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Co-expression of neuropeptide Y Y1 and Y5 receptors results in heterodimerization and altered functional properties

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ABSTRACT

Centrally administered neuropeptide Y (NPY) produces anxiolytic and orexigenic effects by interacting with Y1 and Y5 receptors that are colocalized in many brain regions. Therefore, we tested the hypothesis that co-expression of Y1 and Y5 receptors results in heterodimerization, altered pharmacological properties and altered desensitization. To accomplish this, the carboxyl-termini of Y1 and Y5 receptors were fused with *Renilla* luciferase and green flourescent protein and the proximity of the tagged receptors assessed using bioluminescent resonance energy transfer. Under basal conditions, cotransfection of tagged Y1 receptor and Y5 produced a substantial dimerization signal that was unaffected by the endogenous, nonselective agonists, NPY and peptide YY (PYY). Selective Y5 agonists produced an increase in the dimerization signal while Y5 antagonists also produced a slight but significant increase. In the absence of agonists, selective antagonists decreased dimerization. In functional studies, Y5 agonists produced a greater inhibition of adenylyl cyclase activity in Y1/Y5 cells than cells expressing Y5 alone while NPY and PYY exhibited no difference. With PYY stimulation, the Y1 antagonist became inactive and the Y5 antagonist exhibited uncompetitive kinetics in the Y1/Y5 cell line. In confocal microscopy studies, Y1/Y5 co-expression resulted in increased Y5 signaling following PYY stimulation. Addition of both Y1 and Y5 receptor antagonists was required to significantly decrease PYY-induced internalization.

Therefore, Y1/Y5 co-expression results in heterodimerization, altered agonist and antagonist responses and reduced internalization rate. These results may account for the complex pharmacology observed when assessing the responses to NPY and analogs *in vivo*.

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Abbreviations: ALPHA, amplified luminescent proximity homogenous assay; AV12, Syrian hamster salivary cell line; BIBP3226, *R*-N^2-(diphenylacetyl)-*N*-(4-hydroxyphenyl)-methyl argininamide; BRET, bioluminescence resonance energy transfer; CGP71683A, (*trans*-naphtalene-1-sulphonic acid [4-[(4-amino-quinazolin-2-ylamino)-methyl]-cyclohexylmethyl]-amide hydrochloride); GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; h, human; HEK, human embryonic kidney; LY366258, 1-[3-[1-[2-(4-iodophenyl)ethyl]piperidin-4-yl]propyl]-2-[(phenoxy)methyl]-4-[3-(1-piperidinyl)propoxy]-1H-benzimiazole; Novartis-1, (*trans*-2-nitrobenzene-2-sulfonic acid (4-(2-naphthylmethylamino)methyl) cyclohexyl methyl)amide; PP, pancreatic polypeptide; PYY, peptide YY; RLUC, *Renilla* luciferase; rh, rhesus monkey.

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1. Introduction

Peptide YY (PYY), pancreatic polypeptide (PP) and neuropeptide Y (NPY) constitute a family of endocrine and neuronally derived peptides that have important physiological functions [1]. NPY is found in central and peripheral neurons and is released into the circulation from the adrenals. The effects of these peptides are mediated through G-protein-coupled receptors (class 1) entitled Y1, Y2, Y4 and Y5 (for review see [2]). An additional receptor, y6 has been identified in mice but is not functional in rats and humans [3]. The endogenous ligand for the Y4 receptor appears to be PP while Y1, Y2 and Y5 have similar high affinities for NPY and PYY [2]. Centrally administered NPY and PYY produce a pronounced orexigenic effect, anxiolysis, antiepileptic action, altered hormonal responses, altered diurnal functions and cardiovascular responses. As such, this system has been an important target for further research and pharmaceutical discovery.

A number of studies have been conducted to understand the pharmacological specificity of the central responses to NPY and PYY. In particular, the receptor mediating the orexigenic responses has been pursued by a number of groups. Early studies using peptide analogs indicated an Y1-like pharmacological profile, however, the peptide responses were ''atypical'' [4,5]. The robust activity of the fragment NPY2-36 was particularly puzzling since it had greater efficacy in stimulating the feeding responses than NPY but lower affinity for the Y1 receptor in vitro. Subsequent studies using more specific peptide analogs and antagonists indicated that both Y1 and Y5 receptors could mediate the feeding response. When NPY and PYY are administered to mice with the Y1 or Y5 receptor genes deleted, a partial reduction of the feeding response was observed (for review see [6]. However, these studies are confounded by the expression of a functional y6 receptor in mice that may be involved in the feeding responses observed in this species [3,7]. A similar profile has been observed in anxiety studies where both Y1 and Y5 receptor-selective agonists can produce anxiolytic-like activities after central administration [8]. Taken as a whole, these studies suggest that Y1 and Y5 receptors mediate similar actions in the brain and their combined pharmacologies may account for the ''atypical Y1 receptor-like'' pharmacology observed in earlier studies.

Since Y1 and Y5 receptor agonists produce similar physiological properties after central administration, it would be logical that they would be found in similar brain regions. The Y1 and Y5 receptor genes are found in close proximity but opposite and overlapping orientation on chromosome 11, suggesting coordinate regulation [9]. In the rat brain, Y5 receptor mRNA expression was found to coincide with regions expressing Y1 receptor mRNA [10]. Furthermore, using double label immunohistofluorescence, Wolak et al. [11] demonstrated that the Y1 and Y5 receptor proteins were colocalized in many brain regions and, in many instances, to the same cells. Since both receptors mediate their functions through Gi, it would be interesting to know if co-expression of these receptors in the same cell line would result in enhanced function or altered pharmacological properties. Recent work with a variety G-protein coupled receptors has proven that co-expression can result in heterodimerization [12,13] leading to alterations in pharmacology, desensitization and functional responses [14,15].

A number of techniques have been used to demonstrate G-protein-coupled receptor dimerization (or oligomerization) [16]. Resonance energy transfer methods provide evidence that the receptors are in close proximity, allow for assessment of agonist and antagonist interactions and can be performed in living cells. Therefore, we tested the hypothesis that co-expression leads to a heterodimerization of Y1 and Y5 receptors using the well-established technique of bioluminescence resonance energy transfer (BRET). To accomplish these goals, *Renilla* luciferase (RLUC) and green fluorescent protein (GFP) were attached to the C-terminus of the Y1 and Y5 receptor subtypes, respectively, and the receptor proximity assessed using a BRET assay. Receptor functionality was assessed using binding and functional adenylyl cyclase assays. Receptor internalization was assessed using confocal microscopy.

2. Materials and methods

2.1. Receptor expression construct assembly

The cloning of the Y1 and Y5 receptors from the rhesus monkey was reported previously [17]. Using clones containing the coding sequence of the Y1 and Y5 receptors, one forward primer was synthesized for each: Y1.fH containing a HindIII site with the sequence: (5′-AAGCTTAAGCTTACCATGAATTCAACAT-TATTTTCCC AG-3′) and Y5.fH (5′-AAGCTTAAGCTTACCATG-GATTTAGAGCTCGATGA AT-3′). One reverse primer was also synthesized for each Y1.NSrKS (5′-CCGCGGTACCGAT TCTTTCATTATCATCATTGTTG-3′), and Y5.NSrKS (5′-CCGCG-GTACCCATATG AAGACAGTGTATAAGGGAC) containing a KpnI and a SacI site but lacking the stop codon in order to make the C-terminally tagged constructs. The primer pairs were used in a PCR reaction using genomic rhesus DNA as template. A band of 1.1 and 1.4 kb were generated for the Y1 and Y5 receptors, respectively, and gel was purified. The PCR product and the vectors pGFP-N2 and pRLUC-N2 (PerkinElmer) were cut for 2 h at 37 °C using restriction enzymes HindIII and KpnI (10 U, Invitrogen) and, subsequently, gel was purified. All clones from each construct were sequenced fully to confirm the correct sequence. Clones for the kappa and delta opioid receptors were provided by Dr. Michael Statnick (Lilly Research Laboratories).

