A G Protein–Coupled Receptor Dimer Imaging Assay Reveals Selectively Modified Pharmacology of Neuropeptide Y Y1/Y5 Receptor Heterodimers

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ABSTRACT
The ability of G protein–coupled receptors (GPCRs) to form dimers, and particularly heterodimers, offers potential for targeted therapeutics with improved selectivity. However, studying dimer pharmacology is challenging, because of signaling cross-talk or because dimerization may often be transient in nature. Here we develop a system to isolate the pharmacology of precisely defined GPCR dimers, trapped by bimolecular fluorescence complementation (BiFC). Specific effects of agonist activation on such dimers are quantified using automated imaging and analysis of their internalization, controlled for by simultaneous assessment of endocytosis of one coexpressed protomer population. We applied this BiFC system to study example neuropeptide Y (NPY) Y1 receptor dimers. Incorporation of binding-site or phosphorylation-site mutations into just one protomer of a Y1/Y1 BiFC homodimer had no impact on efficient NPY-stimulated endocytosis, demonstrating that single-site agonist occupancy, and one phosphorylated monomer within this dimer, was sufficient. For two Y1 receptor heterodimer combinations (with the Y4 receptor or β2-adrenoceptor), agonist and antagonist pharmacology was explained by independent actions on the respective orthosteric binding sites. However, Y1/Y5 receptor BiFC dimers, compared with the constituent subtypes, were characterized by reduced potency and efficacy of Y5-selective peptide agonists, the inactivity of Y1-selective antagonists, and a change from surmountable to nonsurmountable antagonism for three unrelated Y5 antagonists. Thus, allosteric interactions between Y1 and Y5 receptors modify the pharmacology of the heterodimer, with implications for potential antiobesity agents that target centrally coexpressed Y1 and Y5 receptors to suppress appetite.

Introduction

G protein–coupled receptor (GPCR) family members are integral to cell–cell communication and transduce signals to a wide range of chemical messengers. Over the past decade, it has become clear that GPCRs can form dimers or oligomers and that this has the potential to modify receptor function (Smith and Milligan, 2010; Ferre et al., 2014). Evidence for GPCR dimerization includes multiple approaches in transfected cells (Guo et al., 2008; Dorsch et al., 2009; Han et al., 2009; May et al., 2011; Comps-Agrar et al., 2012; Corriden et al., 2014) or in tissues (Albizu et al., 2010). Structures have identified potential dimerization interfaces for the largest class A family (Wu et al., 2010; Huang et al., 2013), some of which have been tested (Johnston et al., 2011; Hu et al., 2013). However, the relevance of class A GPCR dimerization to activation of G proteins and other effectors is controversial, particularly as dimer formation may be transient in living cells (Dorsch et al., 2009; Fonseca and Lambert, 2009). For example, evidence for a single heterotrimeric G protein interacting with a GPCR dimer (Damian et al., 2008; Han et al., 2009; Smith and Milligan, 2010) is countered by lipid disc preparations in which defined monomeric GPCRs recruit G proteins and regulatory β-arrestins (Whorton et al., 2007; Bayburt et al., 2011; Damian et al., 2012). Understanding this stoichiometry is crucial to evaluating ligand pharmacology, as GPCR-effector interaction also allosterically governs agonist affinity (Smith and Milligan, 2010).

Moreover, several investigations highlight formation of GPCR heterodimers with pharmacological profiles and signaling patterns that are distinct from those of the parent receptors (Waldhoer et al., 2005; Smith and Milligan, 2010; Urizar et al., 2011; Ward et al., 2011; Watts et al., 2013) and that potentially offer targets with improved therapeutic selectivity and efficacy. However, it remains a challenge to unambiguously assign “heterodimer” pharmacology. In cell population assays examining coexpressed GPCRs, the identification of heterodimers does...
not usually indicate their relative membrane prevalence compared with monomers, homodimers, or other complexes. When novel pharmacology is observed in such systems, it could arise from binding-site interactions within a defined heterodimer, but it is often difficult to exclude other options such as downstream signaling cross-talk or lack of selectivity of the ligand investigated (see, e.g., Chun et al., 2013).

The G1-coupled Y receptors for neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) illustrate particular possibilities for heterodimers. NPY is a widely expressed central nervous system modulator, noted for its role in appetite regulation when released from hypothalamic arcuate neurons (Michel et al., 1998; Brothers and Wahlestedt, 2010). Orexigenic effects of NPY are mediated through a combination of Y1 and Y5 receptor subtypes coexpressed in paraventricular target cells (Gerald et al., 1996; Cabrera et al., 2000; Leonkin et al., 2002; Brothers and Wahlestedt, 2010). Y1 antagonists (e.g., (diphenylacetyl)-N-orthosteric binding sites. receptor heterodimer, but not others, displays modified ligand BiFC homodimer. Moreover, we demonstrate that the Y1/Y5 macology can be altered in cells coexpressing Y1 and Y5 et al., 2007). One study has suggested that antagonist phar- coimmunoprecipitation approaches (Dinger et al., 2003; Gehlert been obtained via fluorescence resonance energy transfer and receptor homodimerization and Y1/Y5 heterodimerization has (Turnbull et al., 2002; George et al., 2014). Evidence for both Y leading candidates have often been set back by lack of efficacy and internalization and compared with simultaneous assess- ments, following the same pharmacological stimulus, of

Materials and Methods

Chemicals. NPY, PYY, and related peptides were purchased from Bachem (Merseyside, UK) and stored in experiment aliquots at ~20°C. BIBO3304 was a gift from Boehringer Ingelheim GmbH (Biberach, Germany), while CGP71683, 5,5-dimethyl-2-2,3,4,9-tetrahydro-3,3- dimethyl-1-oxo-1H-xanthene-9-yl)-1,3-cyclohexadiene (L152,804), N-[4(methyl)-9-(1-methylethyl)-9H-carbazol-3-yl]-4-morpholinocarboxamide (NPY5AR972), isoprenaline, and (S)-propranolol were all from Tocris (Poole, UK). PerkinElmer (Seer Green, UK) provided [35S]PYY (2200 μCi/mmol) and [3H]adenine (25 Ci/mmol), and [14C]AMP (264 mCi/mmol) was from GE Healthcare (Little Chalfont, UK). Receptor cDNA sequences corresponded to the following GenBank reference numbers: Y1 receptor (Z11504), Y4 receptor (NM_005972), Y5 receptor (NM_006174), β2-adrenoceptor (β2AR) (NM_000024), and D2 dopamine receptor long isoform (D2L) (NM_000795). General molecular biology enzymes were obtained from Fermentas (Thermo Fisher Scientific, Loughborough, UK), and other consumables were from Sigma-Aldrich (Poole, UK) or Fisher (Loughborough, UK) unless other- wise stated.

Molecular Biology. The NPY Y1 receptor cDNA sequence, lacking both start and stop codons, was polymerase chain reaction–amplified and cloned between BamH1 and NotI sites in the pcDNA3.1/Zeo(+) mammalian expression vector (Invitrogen, Paisley, UK). To generate the SNAP-Y1-Yn receptor construct, a KpnI/BamH1 DNA fragment was cloned upstream of the Y1 region, which encoded a Kozak sequence (GCCACCC) followed by cDNA for a 5-1H–labeled peptide signal MRLC1PQVLALFSLMTGPEG8S and SNAP-tag (New England Biolabs, Hitchin, UK). The cDNA encoding the Venus YFP N-terminal fragment (Yn, residues 2–173; detailed in Kilpatrick et al., 2010) was cloned in frame with the C-terminus of the Y1 receptor between NotI and XbaI. Receptor constructs were also fused to the complementary BiFC protein, Venus YFP C (Yc, residues 155–238; “receptor–Yc”), as previously described (Kilpatrick et al., 2010). Briefly, receptor cDNAs (Y1, Y4, Y5, β2AR, and D2L) were located between BamH1 and NotI in frame downstream of an N-terminal FLAG-tag (MDYKDDDDPKGS) in pCMV-Tag2B (Agilent Technologies, Wokingham, UK) and upstream of the Yc sequence between NotI and XbaI. The generation of binding-site (Y99A, Bindneg) or phosphodeficient mutants (5A, Phosneg) of the Y1 receptor by QuikChange-based mutagenesis (Agilent Technologies) has been reported earlier (Kilpatrick et al., 2010). For the noncomplemented cointernalization studies, similar methods generated the SNAP-Y1 cDNA construct (with no C-terminal tag) in pcDNA3.1/Zeo(+), and either Y1 or Y1Bindneg in pCMV-Tag2B, fused to full-length green fluorescent protein (GFP) containing key superfolder mutations (Pedelacq et al., 2006; Kilpatrick et al., 2012).

