G Protein Preassembly Rescues Efficacy of W6.48 Toggle Mutations in Neuropeptide Y2 Receptor

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Received September 8, 2017; accepted February 2, 2018

ABSTRACT

Ligand binding and pathway-specific activation of G protein–coupled receptors is currently being studied with great effort. Individual answers may depend on the nature of the ligands and the effector pathway. Recently, we have presented a detailed model of neuropeptide Y bound to the Y2R. Accordingly, the C-terminal part of the peptide binds deeply in the transmembrane bundle and brings the side chain of the most essential Y36 in close proximity to W6.48. Here, we investigate the role of this interaction for ligand binding and activation of this receptor. BRET sensors were used for detailed investigation of effector interaction for ligand binding and activation of the Y2R. It further confirmed ligand-dependent recruitment of arrestin3. Using equally sensitive readouts for G activation and arrestin recruitment as well as quantification with operational models of agonism allowed us to identify a strong inherent bias for G activation over arrestin3 recruitment for the wild-type receptor. By systematic mutagenesis, we found that W6.48 does not contribute to the binding affinity, but acts as an allosteric connector to couple ligand binding to G activation and arrestin3 recruitment. However, even mutagenesis to a small threonine did not lead to a complete loss of signaling. Interestingly, signaling was restored to wild-type levels by ligands that contain a naphthylalanine as the C-terminal residue instead of Y36. Steric and polar contributions of W6.48 for the wild-type receptor. By systematic mutagenesis, we found that W6.48 does not contribute to the binding affinity, but acts as an allosteric connector to couple ligand binding to G activation and arrestin3 recruitment. However, even mutagenesis to a small threonine did not lead to a complete loss of signaling. Interestingly, signaling was restored to wild-type levels by ligands that contain a naphthylalanine as the C-terminal residue instead of Y36. Steric and polar contributions of W6.48 for the wild-type receptor. By systematic mutagenesis, we found that W6.48 does not contribute to the binding affinity, but acts as an allosteric connector to couple ligand binding to G activation and arrestin3 recruitment. However, even mutagenesis to a small threonine did not lead to a complete loss of signaling. Interestingly, signaling was restored to wild-type levels by ligands that contain a naphthylalanine as the C-terminal residue instead of Y36. Steric and polar contributions of W6.48 for the wild-type receptor. By systematic mutagenesis, we found that W6.48 does not contribute to the binding affinity, but acts as an allosteric connector to couple ligand binding to G activation and arrestin3 recruitment. However, even mutagenesis to a small threonine did not lead to a complete loss of signaling. Interestingly, signaling was restored to wild-type levels by ligands that contain a naphthylalanine as the C-terminal residue instead of Y36. Steric and polar contributions of W6.48 for the wild-type receptor.

Introduction

Within the last decades, the view of G protein–coupled receptors (GPCRs) has changed from simple on/off signal transduction switches to multifaceted relays with tight spatiotemporal regulation and multidimensional signaling through various intracellular effectors. Specifically, the discovery of functional selectivity that addresses only a subset of possible effectors for a given receptor holds great promise for tailored pharmaceutical interventions (Rajagopal et al., 2010; Khoury et al., 2014). Only a small fraction of the overall 800 members are currently targeted by pharmaceuticals, yet this accounts for at least 30% of all marketed drugs (Hopkins et al., 2015). Nonetheless, the entity of the structures obtained so far suggests that also peptide-activated GPCRs bind to the suggested common binding crevice, similar to the well-studied GPCRs with much smaller adrenergic or aminergic ligands (Venkatakrishnan et al., 2013).

Peptide-binding receptors make up a significant fraction of the rhodopsin family and are attractive targets, for instance for the treatment of obesity and pain (Wu et al., 2017). Their binding mode and activation, however, has long remained enigmatic (Schwartz and Rosenkilde, 1996). In recent years, crystallographic snapshots could be obtained for some of these receptors, revealing great variability of the binding pockets with respect to charge distribution and depth (Krumm and Grishammer, 2015; Wu et al., 2017). Most of these structures, however, were obtained in complex with nonpeptidic or peptidic antagonists, and the neurotensin receptor 1 remains the only receptor cocrystallized with its peptidic agonist (White et al., 2012; Egloff et al., 2014; Krumm et al., 2015, 2016). Nonetheless, the entity of the structures obtained so far suggests that also peptide-activated GPCRs bind to the suggested common binding crevice, similar to the well-studied GPCRs with much smaller adrenergic or aminergic ligands (Venkatakrishnan et al., 2013).

We have recently presented a model of the neuropeptide Y2 receptor (Y2R) bound to its agonist NPY13–36 by combining NMR, mutagenesis, and molecular modeling (Kaiser et al., 2015). In line with recent crystallographic structures, we

[This article was supported by the German Research Foundation (SFB1052/A3 and BE1264/16), the European Union and Free State of Saxony [Grants 100148835 and 143213128452], and the Max Kade Foundation.]