2.2. Transient transfection and stable cell line expression construction

HEK293 cells were selected for expression of the tagged receptors based on recommendations by the vendor. HEK293 cells were grown in 90 mm Falcon dishes (Fisher Scientific) in a DMEM:F-12 (3:1) mix supplemented with 5% FBS, 20 mM HEPES and 100 μg/ml Penicillin–Streptomycin (Gibco) at 37 °C in 5% CO_2. AV12 cells were grown in 90 mm Falcon dishes in a DMEM with high glucose supplemented with 10% FBS, 25 mM HEPES and 100 μg/ml Penicillin–Streptomycin (Gibco) at 37 °C in 5% CO_2. Two micrograms DNA from the each construct was individually transfected into HEK293 cells for the tagged receptors or AV12 cells for the wild-type receptors using 5 μl FuGENE6 (Roche Diagnostics) diluted in 145 μl OptiMEM

(Gibco) according to the manufacturer's instructions. Seventy-two hours later, the media was replaced with media containing Zeocin (100 μg/ml, Neosystems) for the GFP-tagged constructs, G418 (500 μg/ml, Gibco) for the RLUC-tagged and wild-type rhesus Y5 (in pCI-neo (Promega)) constructs, and 1 μM methotrexate (SigmaAldrich) for the wild-type rhesus Y1 receptor (in $pGTD_{10}$, Lilly) construct.

To produce a stable co-expressing cell line, the stable Y5 expressing cell lines were used as the progenitor cell line (HEK293 for the tagged receptors and AV-12 for the wild-type receptors). After 2–3 weeks, individual clones were randomly picked from the plate using a sterile pipette tip. Subsequently, all clones were tested for receptor expression using [^{125}I]-PYY (PerkinElmer) binding as described below.

2.3. Radioligand-binding studies

AV-12 cells expressing the wild-type receptors were used for comparison. We had previously characterized Y1 and Y5 receptors expressed in this cell line [17]. HEK293 cells expressing the chimeric receptors and AV12 cells expressing the wild-type receptors stably, were washed once with phosphate-buffered saline (PBS) and pelleted in fresh PBS. Radioligand-binding assays were conducted on isolated crude membrane homogenates as previously described [18] using [^{125}I]-PYY as radioligand. Nonspecific binding was defined as the amount of radioactivity remaining on the filter after incubating with 0.1 μM human PYY (Bachem). Various concentrations of human NPY, PYY, PP, NPY (2–36), selective Y5 agonists such as (hPP1–17, Ala^{31}, Aib^{32})hNPY [19] and (cPP1–7, NPY 19–23, Ala^{31}, Aib^{32}, and Gln^{34})hPP [19], BIBP3226 (*R*-N^2-(diphenylacetyl)-*N*-(4-hydroxyphenyl)-methyl argininamide, Bachem), LY366258 (1-[3-[1-[2-(4-iodophenyl)ethyl]piperidin-4-yl]propyl]-2-[(phenoxy)-methyl]-4-[3-(1-piperidinyl)propoxy]-1H-benzimiazole, Lilly) or Novartis-1 ((*trans*-2-nitrobenzene-2-sulphonic acid (4-(2-naphthylmethylamino)methyl) cyclohexyl methyl)amide, Lilly) were added to the incubations to determine binding affinity. Both BIBP3226 [20] and LY366258 [21] were reported to be selective Y1 receptor antagonists. Novartis-1 [22] is a modified version of the Y5-selective antagonist CGP71683A [(*trans*-naphtalene-1-sulphonic acid [4-[(4-amino-quinazolin-2-ylamino)-methyl]-cyclohexylmethyl]-amide hydrochloride)] [23]. For saturation binding analysis, HEK293 and AV12 cell homogenates containing the tagged and wild-type Y1 and Y5 receptors, respectively, were incubated with 12 different concentrations of [^{125}I]-PYY for 2 h at room temperature.

Radioligand-binding studies were performed in duplicate and repeated at least three separate times. All data were calculated, statistically evaluated and plotted using GraphPad Prism (San Diego, CA) software unless otherwise indicated. Protein concentrations were measured using Coomassie Plus® Protein Assay Reagent (Pierce) using bovine serum albumin standards.

2.4. BRET assay

Methods used for BRET studies have been published previously [24,25]. Initial studies comparing the BRET signal with various receptor pairings were conducted using transient transfections. Subsequent studies were performed with Y1 and Y5 using only stably expressing cell lines. HEK293 cells stably transfected with Y1-RLUC and Y5-GFP were plated out in six-well Falcon dishes (Fisher Scientific). When confluent, the cells were treated with various concentrations of peptides, peptide analogs, and antagonists. After 1 h, the cells from each well were detached by washing with 1 ml PBS using a pipette tip, transferred to an 1.5 ml eppendorf tube, spun in a microcentrifuge at 5000 rpm for 2 min, and resuspended in 150 μl PBS (about 10,000 cells/μl). Cell suspension (25 μl) was dispensed into each well of a 96-well plate (OptiPlate96, white, PerkinElmer). Immediately before counting the plate in a Fusion™ Universal Microplate Analyzer (PerkinElmer), 25 μl of modified coelenterazine (DeepBlueC™, PerkinElmer) diluted 1:100 in PBS was added (final concentration 5 μM).

The emission from each well was assessed at λ = 410 nm (RLUC optimum) and λ = 515 nm (GFP optimum) using a Fusion™ Universal Microplate Analyzer. Background luminescence was defined by expressing Y5-GFP and untagged RLUC in HEK293 cells. This typically produced a BRET ratio of 0.05 (data not shown). Thus, this BRET ratio represents what one would expect due to coincidental proximity when co-expressing both fusion proteins in the same cell. HEK293 cell expressing a cytosolic fusion protein, BRET$^+$ (PerkinElmer), was run in each experiment as a positive control. BRET$^+$ usually generates a BRET ratio of 0.4 (data not shown) and therefore represented the maximal BRET ratio in each assay. Each sample was run in quadruplicate and the ratios calculated for each replicate using a Microsoft Excel™ Worksheet. Data obtained from independent experiments were averaged together and expressed as percent control to normalize BRET ratios between experiments. The constitutive BRET ratio in the absence of any drug treatment was considered 100%. Any deviation from this constitutive BRET signal was reported as a percent change. In summary:

$$\text{BRET}^2\ \text{ratio} = \frac{\begin{array}{c}\text{emission at 515 nm (sample)}\\ -\text{emission at 515 nm (untransfected cells)}\end{array}}{\begin{array}{c}\text{emission at 410 nm (sample)}\\ -\text{emission at 410 nm (untransfected cells)}\end{array}}$$

$$\text{corrected BRET}^2\ \text{ratio} = \text{BRET}^2\ \text{ratio} - \text{background}$$

2.5. Amplified luminescent proximity homogenous assay (AlphaScreen™)

To analyze the functional response of the recombinant cell lines, AlphaScreen™, an assay designed to measure concentrations of cAMP, was employed. In general, AV12 cells grown in 30 mm Falcon dishes (Fisher Scientific) were harvested with Versene (Gibco), a non-enzymatic agent and resuspended in HBSS (Gibco) buffer containing 5 mM HEPES, 0.5 mM IBMX and 0.1% BSA (SigmaAldrich). The cells were washed in 1 ml HBSS using a pipette tip, transferred to a 15 ml conical tube, centrifuged at 1000 rpm for 10 min, and resuspended in 1 ml HBSS (about 30,000 cells/ml). Acceptor beads were added to cell the suspension at a concentration of 15 μg/ml. Cell suspension (10 μl) was then dispensed into each well of a Costar 96-well, half area, white plate (Corning). To stimulate cAMP, various concentrations of Forskolin (SigmaAldrich) were added. In

addition, various agonists were co-incubated with the cell suspension to inhibit Forskolin-stimulated cAMP production. Inhibition studies were carried out by pre-incubating the cells for 30 min with the antagonist followed by the addition of PYY and Forskolin. Plates were incubated in the dark at RT for at least 30 min. Following the addition of 30 μl of the detection mix (HBSS buffer containing 0.1% Tween-20, 22.5 μg streptavidin donor beads and 0.1 μM biotinylated-cAMP, 3.5 mls/plate) was immediately added to each well and incubated for an additional 90 min before counting the plate in a Fusion™ Universal Microplate Analyzer.

The captured AlphaScreen™ units were calculated using a Microsoft Excel™ Worksheet. Each sample was run in quadruplicate and the average calculated for each condition. On each plate, AlphaScreen™ units were measured in wells that only contain transfected HEK293 cells with the appropriate receptors represented the maximal response (100%) while baseline (0.0%) was represented by wells containing 0.1 nM Forskolin (the amount needed to stimulate about 50% of the maximal increase in intracellular cAMP). Each EC_{50} value represents an average of at least four independent determinations per compound treatment. These data were expressed as percent control to normalize AlphaScreen™ readings between experiments. To determine the competitive nature of the antagonists, Schild plot analysis was conducted on the results from each experiment [26]. To generate a Schild plot, several concentrations of antagonist were used to inhibit the agonist concentration–response. These data were graphed as log[antagonist] on the *X*-axis and log(dose ratio − 1) on the Y-axis using GraphPad Prism (San Diego, CA). Linear regression analyses were then used to determine slope, intercept and a confidence interval for each antagonist used this study. If the confidence interval for the slope did not include 1.0, the antagonist was ruled to be non-competitive.