Finally, Y1, Y4, or Y5 fusion protein cDNAs, generating receptors tagged at the C-terminus with GFP, were also inserted into the tetracycline-regulated expression vector pcDNA4/TO (Invitrogen), as indicated previously (Kilpatrick et al., 2012).

Cell Culture and Transfection. Cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Sigma-Aldrich) in a humidified atmosphere at 37°C and 5% CO₂. Receptor cDNAs were stably transfected into HEK293T cells, or 293T cells containing the tetracycline repressor protein (Invitrogen), using LipofectAMIN (Invitrogen) followed by selection in the appropriate antibiotic: zeocin (200 μg/ml), gentamicin (0.8 mg/ml), and/or blasticidin (5 μg/ml). For generation of the BiFC dimer cell lines, a single SNAP-Y1-Yn parent line was first generated by dilution cloning in zeocin and colony selection, based on SNAP fluorophore labeling and automated imaging (see below). Various receptor-Yc constructs were then introduced into the chosen SNAP- Y1-Yn clone by stable transfection and additional selection in genetin, using the resultant cotransfected cell lines as a clonal line (Y1/Y5) or mixed populations. Coexpressing SNAP-Y1/Y1GFP and SNAP-Y1/Y1Bindneg-GFP clonal cell lines were established by the same procedure. Y receptor cDNAs fused to full-length GFP in pcDNA4/TO were expressed in 293T cells and mixed populations selected by blasticidin/zeocin resistance, as described in Kilpatrick et al. (2012).

[35S]PYY Competition Binding Studies. Membranes were freshly prepared from all cell lines as described previously (Kilpatrick et al., 2010). Competition binding assays were performed for 90 minutes at 21°C in buffer (25 mM HEPES, 2.5 mM CaCl₂, 1.0 mM MgCl₂, 0.1% bovine serum albumin (BSA), and 0.1 mg/ml bacitracin; pH 7.4) with increasing concentrations of unlabeled ligands (0.1 pM to 1 μM in

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duplicate) and 15 pM [125I]PYY (PerkinElmer). For GTPγS competition, 30 μg/ml saponin was also included in the assay buffer. Membrane-bound radioligand was separated by filtration through Whatman GF/B filters soaked in 3% polyethyleneimine on a Brandel cell harvester (Alpha Biotech, London, UK). Retained radioactivity was detected using a gamma counter (Packard Cobra II; PerkinElmer, Waltham, MA). Nonspecific binding was subtracted from the data and comprised 5% of total counts.

**[3H]cAMP Accumulation Assays.** Cells were seeded onto poly-D-lysine (5 μg/ml)–coated 24-well plates 2 days prior to experimentation at a density of 100,000 cells per well. On the day of the experiment, cells were loaded with [1H]adenine (2 μCi/ml) in serum-free DMEM and incubated for 2 hours at 37°C and 5% CO2. Cells were then washed and the media replaced with serum-free DMEM/0.1% BSA containing 1 mM rolipram (a phosphodiesterase IV inhibitor) and Y receptor antagonist as required (at the concentration indicated; 30 minutes at 37°C). Cells were then preincubated with NPY for 10 minutes, followed by addition of 30 μM forskolin for a further 1 hour at 37°C. Incubations were terminated using concentrated HCl added to all wells, followed by the addition of [14C]cAMP standard (0.5 nCi per well). Assay plates were frozen and stored at −20°C prior to recovery of [3H]cAMP and [14C]cAMP using sequential
Dowex-Alumina column chromatography as described by Donaldson et al. (1988).

Automated Imaging of Receptor Internalization Using a Confocal Plate Reader. BiFC dimer cell lines were seeded (40,000 cells/well) onto poly-L-lysine–coated black-clear-bottom 96-well plates (Greiner Bio-One, Stonehouse, UK) 24 hours prior to experiments. Cells were pretreated with DMEM containing 0.2 μM SNAP-Surface 647 (New England Biolabs) for 30 minutes at 37°C. The medium was then replaced with HEPES-buffered saline solution/0.1% BSA containing agonist, with or without antagonist pretreatment, for the times and concentrations indicated. Incubations were terminated by 3% paraformaldehyde in phosphate-buffered saline (10 minutes at 21°C). Cell nuclei were subsequently stained using bisbenzamide Hoechst 33342 (2 μg/ml in phosphate-buffered saline; Sigma-Aldrich). Confocal images of four central sites per well were acquired using a IX Ultra confocal plate reader (Molecular Devices, San Diego, CA) fitted with a Plan Fluor 40× NA0.6 extra-long working distance objective. Each field of cells was imaged sequentially using the DAPI (405-nm excitation; 495-nm long pass emission; nuclei), FITC (488-nm excitation; 515/25-nm band pass; reconstituted YFP), and Cy5 channels (635 nm; 688-nm long pass; SNAP-Surface 647 label).

A similar protocol was adopted (without SNAP labeling) to image Y receptor–GFP internalization in 293TR cell lines seeded 48 hours prior to experiments, with a tetracycline (1 μg/ml) induction period of 18–21 hours. For assessment of NPY-induced internalization of the noncomplemented SNAP-Y1/Y1-GFP cell lines, the SNAP-Y1 labeling step was substituted with SNAP-Surface AF546 (0.2 μM), and transferrin-AF633 (5 μg/ml) was also included during NPY stimulation in the assay medium as a marker for internal compartments to aid analysis (Valentin-Hansen et al., 2012). During IX Ultra acquisition, the TexasRed channel (561 nm; 593/20-nm band pass) was used to acquire SNAP-Y1 images, and the Cy5 channel visualized transferrin labeling.

Data Analysis. For the majority of experiments, images of receptor internalization were analyzed using a granularity algorithm (MetaXpress 5.0; Molecular Devices), which was set to classify intracellular receptor compartments of 3–15 μm in diameter. Grayscale intensity thresholds for granularity classification were set according to positive and negative plate controls. For the dual-labeled BiFC cell lines, independent granularity analyses were applied to the acquired Cy5 (SNAP-Y1-Yn) and FITC (BiFC dimer) images; these data were therefore obtained from the same cell population identified by each field of view, but were not paired observations from each individual cell. While several granularity parameters (count, area, intensity) yielded equivalent response data, the figures presented are based on average granule count per cell. To measure receptor internalization in the noncomplemented SNAP-Y1/Y1-GFP cell lines, an alternative method quantified either labeled SNAP-Y1 or Y1-GFP fluorescence intensities within the same 3-μm internal compartments identified by transferrin (using the MetaXpress multi-wavelength cell scoring algorithm; Valentin-Hansen et al., 2012). This improved sensitivity of measurement compared with granularity analysis in these experiments (Fig. 3 versus Supplemental Fig. 3).

