells were treated with 100 nM HU404 (kindly provided by A. Buschauer, University of Regensburg, Germany) and incubated for 30 min prior to agonist stimulation.

II Supplementary Figure 1



Supplementary Figure S.1. Endogenous Y_1 receptor expression was confirmed by RT-PCR and Ca^{2+}assay. A) mRNA expression level of mHypoE-N39 cells examined by RT-PCR and detected by 1% Agarose-Gel (1x TAE-buffer, stained with 0.5 µg/ml ethidium bromide). GAPDH expression was

used as a positive control. cDNA negative control (no amplification control, NAC) confirmed the absence of genomic DNA. A negative control without cDNA (NC) of each construct excluded contamination and primer oligomerization. One clear band was detected and matched with the Y_1R and an additional weak band for Y_5R was observed. **B)** Data were verified with the Ca^{2+}flux assay by testing selective peptide agonists for Y_1R (F^7,P^{34}-NPY), Y_2R (AHX^{5-24}-NPY) Y_4R (hPP,) and Y_5R (A^{31},Aib^{32}-NPY). Only stimulation with NPY and F^7,P^{34}-NPY resulted in a robust Ca^{2+}response, whereas PP (selective for Y_4R), Ahx^{5-24}-NPY (Y_2R) and A^{31}, Aib^{32}-NPY (Y_5R) provoked no response even at high concentration. **C)** Data were confirmed by using a Y_1R-specific antagonist HU404 (30 min prior agonist stimulation, 100 nM). Signal transduction was completely suppressed by incubating the cells with HU404 30 min prior to agonist stimulation compared to the control curve without an antagonist. Experiments represent data $n \geq 3$.