2.6. Assessing receptor internalization using confocal microscopy

rhY5-GFP expressing HEK293 cells were grown on poly-lysine-coated chambered coverglass (Lab-Tek) in normal growth media containing 10% FBS at 37 °C for 48 h prior to imaging. Twenty-four hours prior to imaging, the growth media was removed and replaced with serum-free media (Cellgro-Free, Mediatech). Receptor internalization following agonist stimulation was visualized using a confocal microscope 30 min following the addition of 1 μM PYY. Antagonist studies were conducted by the addition of antagonist 15 min prior to the addition of agonist. Visualizations of various intracellular compartments were accomplished using the following dyes: lysosomes-LysoTracker Red DND-99; endosomes-Transferrin Alexa Fluor 568; nuclei-Hoechst 33258. All dyes were obtained from Molecular Probes.

For kinetic studies, the cells were grown on Delta T dishes (Bioptechs Inc.) which allowed a constant temperature of 37 °C to be maintained during imaging. Baseline images were obtained for a select cell group and timed-interval images of the same cell group were acquired following agonist addition. Percent internalization was determined by software analysis of total membrane fluorescence compared to total internal compartment fluorescence at the various time points.

Cells were evaluated using a Bio-Rad MRC1024-UV Confocal System (Bio-Rad, Hemel Hemstead, UK) using a 40× Plan Apo, NA 1.3 oil immersion objective on a Nikon Diaphot 200 inverted microscope. The green, blue and red fluorescent images were collected sequentially with Lasersharp 3.1 acquisition software. Images were collected as a stack in the. PIC format using LaserSharp 2000 acquisition software (Bio-Rad, Hemel Hemstead, UK).

Green fluorescent molecules were excited with a krypton/argon laser at 488 ± 10 nm emission at 522 ± 17 nm detected with a PMT. Red fluorescent molecules were excited with a 568 nm krypton/argon line and the emission greater than 585 nm was detected with a PMT. Blue fluorescent molecules were excited with a 363 nm line of an argon in laser (water cooled) and the emission was detected at 455 ± 15 nm.

3. Results

3.1. Radioligand-binding analysis of wild-type versus tagged receptors

These initial experiments were performed to determine if the addition of the GFP and RLUC tags affected the ligand recognition properties of the Y1 and Y5 receptors in radioligand binding assays (Supplemental Figures 1–7). In these studies, ^{125}I-PYY was found to bind with similar affinity to the wild-type and tagged receptors (Supplemental Figure 1) with specific binding constituting greater than 80% of total binding. Subsequently, the pharmacology of [^{125}I]-PYY binding to the cell lines was examined. When competing 100 pM [^{125}I]-PYY binding from Y1 and Y1-RLUC receptors with various peptides and peptide analogs, a similar rank of potency was observed: NPY > PYY > NPY (2–36) > PP ⋙ (cPP1–7, NPY19–23, Ala^{31}, Aib^{32}, and Gln^{34})hPP ≅ (hPP1–17, Ala^{31}, and Aib^{32})hNPY (Supplemental Figures 2 and 3). Displacement of [^{125}I]-PYY binding from Y5 and Y5-GFP receptors had a rank order of: (cPP1–7, NPY19–23, Ala^{31}, Aib^{32}, and Gln^{34})hPP ≥ NPY ≥ NPY (2-36) > PYY ≥ ≅ (hPP1–17, Ala^{31}, and Aib^{32})hNPY ≥ PP (Supplemental Figures 4 and 5). All displacement curves were best fit to a one-site binding model. The linear correlation coefficient (r^2) of the pEC_{50} values obtained from Y1 and Y1-RLUC receptors was 0.88 (Supplemental Figure 4). The linear correlation coefficient (r^2) of the pEC_{50} values obtained from Y5 and Y5-GFP receptors was 0.81 (Supplemental Figure 7). Subsequent experiments were conducted on the stable cell lines co-expressing the Y1 and Y5 receptors (AV-12) and the Y1-RLUC and Y5-GFP-tagged receptors (HEK293) to determine Y1 and Y5 receptor densities. In these studies, ^{125}I-PYY saturations were conducted in the presence or absence of a 1 μM concentration of the Y1 antagonist BIBP3226. These data are summarized in Table 1. Based on these results, the Y5/Y1 ratio in the WT cell line was 3.3 while the Y5/Y1 ratio in the tagged receptor cell line was 1.0.

3.2. BRET analysis of the Y1-RLUC and Y5-GFP receptors co-expressed in HEK293 cells

BRET analysis was used to evaluate Y receptor dimerization by measuring the proximity of one receptor to another in live cells using Y1- and Y5-tagged receptors with RLUC and a GFP.

Table 1 – Summary of saturation experiments with stably transfected cell lines expressing Y1-Y5 or Y1-RLUC/Y5-GFP

Cell line	Inhibitor	K_d (pM)	B_{max} (fmol/mg protein)
Y1/Y5	–	332 ± 32	2659 ± 110
Y1/Y5	1 μM BIBP3226	679 ± 61	616 ± 25
Y1-RLUC/Y5-GFP	–	730 ± 43	2242 ± 66
Y1-RLUC/Y5-GFP	1 μM BIBP3226	539 ± 39	1102 ± 93

Co-expressing cell lines were incubated with 12 concentrations of ^{125}I-PYY and the resulting binding data analyzed using Graphpad Prism with single site analysis ($N = 4$). K_d and B_{max} were determined in the presence of a saturating concentration of the selective Y1 antagonist, BIBP3226 to determine Y1 binding or in the absence of antagonist to determine Y1 and Y5 binding.

In initial experiments outlined in Fig. 1, transient transfection of several different RLUC tagged receptors into the Y5-GFP expressing cell line was used to assess the relative dimerization signal as described in Section 2. As illustrated in Fig. 1A, co-expressing the Y5 receptor with other members of the Y receptor family and delta and kappa opioid receptors tagged with RLUC produced a significant energy transfer as exhibited by an increase in the BRET ratio. While all Y receptor combinations produced a ratio above background, the co-expressed Y1 and Y5 receptors produced that greatest BRET response (Fig. 1A). In addition, the other Y receptors produced a BRET ratio that was similar to that obtained with the unrelated delta and kappa opioid receptors. Under basal conditions, the BRET ratio obtained with the Y1-RLUC/Y5-GFP pairing produced a consistently greater signal that other receptor pairings, indicating that a constitutive heterodimer exists between both Y1 and Y5 receptor subtypes. This BRET ratio was consistent with other reports from the literature using BRET to study GPCR dimerization [27–31] and was not dependent on the quantity of Y1-RLUC DNA used for the transfection (Fig. 1B). Therefore, the high BRET signal seen with the Y1–Y5 pairing was not due to higher transfection efficiency. In all subsequent studies, the stably expressing cell line described in Section 3.1 was used. To get a broad sense of what pharmacological agents for the Y receptor family could affect the Y1/Y5 dimer pair, cells stably co-expressing these receptors were treated with single concentrations of each agent and then the BRET ratio measured. These data are summarized in Table 2. In general, neither PYY (Table 2, Fig. 2A) nor NPY (Table 2) had any significant effect on the BRET ratio. NPY (2–36) increased the BRET ratio in a concentration-dependent manner with an EC_{50} of 53.1 ± 16.0 nM (Fig. 3A). Selective Y5 agonists such as (cPP1–7, NPY19–23, Ala^{31}, Aib^{32}, and Gln^{34})hPP (Fig. 4A, Table 2) and (hPP1–17, Ala^{31}, and Aib^{32})hNPY (data not shown) also increased the BRET ratio in a concentration-dependent manner with EC_{50} values of 20.6 ± 2.4 and 35.0 ± 6.3 nM, respectively.