Data from triplicate internalization experiments (total of 12 image sites) were normalized to 1 μM NPY responses (Y1/Y5) or 100 nM PP responses (Y4) unless otherwise indicated. [3H]cAMP data were corrected for column recovery of the [14C]cAMP standard and also normalized to the forskolin control (100%). Both competition binding and functional concentration response were fitted to pooled data (expressed as mean ± S.E.M.) by nonlinear least-squares regression (Prism 6.04; GraphPad Software, Inc., La Jolla, CA), using sigmoidal concentration-response relationships with variable Hill slopes. Estimates of the antagonist equilibrium dissociation constant were calculated from the Gaddum equation:

\[ pK_d = \log[(CR-1)/CR] - \log[Antagonist] \]

where CR is defined as the concentration ratio of the agonist pEC50 value obtained in the presence and absence of antagonist. IC50 values for competing PYY in [125I]PYY binding studies from Y1-only cell lines were converted to Kd values using the equation:

\[ K_d = IC_{50} - [PYY^*] \]

[125I]PYY Bmax estimates from homologous displacements were then given by:

\[ B_{max} = TSB \times (1 + (K_d/}[PYY^*]) \]

where Kd and [PYY*] represent the radioligand dissociation constant and concentration and TSB is total specific binding.

Statistical analysis was by two-way analysis of variance (ANOVA) with Bonferroni post-test, to test significance differences between pooled concentration-response and time course relationships; or by Student’s unpaired t test, to assess differences between Rmax or pEC50 values.

Results

BiFC Constrains NPY Y1 Receptors as Dimers of Precise Composition. To establish the BiFC system for Y1 receptor–containing dimers (Fig. 1A), we derived a clonal HEK293T cell line that expressed the Y1 receptor fusion protein with N-terminal SNAP-tag and C-terminal modification with the Yn YFP fragment (SNAP-Y1-Yn). [125I]PYY binding studies indicated that this construct displayed high affinities for the agonist PYY and the nonpeptide Y1 antagonist BIBO3304 (Wieland et al., 1998; Lecklin et al., 2002) (Table 1), equivalent to those we have previously reported for native and fluorescent protein–modified Y1 receptors (Kilpatrick et al., 2010, 2012), and an estimated [125I]PYY binding to SNAP-Y1-Yn membranes was sensitive to GTPyS, indicating receptor coupling to G proteins (Table 1) (Kilpatrick et al., 2010). Automated confocal imaging studies (Fig. 1B) demonstrated that SNAP-Y1-Yn receptors were labeled at the cell surface using a membrane-impermeant SNAP fluorophore (SNAP-Surface 647), and underwent agonist-stimulated internalization in response to either NPY or PYY, previously determined to be a β-arrestin–dependent process (Holliday et al., 2005; Ouedraogo et al., 2008; Kilpatrick et al., 2012). A quantitative measure of internalization was obtained from the images by using granularity analysis to determine receptor accumulation in intracellular compartments. NPY (100 nM) stimulated SNAP-Y1-Yn endocytosis with a half-life (t1/2) of 4.0 ± 1.6 minutes (n = 4) (Supplemental Fig. 1), and NPY and PYY were equipotent in the internalization assay (30-minute stimulation) (Fig. 1C, Table 2), with pEC50 values equivalent to those previously reported for Y1-GFP internalization and Y1 receptor recruitment of β-arrestin-2 (Berglund et al., 2003; Kilpatrick et al., 2010, 2012).

A second FLAG-tagged Y1 receptor cDNA, tagged at the C-terminus with the complementary Ye BiFC fragment (protomer B in Fig. 1A), was then introduced into the HEK SNAP-Y1-Yn line, generating the stable mixed population designated Y1/Y1. Competition for [125I]PYY binding by unlabeled PYY, BIBO3304, or GTPyS was unaltered in Y1/Y1 compared with parent SNAP-Y1-Yn membranes (Table 1), with a similar [125I]PYY Bmax (3.2 ± 0.8 pmol mg⁻¹; n = 4). However, confocal imaging revealed both surface labeling of the SNAP-Y1-Yn population in Y1/Y1 cells (with SNAP-Surface 647) and the generation of complemented YFP fluorescence through irreversible BiFC between the SNAP-Y1-Yn and Y1-Yc receptor.
partners (Fig. 1B). Under control conditions, both SNAP-Y1-Yn–labeled receptors and Y1/Y1 BiFC dimers were present predominantly at the cell surface, with similar extents of internalization to colocalized intracellular compartments following stimulation with a maximal concentration of NPY (Fig. 1B). Using the plate reader imaging format, both the red channel (labeled SNAP-Y1-Yn) and green channel (Y1/Y1 BiFC dimer) cell population images were analyzed independently by granularity analysis. The labeled SNAP-Y1-Yn fluorescence reflected all Y1 receptor complexes containing this protomer—including monomers, oligomers, and the complemented species. In contrast, the visualized BiFC complexes identified those Y1/Y1 dimers trapped by complementation, either as distinct entities or potential participants in higher-order multimeric receptor species (Fig. 1B). The analysis format thus enabled determination of the internalization responses in both these receptor populations to the same agonist challenge. There was no difference in the rate of endocytosis in response to both these receptor populations to the same agonist challenge. Thus, enabled determination of the internalization responses in order multimeric receptor species (Fig. 1B). The analysis format either as distinct entities or potentially participants in higher-

**TABLE 1**
Summary of binding parameters for SNAP-Y1-Yn and Y1 homo- and heterodimer cell lines

<table>
<thead>
<tr>
<th>Competing Ligand</th>
<th>pEC50</th>
<th>pIC50</th>
<th>% Displacement</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNAP-Y1-Yn</td>
<td>9.39 ± 0.09</td>
<td>8.32 ± 0.06</td>
<td>8.40 ± 0.10</td>
</tr>
<tr>
<td>Y1/Y1</td>
<td>9.52 ± 0.07</td>
<td>8.44 ± 0.05</td>
<td>8.48 ± 0.12</td>
</tr>
<tr>
<td>Y1/Y1Bindneg</td>
<td>9.20 ± 0.07</td>
<td>8.40 ± 0.04</td>
<td>8.78 ± 0.14</td>
</tr>
<tr>
<td>Y1/Y1Phosneg</td>
<td>9.39 ± 0.08</td>
<td>8.14 ± 0.08</td>
<td>8.31 ± 0.12</td>
</tr>
<tr>
<td>Y1Phosneg/Y1Bindneg</td>
<td>9.36 ± 0.06</td>
<td>8.38 ± 0.06</td>
<td>8.47 ± 0.17</td>
</tr>
<tr>
<td>Y1/Y4</td>
<td>9.17 ± 0.07</td>
<td>8.39 ± 0.08</td>
<td>8.27 ± 0.10</td>
</tr>
<tr>
<td>Y1/Y5</td>
<td>9.08 ± 0.08</td>
<td>8.31 ± 0.03</td>
<td>8.68 ± 0.13</td>
</tr>
</tbody>
</table>

*Maximum GTPyS-sensitive displacement compared with total specific binding defined in the absence and presence of 1 μM PYY.

**TABLE 2**
Summary of agonist potencies for receptor internalization in SNAP-Y1-Yn and Y1/Y1 dimer cell lines, using simultaneous measurements of SNAP-Y1-Yn and BiFC dimer populations

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>NPY</th>
<th>BiFC Dimer</th>
<th>n</th>
<th>PYY</th>
<th>BiFC Dimer</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAP-Y1-Yn</td>
<td>8.10 ± 0.04</td>
<td>20</td>
<td>8.24 ± 0.13</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1/Y1</td>
<td>7.95 ± 0.09</td>
<td>7.85 ± 0.09</td>
<td>5</td>
<td>8.05 ± 0.09</td>
<td>7.81 ± 0.08</td>
<td>4</td>
</tr>
<tr>
<td>Y1/Y1Bindneg</td>
<td>7.94 ± 0.12</td>
<td>8.06 ± 0.08</td>
<td>4</td>
<td>7.97 ± 0.11</td>
<td>8.25 ± 0.10</td>
<td>4</td>
</tr>
<tr>
<td>Y1/Y1Phosneg</td>
<td>7.84 ± 0.13</td>
<td>8.14 ± 0.12</td>
<td>3</td>
<td>8.38 ± 0.09</td>
<td>8.31 ± 0.22</td>
<td>3</td>
</tr>
</tbody>
</table>
mutant) have been previously shown to abolish β-arrestin recruitment to the Y1 receptor, while preserving agonist binding (Holliday et al., 2005; Ouedraogo et al., 2008; Kilpatrick et al., 2010). However, selective introduction of this mutation into protomer B (the Y1/Y1Phosneg BiFC dimer) was also without inhibitory effect on the time course of 100 nM NPY–induced dimer internalization ($t_{1/2} = 4.2 ± 1.2$ minutes; $n = 3$) (Supplemental Fig. 1) or associated agonist concentration-response relationships (Fig. 2B; Table 2) (two-way ANOVA followed by Bonferroni post-test; $P > 0.05$). Finally, the dual effect of binding- (Bindneg) and phosphosite (Phosneg) mutations was tested by incorporating these substitutions into different protomers of the BiFC dimer. In this case, the 5A Phosneg substitution was generated in the SNAP-Y1-Yn construct (protomer A), and the Y99A Bindneg mutant was present in the Y1-Yc protomer B. As Fig. 2C illustrates, no agonist-stimulated endocytosis was observed in the SNAP-Y1Phosneg/Y1Bindneg-coexpressing cell lines, either for the SNAP-Y1Phosneg-Y1Bindneg BiFC dimer. Thus, the distinct effects of each mutation placed in one protomer (ligand binding or phosphorylation) could not be compensated by preserving this function in the second protomer. For each of the cell lines generated (Y1/Y1Bindneg, Y1/Y1Phosneg, and Y1Phosneg/Y1Bindneg), $[^{125}]$PYY competition binding studies revealed no changes in agonist or antagonist

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**Fig. 2.** Binding or phosphorylation mutants incorporated into a single protomer do not prevent Y1/Y1 BiFC dimer internalization. Mutations adversely affecting ligand binding (Y1/Y1Bindneg; A) or receptor phosphorylation (Y1/Y1Phosneg; B) were selectively introduced into protomer B only (see middle insets) by stably coexpressing the FLAG-tagged mutant receptor–Yc with SNAP-Y1-Yn in HEK293T cells. In automated measurements of internalization, both the constrained receptor BiFC dimer and labeled SNAP-Y1-Yn population were monitored for responses to NPY or PYY, following incubation for 30 minutes. These concentration-response curves provided the potency data given in Table 2. The right-hand panels show representative images of each labeled population, for the Y1/Y1Bindneg and Y1/Y1Phosneg clones, under control or 100 nM NPY–stimulated conditions. (C) Equivalent panels demonstrate the absence of NPY- or PYY-induced internalization of the SNAP-Y1Phosneg-Yn population or the BiFC dimer in Y1Phosneg/ Y1Bindneg cells, in which the binding and functional mutations are incorporated into separate protomers. As a consequence, quantitative data are expressed as % basal rather than as % 1 μM NPY. Pooled data are from 3–5 experiments.
pharmacology compared with Y1/Y1 control membranes, with $B_{\text{max}}$ values between 2.7 and 4.5 pmol mg$^{-1}$ (Table 1).

**Cointernalization of NPY Binding–Deficient Y1-GFP Receptors with SNAP-Y1 Is Also Observed in a Noncomplemented System.** Although BiFC highlights defined Y receptor dimers for investigation, there are also limitations, principally that the irreversibility characteristic of BiFC formation, which allows unambiguous identification of the complex, will also trap dimers as stable entities and perhaps increase their relative prevalence. In noncomplemented systems, dimers might only exist at lower levels compared with other species, such as monomers, and be transient in nature (Dorsch et al., 2009; Fonseca and Lambert, 2009). We therefore sought to detect whether evidence for Y1 receptor homodimer internalization could also be obtained in a system where the complexes were not constrained by complementation. Dual clonal cell lines were generated, using the equivalent expression vectors to the BiFC system, coexpressing SNAP-Y1 receptors lacking C-terminal modification and either Y1-GFP or Y1Bindneg-GFP (Fig. 3). Following stimulation with NPY, receptor-GFP or SNAP-Y1 receptor fluorescence intensities were then measured within internal compartments identified by a transferrin marker (Valentin-Hansen et al., 2012)—an analysis that offered improved sensitivity compared with granularity for both SNAP-Y1 and Y1-GFP responses in these cells (illustrative granularity data are shown in Supplemen-tal Fig. 3). Unsurprisingly, SNAP-Y1 and Y1-GFP receptors internalized in response to NPY in the coexpressing line (Fig. 3, A and C), with respective pEC50s of 7.94 ± 0.08 and 7.86 ± 0.08 (each $n = 5$). However, stimulation of SNAP-Y1 receptor endocytosis also caused cointernalization of the Y1Bindneg-GFP receptor unable to bind agonist (Fig. 3, B and C), and this occurred with similar NPY potency (pEC$_{50}$, 8.06 ± 0.13 for SNAP-Y1 and 7.86 ± 0.17 for Y1Bindneg-GFP; each $n = 4$). As indicated in the representative images (but not evident in the normalized concentration-response data), the maximal level of costimulated endocytosis was greater for Y1-GFP than Y1Bindneg-GFP receptors. This result would be anticipated because the many Y1Bindneg-GFP receptors not complexed with SNAP-Y1 (e.g., monomeric or homodimeric species) would not be expected to undergo internalization. Thus, the ability of single-site agonist occupancy to stimulate endocytosis of a trapped Y1/Y1Bindneg BiFC dimer is reflected by similar behavior of the respective noncomplemented receptors.

**Probing Y1 Receptor–Containing Heterodimers Using the BiFC System—Agonist Pharmacology.** To investigate the pharmacology of defined Y1 heterodimers, the Y4 or Y5 receptor subtypes, or the less closely related class A D2L or β2AR, were each cotransfected as Yc fusion proteins (protomer B; Fig. 1B) into the SNAP-Y1-Yn clone—generating Y1/Y4, Y1/Y5, Y1/β2AR, or Y1/D2L cell lines. Three of these lines (Y1/Y4, Y1/D2L, and β1/β2AR) were used as stable mixed expression vectors for the receptor-Yc construct, with BiFC fluorescence resulting from cotransfection identified in the majority of cells. For the Y1/Y5 line (the subsequent focus of heterodimer investigations), a second round of dilution cloning minimized cell–cell variability in SNAP-Y1-Yn and BiFC dimer expression (Fig. 4). Y1/Y4, Y1/Y5, and β1/β2AR combinations all generated BiFC receptor heterodimers localized predominantly at the plasma membrane under control conditions (Fig. 4; Supplemental Fig. 4). However, BiFC fluorescence in the Y1/D2L cell line was almost exclusively intracellular (Supplemental Fig. 4), indicating that the constrained heterodimer was unable to traffic appropriately to the cell surface. The Y1/D2L receptor combination was therefore not considered further.

For the remaining heterodimer cell lines ([125I]PYY binding data in Table 1), the effect of different agonists on internalization of the BiFC dimer was examined, in comparison with endocytosis of the SNAP-Y1-Yn population measured simultaneously in the same experiment, or to internalization of GFP-tagged Y4 or Y5 receptors expressed alone. When considering Y receptor dimer combinations, we first made use of the selectivity of the endogenous NPY-related peptide PP for Y4 and Y5 receptors over the Y1 subtype, and the availability of a highly Y5-selective synthetic peptide agonist, cPP(1–17) (Ala$^{31}$, Aib$^{32}$) NPY(18–36) (PP-Aib-NPY) (Michel
et al., 1998; Cabrele et al., 2000). Figure 5 demonstrates the pharmacology of these two peptides in comparison with NPY when measuring endocytosis of SNAP-Y1-Yn receptors expressed alone (Fig. 5A) or in combination with Y1-Yc (Fig. 5B) or of the Y1/Y1 BiFC dimer (Fig. 5B). In all three cases, PP-Aib-NPY was inactive at concentrations up to 1 μM, while PP was a low-potency partial agonist compared with NPY (Table 3).