The Y1 receptor antagonists, BIBP3226 (Fig. 5) and LY366258 (data not shown) both decreased the BRET ratio in a concentration-dependent manner with IC_{50} values of 8.7 ± 1.4 and 6.2 ± 0.8 nM, respectively. The less-active enantiomer to BIBP3226, BIBP3435 ((*S*)-*N*^{2}-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-D-arginine amide) [20] had no statistically significant effect on the BRET ratio at 10 μM (Fig. 5). The Y5-selective antagonist, Novartis-1, produced a slight but statistically significant increase in the BRET ratio (Table 2). In summary, some agonists and antagonists produced shifts in the energy transfer from RLUC to GFP in a concentration-dependent manner.

3.3. *Functional analysis of wild-type Y1 and Y5 receptors co-expressed in AV12 cells*

In this study, a whole cell cAMP-based assay was used to determine the functional consequences of co-expressing Y1

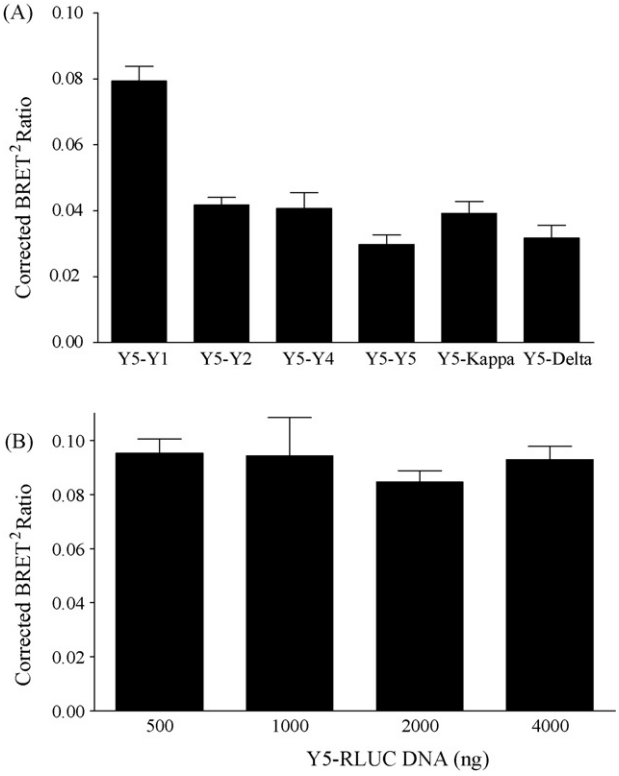


Fig. 1 – Dimerization of Y1 or Y5 receptor tagged with GFP and co-expressed with Y receptors tagged with RLUC. (A) Dimerization of Y1-GFP receptors when co-expressed with Y5-RLUC. Different amounts of Y5-RLUC tagged receptor DNA was transiently transfected into HEK293 cells expressing the Y1-GFP receptor. The bar graph represents an average (mean ± S.E.M.) of four determinations performed in quadruplicate and expressed as BRET ratios that have been corrected for background luminescence. (B) Dimerization of Y5-GFP receptors when co-expressed with other members of the Y receptor family tagged with RLUC. The RLUC-tagged receptors were transiently transfected into HEK293 cells expressing the Y5-GFP receptor. The bar graph with represents an average (mean ± S.E.M.) of four determinations performed in quadruplicate and expressed as BRET ratios that have been corrected for background luminescence.

Table 2 – Effect of various pharmacological agents on dimerization of Y1-RLUC and Y5-GFP receptors co-expressed in HEK293 cells

Pharmacological agent	Corrected BRET ratio
Vehicle	0.115 ± 0.004
1 μM PYY	0.127 ± 0.006 (n.s.)
1 μM NPY	0.130 ± 0.001 (n.s.)
1 μM NPY (2–36)	0.174 ± 0.006**
10 μM LY366258	0.064 ± 0.004**
10 μM BIBP3226	0.081 ± 0.002**
1 μM (hPP1–17, Ala^{31}, Aib^{32})hNPY	0.169 ± 0.004**
1 μM (cPP1–7, NPY19–23, Ala^{31}, Aib^{32}, and Gln^{34})hPP	0.184 ± 0.006**
10 μM Novartis-1	0.148 ± 0.004*

The effect various pharmacological agents have on BRET ratios in HEK293 cells co-expressing Y1-RLUC and Y5-GFP receptors. The BRET ratios were determined using Microsoft Excel™ software and have been corrected for background luminescence. Each value represents an average of six determinations performed in quadruplicate (means ± S.E.M.). *P* values were calculated using a Student's *t*-test (GraphPad Prism software) **, $P < 0.0001$; *, $P < 0.001$; n.s., not significantly different from vehicle.

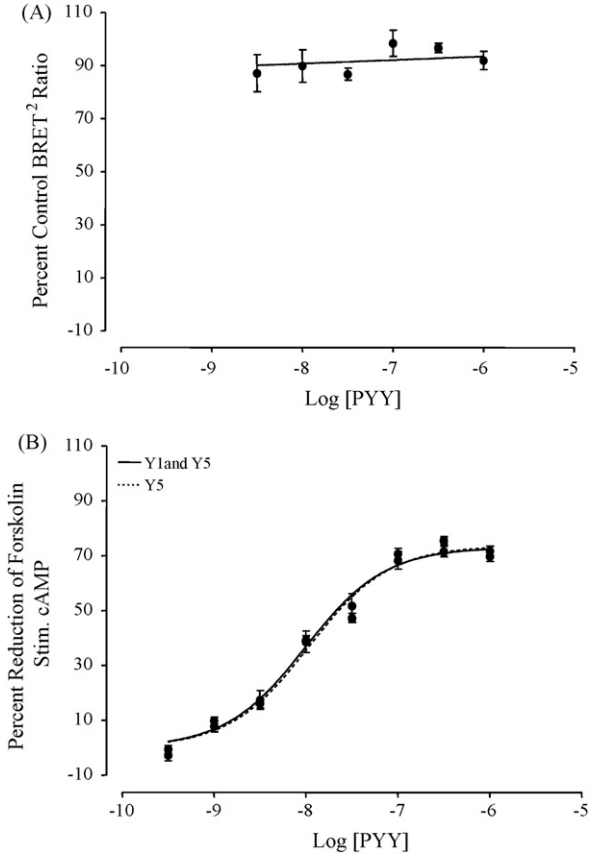


Fig. 2 – The effect of human PYY on cell signaling and dimerization when Y1 receptor Y1 and Y5 receptors are co-expressed. (A) Concentration-dependent effect of PYY on Y1-RLUC and Y5-GFP receptor dimerization in HEK293 cells as measured by a corrected BRET ratio. Data were obtained from three independent experiments performed in quadruplicate and expressed as % maximal response to normalize BRET ratios between experiments. (B) AlphaScreen™ analysis of PYY in AV12 cell lines expressing either Y5 or co-expressing Y1 and Y5 receptors. In the Y1 and Y5 receptors co-expressing cell line, after 30 min, PYY produced a concentration-dependent reduction of 0.1 μM Forskolin-stimulated adenylyl cyclase with an EC_{50} of 11.7 ± 2.8 and 10.7 ± 0.9 nM in the Y5 only cell line. These AlphaScreen™ data were expressed as percent control to normalize readings between experiments. Each point represents the mean of six independent determinations performed in quadruplicate (mean ± S.E.M.).