Both PP and PP-Aib-NPY were potent full agonists when stimulating Y5-GFP internalization (respective pEC50s of 8.33 ± 0.05 and 9.04 ± 0.15; n = 4) compared with NPY (pEC50, 8.13 ± 0.09; n = 5) (Fig. 5C). Y5-GFP internalization proceeded at a somewhat slower rate than SNAP-Y1-Yn endocytosis, and we measured these agonist concentration-response curves at 60 minutes for an improved response window. On the other hand, 100 nM NPY–stimulated endocytosis of the SNAP-Y1-Yn and BiFC dimer populations in Y1/Y5 cells was characterized by similar time courses (Supplemental Fig. 1C) (respective t1/2s of 9.4 ± 2.7 minutes and 9.9 ± 1.8 minutes; n = 3), and the data represent 30-minute agonist stimulation in this clone for consistency with other BiFC dimer lines. However, equivalent findings for Y1/Y5 agonist pharmacology were obtained at the 60-minute time point (data not shown). As expected, PP and PP-Aib-NPY were inactive when monitoring the internalization of the SNAP-Y1-Yn population in Y1/Y5 cells, but the same treatments promoted Y1/Y5 BiFC heterodimer endocytosis measured simultaneously (Fig. 5D). However, compared with their effects on Y5-GFP internalization, both PP and PP-Aib-NPY were markedly less potent than NPY in stimulating Y1/Y5 dimer responses (100- to 300-fold compared with Y5-GFP) and also became partial agonists, with maxima ~50% of the 1 μM NPY response (Table 3). As Supplemental Fig. 2B illustrates, other peptides with reported selectivity for Y5 over the Y1 subtypes, including NPY(3-36) and d-Trp32-NPY (Michel et al., 1998), displayed low-potency partial agonism when measuring Y1/Y5 BiFC dimer endocytosis.

PP also stimulated Y4-GFP internalization (30 minutes) with a pEC50 of 9.37 ± 0.07 (n = 9), while NPY was a low-potency partial agonist (Fig. 5E). Conversely, PP had expected low efficacy compared with NPY in promoting SNAP-Y1-Yn endocytosis in the Y1/Y4 BiFC heterodimer cells (Fig. 5F). However, both NPY and PP induced Y1/Y4 BiFC dimer internalization, with respective pEC50s of 7.85 ± 0.07 and 7.57 ± 0.13 (n = 4). PP was therefore 63-fold less potent in stimulating Y1/Y4 dimer internalization compared with Y4-GFP responses (P < 0.001 comparing pEC50 values, Student’s t test), but the maximum response observed was almost equivalent to that observed to NPY (78.9 ± 4.4%; n = 4; comparing 1 μM responses). Finally, we examined the ability of structurally unrelated agonists (NPY and isoprenaline) to stimulate endocytosis of the Y1/β2AR BiFC dimer, and in this case both were full agonists (Supplemental Fig. 5), with respective potencies that did not differ significantly from the SNAP-Y1-Yn population responses (NPY) or our previous assessment of isoprenaline-induced internalization of SNAP-tagged β2ARs (Valentin-Hansen et al., 2012).

The Effects of Nonpeptide Selective Antagonists Confirm the Altered Pharmacology of the Y1/Y5 Heterodimer.

Given that a single binding site appeared sufficient for the internalization of Y1-containing dimers (from the studies in Y1/Y1 bindneg cells; Fig. 2A), the markedly reduced potency and relative efficacy of Y5-selective agonists in stimulating endocytosis of Y1/Y5 BiFC heterodimers suggested that constrained dimerization had an impact on their pharmacology, though alternative explanations were also possible (for example, subtype differences in receptor internalization rates). Thus, to determine whether allosteric interactions between the receptor protomers were occurring, the effects of Y1- or Y5-selective antagonists were assessed in Y1/Y5 cells, compared with the Y1/Y1, Y1/Y4, or single expressed GFP-tagged Y receptor lines.

Figure 6A illustrates that the Y1 ligand BIBO3304 was a surmountable antagonist of NPY responses in Y1/Y1 cells at up to 300 nM, whether internalization of the SNAP-Y1-Yn population or the Y1/Y1 BiFC dimer was measured. From the parallel rightward shifts of the NPY concentration-response curves following treatment with 30 nM BIBO3304, antagonist pKb estimates were calculated as 8.5 ± 0.3 (SNAP-Y1-Yn) and 8.1 ± 0.2 (Y1/Y1 BiFC dimer; n = 3), similar to the pIC50 from [125I]YPP binding studies (Table 1) and effects on NPY-induced Y1-GFP receptor internalization (pKb, 8.6 ± 0.2; n = 4; data not shown). Moreover, the effects of BIBO3304 on NPY responses were preserved when examining the behavior of the Y1/Y4 heterodimer. In the presence of 30 nM BIBO3304, NPY stimulated Y1/Y4 BiFC heterodimer internalization with 10-fold-lower potency than control, yielding a pKb of 8.5 ± 0.2 (n = 4) that was indistinguishable from that derived from SNAP-Y1-Yn receptor responses measured simultaneously (pKb, 8.4 ± 0.2; Fig. 6B). However, in these cells, BIBO3304 had no effect on PP-stimulated Y1/Y4 dimer internalization (Fig. 6C), consistent with its reported lack of affinity for the Y4 binding site (Wieland et al., 1998) and our observations that this antagonist failed to prevent PP-stimulated Y4-GFP endocytosis (data not shown).
Thus, the effects of BIBO3304 on Y1/Y4 BiFC dimer responses could be explained sufficiently by known ligand selectivity of the Y1 and Y4 orthosteric binding sites, and the assumption that these acted independently to drive PP- or NPY-stimulated internalization. Equally, NPY- or isoprenaline-stimulated internalization of the Y1/\(\beta\)2AR BiFC dimer was inhibited in an agonist-selective manner by either BIBO3304 or the \(\beta\)-blocker propranolol (Supplemental Fig. 5).

Fig. 5. Agonist pharmacology assessed by internalization of Y1-containing BiFC homodimers or heterodimers. Stably transfected HEK293T cell lines were labeled with SNAP-Surface 647 (if appropriate) and stimulated with NPY, PP, or PP-Aib-NPY prior to fixation and imaging on the IX Ultra plate reader. In each case, granularity was used to quantify the extent of agonist-promoted endocytosis normalized to 1 \(\mu\)M NPY or 100 nM PP (Y4-GFP). The pooled concentration-response data (at 30 minutes, or 60 minutes for Y5-GFP) represent mean \(\pm\) S.E.M. for at least 3–20 experiments. (A), (C), and (E) illustrate control experiments in cells expressing a single receptor subtype [the SNAP-Y1-Yn (A), Y5-GFP (C), or Y4-GFP (E) lines]. For coexpressing homodimer and heterodimer lines Y1/Y1 (B), Y1/Y5 (D), and Y1/Y4 (F), internalization of both the labeled SNAP-Y1-Yn and BiFC dimer populations was assessed in response to the same agonist challenge, yielding the paired concentration-response relationships illustrated. pEC\textsubscript{50} estimates obtained from these data are given in the text and Table 3.
In contrast, the participation of Y1 receptors in a Y1/Y5 BiFC heterodimer led to profound changes in the action of BIBO3304 (Fig. 6D). In the Y1/Y5 cell line, increasing concentrations of BIBO3304 produced the expected surmountable antagonism of NPY-stimulated SNAP-Y1-Yn endocytosis (with $pK_a$ of $8.4 \pm 0.2$; $n = 5$; from 30 nM curve shifts). However, in the same experiments, BIBO3304 had no significant effect on Y1/Y5 dimer internalization (Fig. 6D) at concentrations up to 100 times the measured $pK_a$ for the Y1 binding site (300 nM)—with the potency and maximum response to NPY being unchanged in the presence of the antagonist. Within the Y1/Y5 dimer (unlike Y1/Y4 or Y1/2AR), the Y5 binding site would also be available for activation by NPY, and this could continue to promote endocytosis despite occupancy of the Y1 protomer by BIBO3304. If this were the case, however, modified pharmacological properties of the Y1/Y5 BiFC dimer would still be a necessary explanation for the preservation of NPY efficacy (potent full agonism) compared with the reduced-potency partial agonism of Y5-selective agonist peptides (Fig. 5D; Supplemental Fig. 2B).

Furthermore, given the lack of effect of BIBO3304 on NPY-stimulated Y1/Y5 BiFC dimer internalization, it might be expected that the reciprocal experiment, using a Y5 antagonist, would also be ineffective in preventing NPY responses. To this end, the selective Y5 antagonist CGP71683, a structural homolog of velneperit, was first investigated. CGP71683 has previously been reported as a competitive reversible antagonist of Y5 receptor signaling with nanomolar affinity (Criscone et al., 1998; Lecklin et al., 2002), and as Fig. 7A illustrates, surmountable antagonism of NPY-induced Y5-GFP internalization was indeed observed ($pK_a = 8.4 \pm 0.1$; $n = 3$). Equally, in Y1/Y5 cells, CGP71683 had no significant effect on endocytosis of the reference SNAP-Y1-Yn population at concentrations up to 300 nM (Fig. 7B), confirming its lack of affinity for the Y1 receptor. Surprisingly, however, the presence of CGP71683 significantly inhibited maximal agonist-promoted Y1/Y5 dimer internalization (1 μM NPY in the presence of 30 nM CGP71683, $44.0 \pm 7.6\%$; $P < 0.001$ compared with 1 μM control NPY; Student’s t test; $n = 6$). The nonsurmountable inhibition by CGP71683 was also saturable, with no further decrease in the maximum NPY response observed as the antagonist concentration was raised from 30 to 300 nM (Fig. 7C). To investigate whether this change from competitive (Y5-GFP) to noncompetitive inhibition (Y1/Y5 BiFC dimer) was related to a particular class of antagonist, we considered two further structurally unrelated nonpeptide Y5 antagonists, NPY5RA972 (Turnbull et al., 2002) and L152,804 (Kanatani et al., 2000). Both antagonists (at 30 nM) were characterized by an absence of an effect on agonist-promoted SNAP-Y1-Yn endocytosis in Y1/Y5 cells. As for CGP71683, however, both compounds were nonsurmountable antagonists of NPY Y1/Y5 BiFC dimer responses (Fig. 8).

**Discussion**

Exploitation of GPCR dimers, and particularly heterodimers, remains an exciting avenue for selective therapeutic approaches. The demonstration of surmountable antagonism of NPY-stimulated Y1/Y5 BiFC dimer internalization, and the differences in the pharmacological properties of the Y1/Y5 heterodimer compared with Y1/Y1 and Y1/Y5 cells, provides a means to study the resulting pharmacology of this molecular species specifically. However, such identified dimers represent a minority among a cellular traffic that may reside at the cell surface and elsewhere. To gauge this influence on cell population–based experiments, standard $[125I]$PYY competition and $[3H]$cAMP accumulation assays were also performed on Y1/Y1 and Y1/Y5 cells for comparison. In Y1/Y1 and Y1/Y5 membranes, both PYY and BIBO3304 fully competed for $[125I]$PYY binding (Fig. 9A; Table 1), whereas in Y1/Y4 membranes, only partial $[125I]$PYY displacement was observed (Table 1). However, despite the ability of $[125I]$PYY to label Y5 receptors with high affinity (Gerald et al., 1996), neither PP-Aib-NPY nor CGP71683 displaced >20% of the Y1/Y5 membrane binding sensitive to 1 μM PYY (Fig. 9A). Equally, NPY inhibited forskolin-stimulated cAMP accumulation in both Y1/Y1 and Y1/Y5 cells (with respective $pEC_{50}$s of 9.74 ± 0.03 and 9.42 ± 0.05; $n = 3–7$), further confirming G protein coupling and downstream function of the expressed receptors (Fig. 9, B and C). In both Y1/Y1 and Y1/Y5 cells, surmountable antagonism by BIBO3304 was observed (pK$\alpha$ calculated from pooled 30 nM antagonist shifts: 7.9 and 7.7, respectively), but in each case the Y5 antagonist CGP71683 was ineffective. Both binding data and cAMP accumulation data therefore indicated that in Y1/Y5 cells, the expression of transfected Y1 receptors was significantly greater than the Y5 subtype, with Y1-like pharmacology predominating. This is consistent with the Y1/Y5 internalization data for the SNAP-Y1 population—reflecting all monomeric and homodimeric complexes containing this protomer—demonstrating agonist and antagonist behavior expected for the Y1 subtype.

### Table 3

Comparison of agonist potencies and 1 μM internalization responses (relative to 1 μM NPY) in SNAP-Y1, Y1/Y1, and Y1/Y5 dimer cell lines.

<table>
<thead>
<tr>
<th>Cells/Channel$^a$</th>
<th>NPY</th>
<th>PP</th>
<th>PP-Aib-NPY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pEC_{50}$</td>
<td>$pEC_{50}$</td>
<td>% 1 μM NPY</td>
</tr>
<tr>
<td>SNAP-Y1-Yn</td>
<td>8.10 ± 0.04</td>
<td>&lt;7.10</td>
<td>44.9 ± 8.9</td>
</tr>
<tr>
<td>Y1/Y1</td>
<td>7.95 ± 0.09</td>
<td>&lt;6.10</td>
<td>23.1 ± 2.7</td>
</tr>
<tr>
<td>SNAP-Y1-Yn</td>
<td>7.85 ± 0.09</td>
<td>&lt;6.10</td>
<td>26.6 ± 4.8</td>
</tr>
<tr>
<td>Y1/Y5</td>
<td>7.95 ± 0.04</td>
<td>&lt;6.10</td>
<td>23.5 ± 7.3</td>
</tr>
<tr>
<td>SNAP-Y1-Yn</td>
<td>7.71 ± 0.04</td>
<td>6.91 ± 0.15</td>
<td>57.4 ± 9.0</td>
</tr>
</tbody>
</table>

*Channel refers to responses from the BiFC dimer or SNAP-Y1-Yn populations measured simultaneously in the same experiment (30-minute agonist treatment).
agents (Smith and Milligan, 2010; Ferre et al., 2014). Bioluminescence resonance energy transfer–based approaches (Urizar et al., 2011; Watts et al., 2013), modified GPCR–G protein fusion systems (Han et al., 2009; van Rijn et al., 2013), or bivalent ligands (Waldhoer et al., 2005; Ferre et al., 2014) have all been used to assign pharmacology to defined heterodimers. Such investigations can depend on ligand selectivity, cross-talk between signaling pathways and the allosteric influence of coexpressed effector proteins, and the potential for receptor dimers to be transient in nature (Dorsch et al., 2009; Fonseca and Lambert, 2009). Thus, we designed a system to address GPCR dimer pharmacology constrained irreversibly by BiFC, using example class A NPY receptors. This process traps the dimer artificially, but it means that the molecular unit under investigation, detectable by a single wavelength readout (β-arrestin–dependent internalization), has known stable protomer composition—with the proviso that such BiFC dimers might also participate in larger oligomers. Y receptor BiFC dimers undergo agonist-stimulated intracellular trafficking, capable of being driven by single NPY-bound or phosphorylated protomers. Moreover, the Y1/Y5 heterodimer, but not others investigated, exhibits modified pharmacology to both agonist and antagonist ligands. These unique properties are relevant to potential antiobesity Y5 antagonists, such as velneperit (Brothers and Wahlestedt, 2010; George et al., 2014), given functional interplay between Y1 and Y5 receptors in the hypothalamic appetite neurons responsive to NPY.