and Y5 receptors. In initial studies, the tagged receptors were found to poorly couple to adenylyl cyclase using a variety of assay types (data not shown), so a cell line stably expressing the wild-type Y1 and Y5 receptors was used instead. Binding data with this cell line is summarized in Table 1 and Supplementary Table 1. AlphaScreen™, a bead based non-radioactive amplified luminescent proximity homogenous assay was used to assess the functional properties of various pharmacological agents following Forskolin stimulation of adenylyl cyclase activity. Forskolin concentration was optimized in preliminary experiments (Supplementary Figure 8). When Y1 and Y5 receptors were co-expressed, PYY inhibited intracellular cAMP induced by the addition of 0.1 μM Forskolin in a concentration-dependent manner with an EC_{50} of 11.7 ± 2.8 nM (Fig. 2B). In an AV12 cell line that only expressed Y5 receptors, PYY inhibited the increase in cAMP induced by 0.1 μM Forskolin in concentration-dependent manner with an EC_{50} of 10.7 ± 0.9 nM, similar to what was observed in the co-expressed cell line (Fig. 2B). Like PYY, NPY inhibited 0.1 μM Forskolin activated adenylyl cyclase activity in a concentration-dependent manner in the co-expressed cell line, but with a slightly lower EC_{50} of 32.3 ± 1.3 nM (data not shown). In the Y5 only cell line, NPY inhibited 0.1 μM Forskolin-stimulated cAMP production with similar affinity and efficacy to the co-expressed cell line. NPY (2–36) had an almost twofold greater efficacy in the Y1 and Y5 receptors co-expressing cell line compared to the efficacy in the cells that only express Y5 receptors with similar EC_{50} values (Fig. 3B). The Y5 agonist, (cPP1–7, NPY19–23, Ala^{31}, Aib^{32}, and Gln^{34})hPP inhibited 0.1 μM Forskolin-stimulated cAMP production with an EC_{50} of 6.6 ± 0.5 nM for the Y5 only cell line and 9.7 ± 0.8 nM for the Y1 and Y5 receptors co-expressing cell line (Fig. 4B). Analogous to NPY (2–36), (cPP1–7, NPY19–23, Ala^{31}, Aib^{32}, and Gln^{34})hPP inhibited 0.1 μM Forskolin activated adenylyl cyclase with almost twofold greater efficacy when Y1 and Y5 receptors were expressed in the same cell. Another selective, but structurally distinct Y5 agonist, human (hPP1–17, Ala^{31}, and Aib^{32}) hNPY inhibited cAMP induced by 0.1 μM Forskolin with an EC_{50} of 10.7 ± 2.0 nM in the Y5 only cell line (data not shown). When evaluated in the co-expressed cell line, (hPP1–17, Ala^{31}, and Aib^{32}) hNPY was also able to inhibit cAMP induced by Forskolin with similar affinity, but with twofold greater efficacy (data not shown). Overall, Y5-selective agonists as well as NPY (2–36) exhibited greater efficacy when Y1 and Y5 receptors are co-expressed in the same cell. Non-selective agonists such as NPY or PYY do not show any

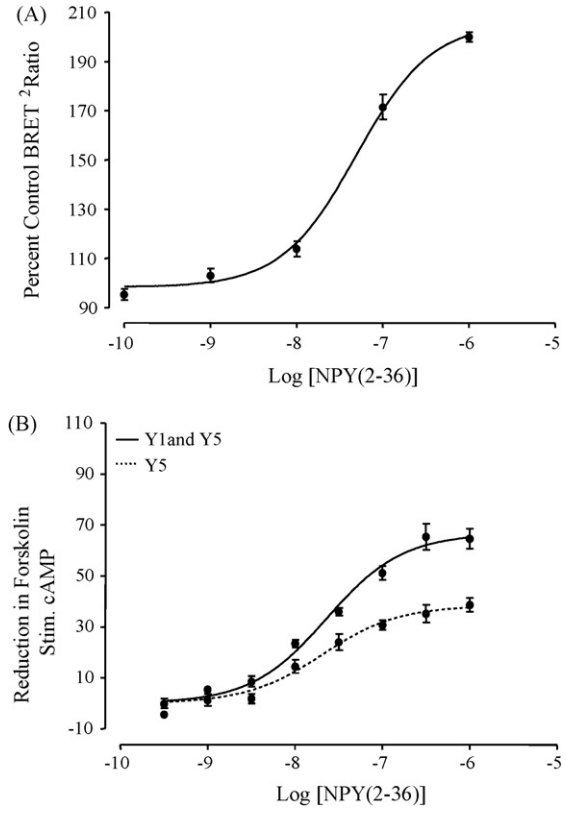


Fig. 3 – The effect of human NPY (2–36) on signaling and dimerization in cell lines co-expressing the Y1 and Y5 receptors. (A) Human NPY (2–36) increased in a concentration-dependent manner the BRET ratio with an EC_{50} of 53.1 ± 1.6 nM after 60 min. Data were obtained from three independent experiments performed in quadruplicate and expressed as percent control to normalize BRET ratios between experiments. (B) In the Y5 expressing and Y1 and Y5 co-expressing cell lines, NPY (2–36) produced a concentration-dependent reduction of 0.1 μM Forskolin-stimulated adenylyl cyclase activity with an EC_{50} of 17.0 ± 2.4 and 24.4 ± 1.6 nM after 30 min, respectively. These AlphaScreen™ data were expressed as percent control to normalize readings between experiments. Each point represents the mean of six independent determinations performed in quadruplicate (mean ± S.E.M.).

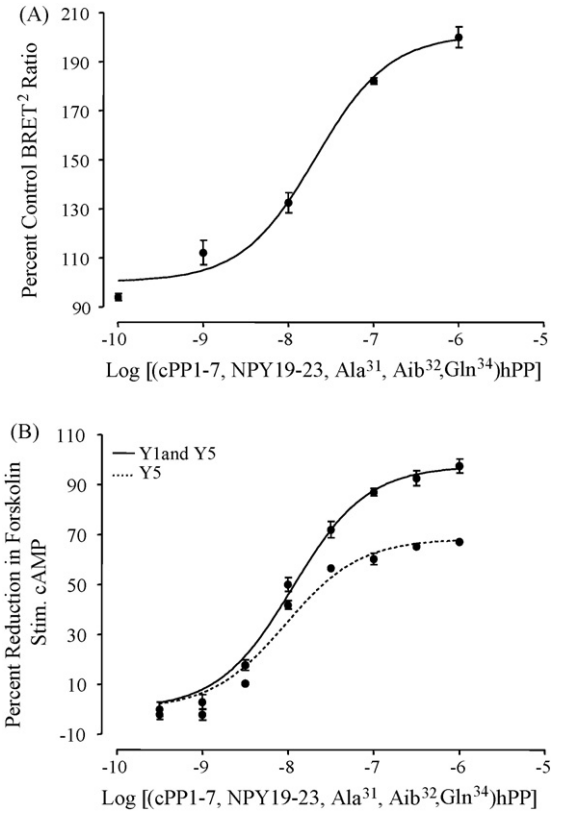


Fig. 4 – The effect of human (cPP1–7, NPY 19–23, Ala31, Aib32, and Gln34)hPP on signaling and dimerization in cell lines co-expressing the Y1 and Y5 receptors. (A) The effect of (cPP1–7, NPY19–23, Ala^{31}, Aib^{32}, and Gln^{34})hPP on Y1-RLUC and Y5-GFP receptor dimerization in HEK293 cells measured using BRET. Data were obtained from three independent experiments performed in quadruplicate and expressed as percent control to normalize BRET ratios between experiments. (cPP1–7, NPY 19–23, Ala^{31}, Aib^{32}, and Gln^{34})hPP increased in a concentration-dependent manner the BRET ratio with an EC_{50} of 20.6 ± 2.4 nM after 60 min. (B) In the Y5 expressing and Y1 and Y5 receptors co-expressing cell lines, (cPP1–7, NPY19–23, Ala^{31}, Aib^{32}, and Gln^{34})hPP produced a concentration-dependent reduction of 0.1 μM Forskolin-stimulated adenylyl cyclase activity with an EC_{50} of 6.6 ± 0.5 and 9.7 ± 0.8 nM after 30 min, respectively. These AlphaScreen™ data were expressed as percent control to normalize readings between experiments. Each point represents the mean of four independent determinations performed in quadruplicate (mean ± S.E.M.).

differences in efficacy between Y1 and Y5 receptors expressed either alone or together.

The following experiments evaluated Y1 and Y5 receptor specific non-peptide small molecule antagonists by antagonizing agonist-induced responses using Schild plot analysis. The Y1 receptor antagonist BIBP3226 shifted, in parallel fashion, the PYY dose–response curve to the right without a change in the maximal response from AV12 cells expressing the Y1 receptor (Fig. 6A). When the antagonist potency was calculated using Schild plot analysis, BIBP3226 had a pA_2 value of 8.52 and a slope that statistically did not differ from unity (Fig. 6B). Therefore, BIBP3226 was shown to be competitive reversible antagonist for the Y1 receptor when expressed alone. However, in the Y1 and Y5 receptors containing cell line, BIBP3226 did not antagonize the PYY inhibition of Forskolin-stimulated adenylyl cyclase activity (Fig. 6C). In fact, the pEC_{50} values were no different from PYY alone (Fig. 6D). Similarly, the chemically distinct Y1 receptor antagonist LY366258, also blocked the effect of PYY in the Y1 receptor cell line (data not shown) with a pA_2 and slope of 7.88 and 1.02, respectively. Comparable to BIBP3226, increasing the concentration of LY366258 did not shift the PYY

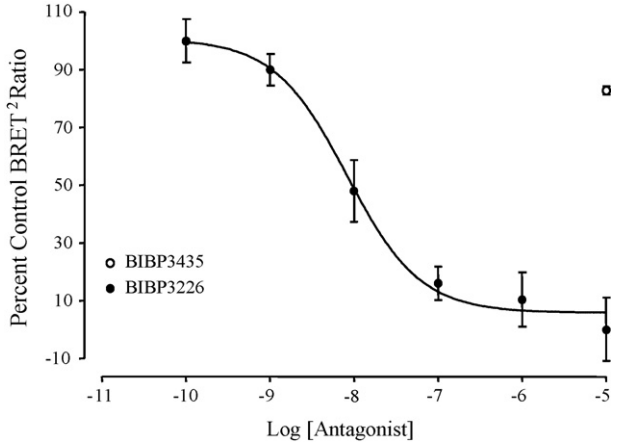


Fig. 5 – Concentration-dependent effect of Y1 receptor antagonists on Y1-RLUC and Y5-GFP receptor dimerization in HEK293 cells as measured by BRET. BIBP3226 decreased in a concentration-dependent manner the corrected BRET ratio with an IC_{50} of 8.76 ± 1.4 nM after 60 min. At 10 μM, BIBP3435 (the less-active enantiomer) decreased the corrected BRET ratio, but to a much lesser extent than BIBP3226 at the same concentration. LY366258 also decreased in a concentration-dependent manner the BRET ratio with an IC_{50} of 6.2 ± 0.8 nM after 60 min (data not shown). Data were obtained from three independent experiments performed in quadruplicate and expressed as percent control to normalize BRET ratios between experiments.

dose–response curve to the right nor did it depress the maximal response to the agonist when co-expressing Y1 and Y5 receptors.

The Y5-selective antagonist, Novartis-1, produced a parallel shift to the right in the PYY dose–response curve without depressing in the maximal response to the agonist in AV12 cells only expressing the wild-type Y5 receptors (Fig. 7A). Schild plot analysis of the Novartis-1 compound revealed that it had high affinity for Y5 receptors with a pA_2 of 7.77 (Fig. 7B). Novartis-1 was also a reversible, competitive antagonist when tested in this same cell line with a slope of 1.2 (Fig. 7B). However, in the Y1 and Y5 receptors co-expressing AV12 cell line, Novartis-1 depressed the maximal response of the PYY dose–response curve in a concentration-dependent manner (Fig. 7C). This response is characteristic for antagonists that are insurmountable in nature. In addition, the overall affinity for PYY did not change in the co-expressed cell line by comparing the pEC_{50} at the different concentration for the Novartis-1 compound (Fig. 7D).

3.4. *Assessing receptor internalization using confocal microscopy*

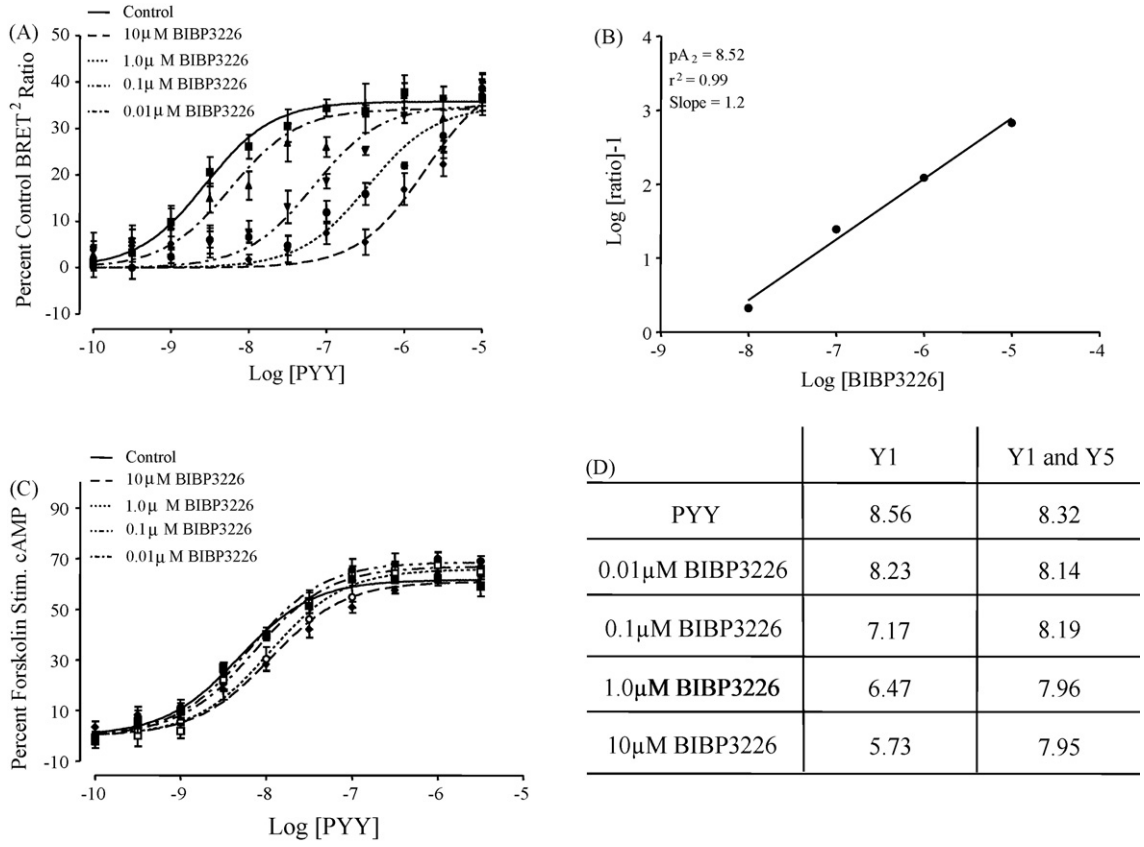
In confocal microscopy studies, rhY5-GFP fluorescence was found to be concentrated in the plasma membranes (Fig. 8). The addition of 1 μM PYY produced a rapid and robust internalization of the fluorescence (Fig. 8B) that colocalized with the lysosomal dye LysoTracker red (Fig. 9). Co-expression of Y5-GFP with Y1-RLUC resulted in an increased rate of Y5 internalization and an increased accumulation of the fluorescence within the cell (Fig. 8A–C). In the co-expressing cell line, PYY-induced internalization of fluorescence in the co-expressing cell line was not significantly affected by the Y1 or Y5 receptor antagonists alone but was substantially reduced by the addition of both antagonists (Fig. 8D).

4. Discussion

In the present study, we have demonstrated that the Y1 and Y5 receptors exhibit profound interactions when co-expressed. When expressed at similar densities as described in Section 3, tagged Y1 and Y5 receptors exhibit an increased BRET ratio compared to other Y receptor pairings. The endogenous agonists, NPY and PYY, had no effect on the BRET ratio and exhibited similar levels of potency when comparing the Y5 cell line with the Y1/Y5 cell line in binding assays. On the other hand, Y5 agonists increased the BRET ratio, produced a similar potency but a higher efficacy in the Y1/Y5 cell line. The increased BRET ratio can represent two different phenomena or a combination of both. There may be an increased proximity of the tags between the receptors or there could be a greater number of tagged receptors in close proximity [16]. For the purposes of the discussion, we will refer to this process collectively as ''dimerization''. Y1 receptor antagonists decreased the dimerization and Y5 antagonist produced a slight, but statistically significant increase. In the Y1/Y5 cell line, the Y1 receptor antagonist did not antagonize PYY-mediated inhibition of Forskolin-stimulated cAMP synthesis while the Y5 antagonist became uncompetitive. This indicates that the receptor complexes can be stimulated through agonist occupancy at with receptor and the Y5 receptor has now become an allosteric regulator of Y1 receptor signaling.

Another prominent feature of G-protein-coupled receptor heterodimerization is an alteration in the measured agonist induced sensitization or internalization [32]. Therefore, the effect of Y1/Y5 co-expression on internalization was assessed. In these studies, cells expressing Y5-GFP alone or with Y1 receptor expression were examined using confocal microscopy. In previous studies, the Y5 receptor was shown to internalize at a relatively slow rate [33] compared to the Y1 receptor which rapidly internalized upon agonist stimulation [34]. Addition of PYY produced a time dependent migration of Y5-GFP to the intracellular compartment. Colocalization of rhY5-GFP fluorescence with the LysoTracker Red dye indicates lysosomes are the primary intracellular destination for Y5-GFP receptors following agonist stimulation. Co-expression with the Y1 receptor increased internalization indicating the Y1 receptor participated in the desensitization of the heterodimer with no change in the intracellular compartment. Interestingly, both Y1 and Y5 receptor antagonists were required to prevent agonist-induced internalization indicating that internalization was dependent on agonist occupancy of both receptor subtypes. The decreased internalization of the dimmer complex may also contribute to the increased efficacy seen with certain peptides in the cell-based functional studies.