It is important to recognize that the irreversible nature of BiFC (Kerppola, 2008) is both an advantage, for identifying defined dimers, and a limitation to our approach. This technique has previously supported the existence of particular GPCR oligomers (Briddon et al., 2008; Guo et al., 2008; Vidi et al., 2008), but complementation might equally trap transient dimers in recombinant systems by unnaturally stabilizing their association (Kerppola, 2008). For receptors where dimerization is short-lived (Dorsch et al., 2009; Fonseca and Lambert, 2009), this artificial stability might lead to altered pharmacological properties. We found, for example, that plasma membrane expression of Y1 BiFC dimers was possible with a variety of partners (Y1, Y4, Y5, and β2AR), though not all (Y1/D2L). Thus, we focused particular efforts on characterizing Y1 receptor BiFC systems, for which previous evidence exists for oligomers using noncomplemented approaches (Dinger et al., 2003; Gehlert et al., 2007), and bearing in mind that such BiFC responses represent the pharmacology of a constrained dimer.

Y1/Y1 BiFC homodimers underwent agonist-promoted endocytosis unchanged from the SNAP-Y1 protomer population overall, identified by simultaneous SNAP-labeling, or indeed earlier Y1 receptor investigations (Kilpatrick et al., 2012). Several studies suggest coordinated trafficking of stable GPCR dimers (Sartania et al., 2007; Smith and Milligan, 2010; Ward et al., 2011), but these are offset by others that suggest monomeric receptors predominate following internalization (Lan et al., 2011). Because GPCR BiFC dimers are artificially constrained by complementation, our curves were constructed from these data, normalized to the control maximal agonist responses or vehicle controls as required. Where appropriate, estimates of BIBO3304 affinity were calculated as pK₈ values, which are given in the text.

Fig. 6. Y1/Y5 BiFC dimers display selectively modified pharmacology to the Y1 receptor antagonist BIBO3304. After SNAP fluorophore labeling, the Y1/Y1 (A; n = 4–7), Y1/Y4 (B and C; n = 4–6) or Y1/Y5 (D; n = 3–7) cell lines were pretreated with vehicle or 30 or 300 nM BIBO3304 for 30 minutes, followed by NPY (A, B, and D) or PP (C). Internalization following 30-minute agonist treatment was assessed from granularity analysis of paired plate reader images of the SNAP-Y1-Yn receptor population and BiFC constrained dimers. Pooled concentration-response
data do not resolve whether native monomeric or oligomeric Y receptors enter the clathrin-mediated endocytosis pathway (Ouedraogo et al., 2008), but internalization of stabilized oligomeric Y1 receptors is clearly possible. This finding was supported by cointernalization of non–agonist-binding Y1Bindneg-GFP receptors after stimulation of coexpressed, but noncomplemented, SNAP-Y1 receptors, as for investigations of β2AR dimers (Sartania et al., 2007).

For the Y receptor family, the key effector proteins driving internalization are β-arrestins (Berglund et al., 2003; Holliday et al., 2005; Ouedraogo et al., 2008; Kilpatrick et al., 2010), and it is unresolved whether or how β-arrestins engage GPCR dimers. Arrestin sensors detect both the activated conformation and the phosphorylated state for agonist-stimulated GPCRs (Gurevich and Gurevich, 2006). Studies of rhodopsin dimer–arrestin interactions (Liang et al., 2003; Sommer et al., 2012) have drawn parallels with the proposed asymmetric mode of G protein heterotrimer binding to a GPCR dimer (Damian et al., 2008), in which each sensor of arrestin may interact with a distinct receptor protomer. However, other experimental evidence, even within the rhodopsin system, indicates 1:1 (Hanson et al., 2007; Bayburt et al., 2011) or changing stoichiometries (Sommer et al., 2011). We tested how arrestin might recognize the Y1/Y1 BiFC dimer by incorporating NPY binding- or phospho-site mutations into one protomer only and monitoring consequences for arrestin-dependent endocytosis. These targeted mutations indicated that one agonist-binding site was sufficient for Y1 BiFC dimer internalization, findings that could be reproduced with coexpressed and noncomplemented SNAP-Y1 and Y1Bindneg-GFP receptors. Equally, ablation of the key Y1 receptor C-terminal phosphorylation sites, which abolishes Y1 receptor internalization overall, had no effect when these mutations were targeted to just one protomer. We predicted that if each arrestin sensor engaged distinct protomers within the dimer, binding- and phospho-site mutations in different protomers might complement each other and so preserve functional internalization. Instead, agonist-promoted internalization of the Y1Phosneg/Y1Bindneg dimer was abolished. This argues against a 2:1 stoichiometry model proposed by Liang et al. (2003), though there are limitations—in addition to the use of a stabilized complementation system, the ability of G protein receptor kinases to phosphorylate the Y1Bindneg dimer might also be compromised. However, our results suggest that at least one protomer within the BiFC dimer must be both agonist occupied and phosphorylated to drive internalization via β-arrestins.

Neither agonist nor antagonist (BIBO3304) pharmacology was altered by trapping Y1/Y1 receptors as BiFC dimers. One caveat to detecting the influence of such forced homodimers is that composition of the control SNAP-Y1-Yn population—in terms of monomers, dimers, or higher-order oligomers—is unknown, and will also include the SNAP-labeled BiFC complexes. If this reference population naturally existed as stable dimers, the further impact of BiFC trapping would be limited. Thus, to detect possible allosteric interactions between the protomer-binding sites, three Y1-containing

![Fig. 7.](https://molpharm.aspetjournals.org/)

**Fig. 7.** CGP71683 exhibits an altered mode for antagonism of NPY-stimulated Y1/Y5 heterodimer internalization. Pooled concentration-response relationships in (A) demonstrate the surmountable antagonist action of CGP71683 (structure shown in inset; 30 or 300 nM, 30-minute pretreatment) in inhibiting NPY-induced Y5-GFP endocytosis over 60 minutes in 293TR cells (n = 3–5). In SNAP fluorophore–labeled Y1/Y5 cells using the same experimental conditions (n = 3–9), CGP71683 had no significant effect on NPY concentration-response curves measured by plate reader imaging of the SNAP-Y1-Yn population at 30 minutes (B). However, maximal NPY-stimulated internalization of the Y1/Y5 BiFC dimer (C), but not NPY potency, was significantly inhibited. **P < 0.001 compared with 1 μM NPY control (Student’s unpaired t test).
heterodimers were considered, for which protomer-selective agonists and antagonists are available. The behavior of the Y1/Y4 and the Y1/β2AR BiFC heterodimers was sufficiently explained by the known pharmacology of the constituent orthosteric binding sites. For example, BIBO3304 remained a surmountable antagonist of Y1/Y4 dimer internalization stimulated by NPY (acting only on the Y1 binding site), but had no influence on responses mediated by the Y4-selective agonist PP. There was therefore no evidence for allosteric communication between the Y1 and Y4 (or β2AR) protomers—the behavior of these BiFC complexes was as expected for each individual receptor protomer acting independently.

Given these control Y1 heterodimer combinations, without detectable interactions between protomers, the novel pharmacology observed for Y1/Y5 heterodimers is most likely to arise from allosteric communication between binding sites within this dimer, rather than a general artifact of the complementation technique. Combined impacts on ligand pharmacology were observed—not only the reduced potency and efficacy of Y5-selective agonists relative to NPY, but also nonreciprocal effects on selective antagonists, in which Y1 antagonists became inactive—but the mode of Y5 antagonism (using three structurally distinct example compounds) changed from surmountable to nonsurmountable. Overall, these changes can only be reconciled if the Y1 and Y5 protomers within the constrained dimer no longer behave independently, via possible allosteric mechanisms that include direct interaction between the receptor protomers or the indirect effects

![Figure 8](https://www.molpharm.org/content/doi/10.1124/mol.117.103424)

Fig. 8. Nonsurmountable inhibition of Y1/Y5 BiFC dimer internalization by structurally distinct Y5 antagonists. Y1/Y5 cells were labeled with SNAP-Surface 647 and pretreated for 30 minutes with the antagonists L152,804 (A) or NPY5RA972 (B) at 30 nM. Internalization following 30-minute NPY stimulation was then assessed for the SNAP-Y1-Yn and BiFC dimer populations by imaging and granularity analysis. Pooled concentration-response curves, normalized to the 1 μM NPY control response, were obtained from five experiments in each case. ***P < 0.001 compared with 1 μM NPY control (Student’s unpaired t test).

![Figure 9](https://www.molpharm.org/content/doi/10.1124/mol.117.103424)

Fig. 9. Y1-like pharmacology of the Y1/Y5 cell line in [125I]PYY and [3H]cAMP accumulation assays. Competition binding studies in Y1/Y5 membrane preparations (A) using 15 pM [125I]PYY are pooled from at least three experiments, with specific binding defined by the absence and presence of 1 μM PYY. pIC50 determinations for PYY and BIBO3304 are quoted in Table 1. NPY-mediated inhibition of 30 μM forskolin–stimulated cAMP accumulation was assessed in Y1/Y1 (B; n = 2–7) and Y1/Y5 (C; n = 3) cells in the absence or presence of BIBO3304 or CGP71683 as indicated. NPY pEC50 and BIBO3304 pKb values are quoted in the text.
of recruited effector proteins to the dimer (Smith and Milligan, 2010). Interestingly, transient coexpression studies of non-complemented Y1 and Y5 receptors have previously implied that such changes occur following heterodimerization. Gehlert et al. (2007) observed the surmountable-to-non-surmountable transition for Y5 antagonists in this system, while Y1 antagonists (BBBP3226) also became inactive in second messenger assays. We believe that Gehlert et al. (2007) were better able to match relative Y1 and Y5 receptor expression in a transient system, revealing modified Y1/Y5 pharmacology in cell population assays, rather than the Y1-like pharmacology we observed in cAMP experiments. However, our assay based on BiFC complexes pinpoints the origin of such changes to the molecular properties of defined Y1/Y5 receptor heterodimers. Although the evidence for coexpressed Y1 and Y5 receptor heterodimerization in hypothalamic appetite neurons in vivo is still circumstantial, the effects of such dimers on pharmacology may be one contributory factor to the checkered history of highly selective Y1 and Y5 antagonists as potential obesity agents (Crisicone et al., 1998; Turnbull et al., 2002; Brothers and Walhede, 2010), as well as the more general requirement for combined Y1/Y5 gene deletion to inhibit NPY effects on food intake and energy homeostasis, compared with sometimes paradoxical observations with single Y receptor knockouts (Nguyen et al., 2012).

The use of BiFC combined with automated measurements of receptor trafficking thus provides a straightforward functional assay that can be generally applied to extract the pharmacology of molecularly defined and stabilized class A GPCR dimers. Our findings reveal allosteric interactions within Y1/Y5 receptor heterodimers that influence the mode of action of subtype-selective antagonists previously developed to inhibit NPY-stimulated appetite, and they also indicate a screening mechanism for selective agents at this receptor combination in the future.

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Authorship Contributions

Participated in research design: Holliday, Kilpatrick.
Conducted experiments: Kilpatrick, Humphreys.
Performed data analysis: Kilpatrick, Humphreys.
Wrote or contributed to the writing of the manuscript: Holliday, Kilpatrick.

References


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A G protein coupled receptor dimer imaging assay reveals selectively modified pharmacology of Neuropeptide Y Y1 / Y5 receptor heterodimers

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Supplemental Figure 1. Time-course data for NPY-induced internalisation measured in SnapY1-Yn (A), Y1 / Y1 homodimer (B, D, E) and Y1 / Y5 heterodimer (C) cell lines. Cells were labelled with SNAPsurface 647, and then treated with 100 nM NPY for various times prior to fixation, as described in Materials and Methods. Granularity analysis measured NPY-stimulated intracellular trafficking using paired images of the SnapY1-Yn labelled receptor population (red channel) and, where appropriate the constrained BiFC dimer (green channel). Pooled data represent 3 – 5 experiments, with mean t_{1/2} values (calculated from each individual experiment) quoted in the text.
Supplemental Figure 2. Agonist concentration response curves for internalisation responses in the Y1 / Y1 (A, n = 3 - 20) and Y1 / Y5 (B, n = 3 - 14) cell lines, comparing SnapY1-Yn and BiFC dimer populations. Cells were labelled with SNAPsurface 647 as described in Materials and Methods, followed by 30 min stimulation with the peptide ligands indicated. NPY, PYY, PP and cPP (1-17)(Ala^{31}, Aib^{32}) NPY (18-36) (PP-Aib-NPY) pEC_{50} values are quoted at relevant points in the text and Tables 2 and 3. Other pEC_{50} values were: [Leu^{31}, Pro^{34}]NPY 7.73 ± 0.09 (Y1 / Y1, SnapY1-Yn), 7.50 ± 0.08 (Y1 / Y1, BiFC dimer), 8.05 ± 0.12 (Y1 / Y5, SnapY1-Yn), 7.26 ± 0.08 (Y1 / Y5, BiFC dimer); D-Trp^{32} NPY 6.69 ± 0.16 (Y1 / Y5, BiFC dimer).
Supplemental Figure 3. Granularity analysis of co-internalisation of non-complemented SnapY1 and Y1-GFP receptors. Data (mean ± s.e.m.) are taken from the individual triplicate experiment shown in Fig. 3A, in which cells were stimulated for 30 min with NPY following labelling with SNAPsurface 647. Upper panels represent granularity analysis for the Y1-GFP (NPY pEC\textsubscript{50} 8.54) or Y1Bindneg-GFP (NPY pEC\textsubscript{50} 8.96) receptor populations in the co-expressing cell lines; lower panels present the quantitation of the SnapY1 receptor images. Four data points with no error bars represent mean only (n = 2), after removal of one outlying replicate.
Supplemental Figure 4. Representative images of the Y1 / β2AR and Y1 / D2L cell lines (IX Ultra platereader, cropped to 300 by 300 pixels from original) under vehicle or NPY stimulated (100 nM, 30 min) conditions. The SnapY1-Yn receptor population was identified by SNAPsurface 647 labelling (red channel) for comparison with complemented YFP fluorescence (green channel) identifying the subcellular localisation of the constrained BiFC dimer.
Supplemental Figure 5. Orthosteric binding site pharmacology of the Y1 / β2AR BiFC dimer. NPY or isoprenaline (ISO) stimulated endocytosis (30 min) was assessed for the SnapY1-Yn receptor populations or the BiFC heterodimer under control conditions or following 30 min pretreatment with 10 nM propranolol (A, n = 4) or 30 nM BIBO3304 (B, n = 4). Concentration response curves are expressed as a percentage of the maximal agonist concentration, or if no isoprenaline response was observed, as a percentage of vehicle controls. pK_B estimates of antagonist affinity could be calculated from the following curve shifts: propranolol pK_B 9.2 ± 0.2 (Y1 / β2AR BiFC dimer, isoprenaline); BIBO3304 pK_B 8.1 ± 0.1 (Y1 / β2AR BiFC dimer, NPY) and 8.2 ± 0.1 (Y1 / β2AR SnapY1-Yn population, NPY).