These results are particularly intriguing in the context of findings in the neuroanatomical localization of Y5. The genes

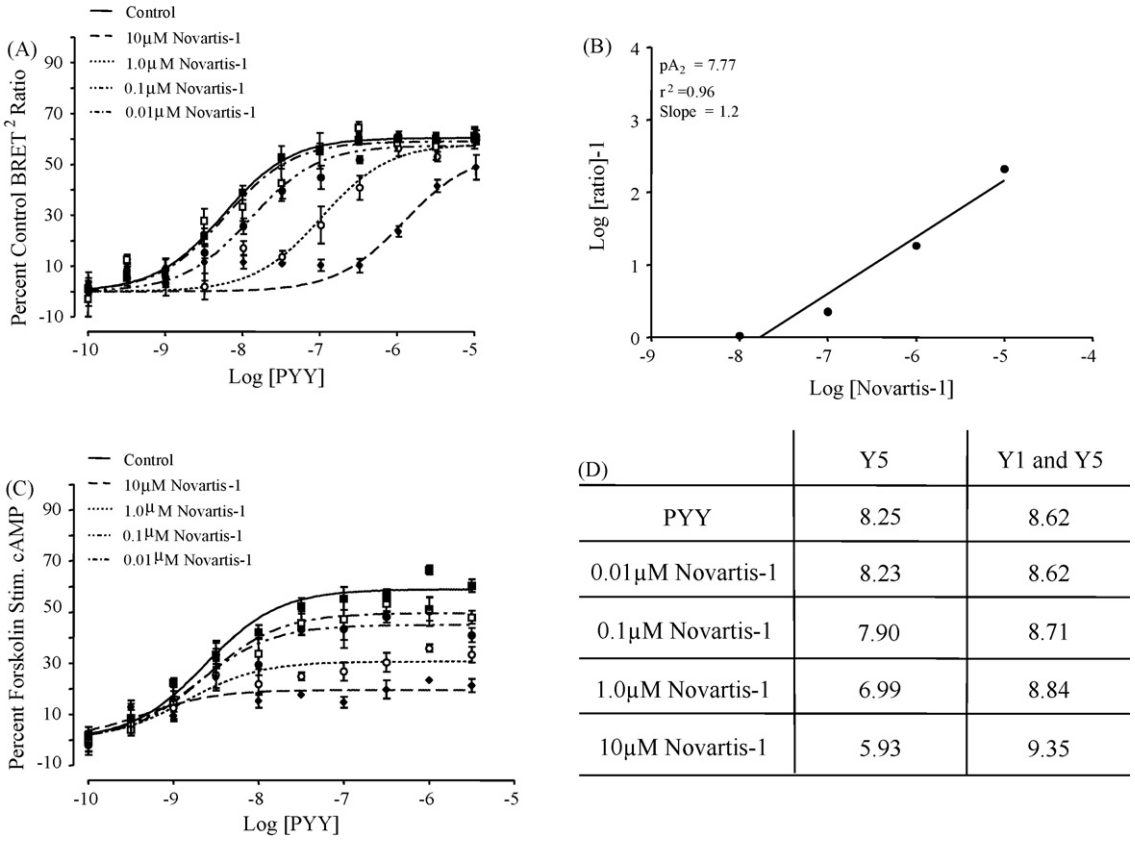


(D)	Y1	Y1 and Y5
PYY	8.56	8.32
0.01μM BIBP3226	8.23	8.14
0.1μM BIBP3226	7.17	8.19
1.0μM BIBP3226	6.47	7.96
10μM BIBP3226	5.73	7.95

Fig. 6 – The effect of BIBP3226 on cell signaling when Y1 and Y5 receptors were co-expressed. (A) Antagonism of PYY by BIBP3226 in the wild-type Y1 receptors expressed in AV12 cells. BIBP3226 at 10, 1.0, 0.1 and 0.01 μM produced a shift in the concentration–response curve of PYY at the Y1 receptor to the right. (B) This graph illustrates Schild regression transformation of the data from panel A and illustrates the effect of BIBP3226 on PYY concentration–response curves from AV12 cell line expressing Y1 receptors. (C) Effect of BIBP3226 on PYY concentration–response curves in an AV12 cell line co-expressing wild-type Y1 and Y5 receptors and measured using AlphaScreen™. BIBP3226 at 10, 1.0, 0.1 and 0.01 μM produced no shift in the concentration–response curve of PYY in the co-expressed cell line. (D) This table shows the pEC_{50} for PYY in the presence of various concentration of BIBP3226 on cells expressing only Y1 receptor or co-expressing Y1 and Y5 receptors. Each value represents an average of four independent determinations performed in quadruplicate (mean ± S.E.M.) and these data were expressed as percent control to normalize AlphaScreen™ readings between experiments.

encoding Y1 and Y5 receptors are found overlapping on the same chromosome but in opposite orientation suggesting coordinate regulation [9]. In the rat, Y1 and Y5 receptor encoding messenger RNAs were observed in numerous brain regions and have been colocalized to the same cells in many brain regions. Using double label immunohistofluorescence, Wolak et al. [11] demonstrated that the Y1 and Y5 receptor proteins were colocalized in many brain regions and, in many instances, to the same cells. In the same study, two bands of ~42 and ~85 kDa were detected in Western blots prepared from rat hypothalamus using specific antisera for the Y1 receptor and the Y5 receptor. These two bands were found in the membrane-enriched fraction and not the cytosol-enriched fraction suggesting that the ~42 kDa band represents a monomeric form of the Y1 receptor whereas the higher molecular weight band may represent an oligomer. Because this was not a co-immunoprecipatation experiment, the exact make-up of this oligomeric complex remains unknown. Nevertheless, the presence of higher molecular weight band in the membrane-enriched fraction of the hypothalamus supports the potential of Y receptor dimers *in vivo*.

In addition to evidence that Y1 and Y5 receptors can heterodimerize in vitro, there is intriguing evidence that NPY can dimerize in vitro as well (for review see [35]). The original solution structure of this peptide family was based on the crystal structure of avian PP and consisted of an alpha helix involving residues 14–31 connected via a beta turn to an N-terminal polyproline II helix and this has been termed the PP-fold. From the crystal, avian PP was found to exist as a dimer with extensive contacts between the alpha helices and the N-terminal polyproline II helix. In solution, salmon PP was a mixture of monomers and dimers dependent on temperature, pH and concentration [35]. In solution studies, NPY has also been found to exist as a dimer with the alpha helicies making numerous contacts and the N-terminal portions of the peptides free to interact with the receptors [36]. These dimers



(D)	Y5	Y1 and Y5
PYY	8.25	8.62
0.01μM Novartis-1	8.23	8.62
0.1μM Novartis-1	7.90	8.71
1.0μM Novartis-1	6.99	8.84
10μM Novartis-1	5.93	9.35

Fig. 7 – The effect of Novartis-1 on cell signaling when Y1 and Y5 receptors were co-expressed. (A) Antagonism of PYY by Novartis-1 from AV12 cells expressing the wild-type Y5 receptor. Novartis-1 at 10, 1.0, 0.1 and 0.01 μM exhibited a shift in the concentration–response curve of PYY at the Y5 receptor to the right. (B) This graph illustrates Schild regression transformation of the data from panel A, the effect Novartis-1 has on PYY concentration–response curves from AV12 cell line expressing Y5 receptors. (C) Effect of Novartis-1 on PYY concentration–response curves in an AV12 cell line co-expressing wild-type Y1 and Y5 receptors and measured using AlphaScreen™. Novartis-1 at 10, 1.0, 0.1 and 0.01 μM exhibited a downward shift in the concentration–response curve of PYY in the co-expressed cell line. (D) This table shows the pEC_{50} for PYY in the presence of various concentration of Novartis-1 on cells expressing only Y5 or co-expressing Y1 and Y5 receptors. Each value represents an average of four independent determinations performed in quadruplicate (mean ± S.E.M.) and these data were expressed as percent control to normalize AlphaScreen™ readings between experiments.

can be disrupted with mutations in various regions of the peptide. Given the high concentrations of peptides found in synaptic vesicles, it is intriguing to consider the released form of the peptide may be a dimer that is preformed to interact with the dimerized receptor.

Given the co-expression of Y1 and Y5 receptors in the rat brain, the dimerization of the peptide in solution, the similarity of behavioral responses elicited by selective agonists and the inability to eliminate the responses to selective agonists after gene deletion, we propose that Y1 and Y5 receptor heterodimerization is important to the central affects of NPY *in vivo*. Co-expression of a number of G-protein-coupled receptors has been shown to result in heterodimerization both within and outside a hormone or neurotransmitter family (for review see [14,37]). Heterodimerization can result in a number of changes in the properties of receptors compared to expression of the single receptor subtype. In many cases, there is a profound change in the radioligand binding or pharmacological properties of the dimer [14,37]. Unlike many G-protein-coupled receptor pairings, we did not observe binding changes in the present study. The binding properties did not differ from what one would expect from a mixture of Y1 and Y5 receptors. However, there are a limited number of radioligands currently available to study the NPY receptors and these are all agonist peptides. It would be interesting to assess the binding of a radiolabeled antagonist based on our observations in the functional assays.

Perhaps the most important finding in the present study is the effects that Y1/Y5 heterodimers demonstrated in functional studies. In general, Y5 agonist efficacy was increased in the heterodimer when compared to Y5 alone. This is similar to what has been observed with the dopamine D2/SSTR5 heterodimer [38]. In those studies, the maximal inhibition of Forskolin-stimulated cAMP levels in the D2/SSTR5 cell line was greater with SST-14 and quinpirole than observed with either agonist alone. Interestingly, we extend this finding to

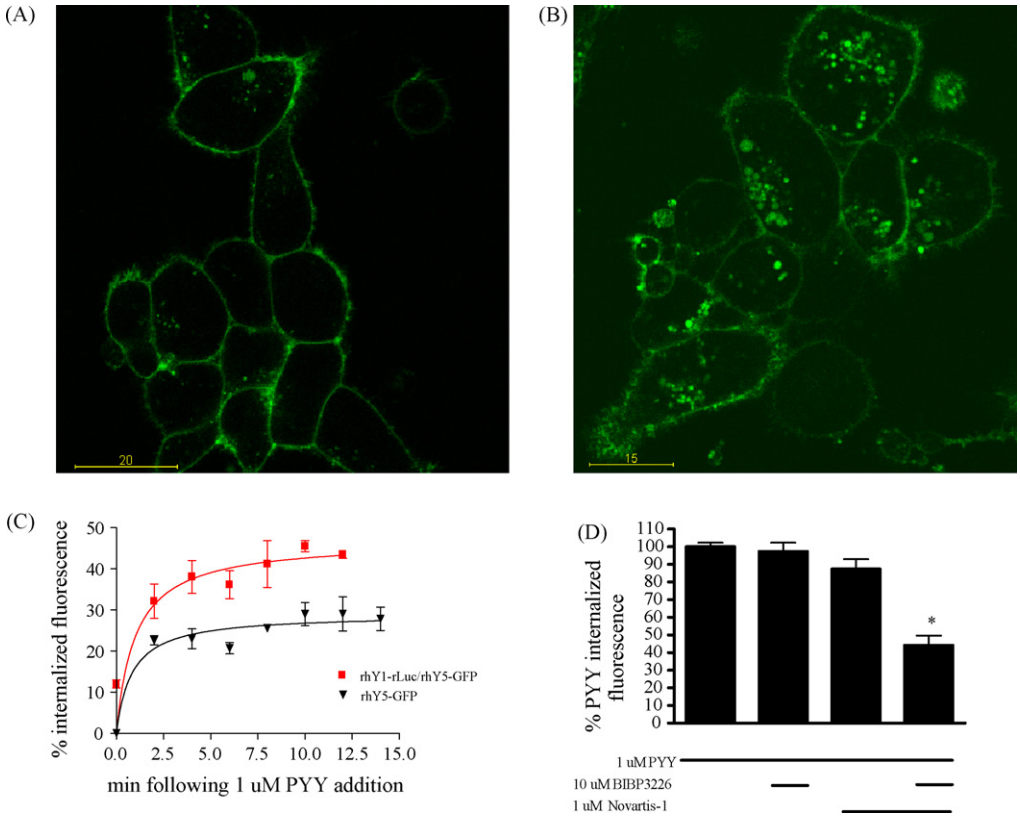


Fig. 8 – Confocal microscopy of agonist-induced internalization of Y5-GFP. (A) Cells expressing Y5-GFP were subjected to confocal microscopy and the fluorescence localized to the plasma membrane. (B) Following stimulation with 1 μM PYY was a translocation of the fluorescence to the interior of the cell. (C) Quantitation of the time course of fluorescence translocation following agonist stimulation. (D) Addition of the Y1 receptor antagonist BIBP3226 or the Y5 antagonist Novartis-1 did not significantly affect agonist-induced Y5-GFP translocation. Addition of both the Y1 and Y5 antagonists prevented agonist-induced translocation. Bars = 10 μM.

show that the heterodimer renders Y1 receptor antagonist impotent and Y5 antagonists change from competitive to uncompetitive. Since the Y1 receptor antagonist produced a reduction in the dimerization signal, it would reason that the dissociated heterodimer was signaling through Y5 alone. Since the Y5 antagonist did not substantially affect the dimerization signal, it is likely acting as an allosteric regulator and, as such, displays uncompetitive kinetics. Further studies will need to look at these behaviors with selective Y5 and Y1 receptor agonists to confirm these hypotheses. Particularly interesting was the finding that NPY2-36 also exhibited increased efficacy in the Y1/Y5 co-expressors. Central administration of this peptide was previously should to have greater efficacy that NPY in feeding studies leading to the suggestion

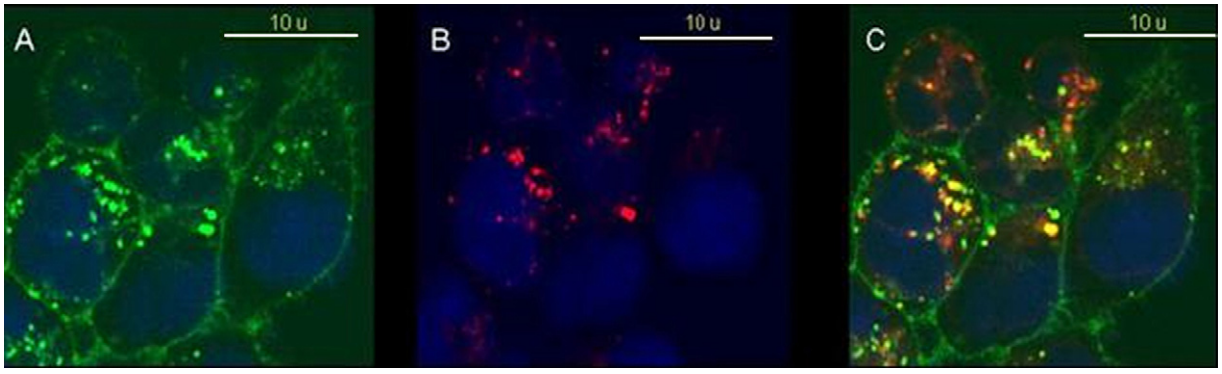


Fig. 9 – Y5-GFP migrates to lysosomes following agonist stimulation. (A) rhY5-GFP fluorescence and (B) lysosomes stained red with LysoTracker Red DND-99. (C) Overlay of rhY5-GFP and LysoTracker Red fluorescence to show colocalization (yellow). Nuclei were visualized with Hoechst 33258.

that NPY-induced feeding was mediated by an ''atypical-Y1'' receptor. The greater efficacy observed in vitro with NPY2-36 may account for these *in vivo* results.

In conclusion, we have demonstrated that co-expression of Y1 and Y5 receptors at similar high densities results in the formation of a constituitive heterodimer. This dimerization is regulated by selective agonists and antagonists. Co-expression also increases the ability of Y5 agonists, but no nonselective agonists, to decrease Forskolin-stimulated cAMP accumulation. The heterodimerization also appears to increase the internalization of the Y5 receptor following agonist stimulation and profoundly alter the pharmacology of antagonist interaction. These findings have important implications for our understanding of role for Y receptors in the central actions of NPY and future drug discovery.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.08.017.

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