Supplemental material to this article can be found at http://molpharm.aspetjournals.org/content/suppl/2018/02/07/mol.117.110544.DC1

ABBREVIATIONS: BRET, bioluminescence resonance energy transfer; BRET50, half-maximal bioluminescence resonance energy transfer; CI, confidence interval; CRE, cAMP response element; Emax, maximal effect; eYFP, enhanced yellow fluorescent protein; F/L, acceptor to donor ratio; GPCR, G protein–coupled receptor; HEK293, human embryonic kidney 293; IP, inositol phosphate; KA, functional affinity constant; koff, dissociation rate constant; kassoc, association rate constant; Nal, naphthylalanine; NPY, neuropeptide Y; PBS, phosphate-buffered saline; Rluc8, Renilla luciferase 8; TM, transmembrane helix; t, transducer constant; WT, wild type; Y2R, neuropeptide Y receptor subtype 2.
demonstrated that, despite its size, NPY binds deeply in the transmembrane bundle guided by hydrophobic contacts of its amphipathic helix to the second extracellular loop, and unwinds its five C-terminal residues from the α-helix to make optimal contacts in the deep binding pocket (Kaiser et al., 2015). The Y2R is part of the NPY multiligand-multireceptor family, and is activated by the 36-amino acid C-terminally amidated NPY and peptide YY as well as N-terminally truncated peptide variants (Pedragosa-Badia et al., 2013). It couples with the inhibitory family of G proteins (G\(_i\)) (Michel et al., 1998) and has also been shown to recruit arrestin3 (Berglund et al., 2003; Kilpatrick et al., 2010; Walther et al., 2010; Gimenez et al., 2014). The individual contributions of these pathways for physiologic function (anorectic effects, neuroprotection, vascularization), however, have not yet been characterized (Babilon et al., 2013). Thus, detailed understanding of the prerequisites for binding and activation of either signaling pathway will help to develop (pathway) specific ligands, and rationalize antagonistic and agonistic ligand properties.

Interestingly, in the Y2R model (Kaiser et al., 2015) the side chain of the essential Y\(_{281}\) of the ligand is pointing toward W\(_{6.48}\) of the receptor (nomenclature according to Ballesteros and Weinstein (1995)). This position had been proposed as part of a global toggle-switch mechanism in GPCR activation (Schwartz et al., 2006; Holst et al., 2010; Katritch et al., 2013), and the exchange of this residue leads to a complete loss of G\(_i\) activation of several receptors (Holst et al., 2010), including two peptide-activated receptors.

Thus, we investigated this position for binding and activation of the Y2R. We systematically mutated the tryptophan side chain and analyzed coupling of G\(_i\), a chimeric G\(_i0\) protein as well as arrestin3 by second-messenger assays and live-cell fluorescence microscopy, as well as by bioluminescence resonance energy transfer (BRET). The use of BRET sensors allowed for a detailed investigation of the effector coupling mechanism, and revealed significant preassembly of Y2R-G\(_i\) in the absence of ligand. Mutagenesis of W\(_{6.48}\) had detrimental effects for all signaling pathways, but mutants still displayed significant activity even with a small threonine side chain. Steric and polar contributions of the C-terminal residue of the ligand is pointing toward W\(_{6.48}\) of the receptor (nomenclature according to Ballesteros and Weinstein, 1995) introducing Rluc8 or a monomeric variant (A206K) (Zacharias et al., 2015) into the helical domain next to M\(_{1}\) (Gn1; Y\(_{281}\)-chimeric G\(_{i0/4}\)myr) by using a Ser-Gly-Gly-Gly-Ser-linker. Human G\(_{i0}\) in pcDNA3 was obtained from UMR cDNA Resource Center (University of Missouri-Rolla, Rolla, MO), and chimeric G\(_{i0/4}\)myr (Kostenis et al., 2005) was provided by E. Kostenis (Dept of Pharmaceutical Biology, University of Bonn, Germany). Cloning was performed by polymerase chain reaction overlap extension using Pfu polymerase, and fusion proteins were inserted into pcDNA3 vector by using HindIII (5′) and XhoI (3′) restriction sites (both Fermentas enzymes were from Thermo Fisher Scientific, Waltham, MA). Construct identity was verified by Sanger dideoxy sequencing.

**125I-PYY Binding Assays.** Binding assays were performed with membrane preparations of transiently transfected HEK293 cells. Membranes were prepared according to Beck-Sickinger et al. (1994) with some modification. Cells were detached by washing with Ca\(^2+\)/Mg\(^2+\)-free phosphate-buffered saline (PBS; PAA Laboratories, Pasching, Austria), collected by centrifugation at 800g for 10 minutes, frozen in liquid nitrogen, and stored at −80°C. Cell pellets were taken up in 50 mM Tris-Cl buffer, pH 7.5 (containing protease inhibitors: 50 μM Pefabloc SC (Sigma-Aldrich, St. Louis, MO) and homogenized 25 times with a Potter S (B. Braun International, Melsungen, Germany) under ice cooling. The suspension was then centrifuged at 1°C and 740g for 10 minutes to remove cell debris, and the supernatant was centrifuged at 4°C and 18,500g for 60 minutes. The resulting pellet containing the microsomal membrane preparation was resuspended in 25 mM HEPES, 25 mM CaCl\(_2\), and 1 mM MgCl\(_2\), pH 7.4, containing 50 μM Pefabloc, and homogenized again 25 times in an ice bath. The suspension was centrifuged at 4°C, 18,500g for 120 minutes, and the pellet was resuspended in HEPES buffer without protease inhibitors for the determination of protein concentration. Subsequently, Pefabloc was added to a final concentration of 50 μM, and membrane preparations were stored in aliquots at −80°C. 125I-PYY (81.4 TBq/mmol; NEX240) was obtained from PerkinElmer (Waltham, MA). Radioligand-binding experiments were performed in 100 μl total volume in 96-well plates in HEPES/Ca\(^2+\)/Mg\(^2+\)-free buffer containing 1% (w/v) bovine serum albumin, using 0.5 μg total protein per well. Unspecific binding was measured in the presence of 1 μM NPY and subtracted from data. Saturation binding experiments were incubated under gentle agitation for 3 hours at room temperature. In dissociation experiments, NPY was added to a final concentration of 1 μM after 3 hours. The remaining bound radioactivity was subsequently determined at several time points to obtain an estimate of the dissociation rate constant (k\(_{\text{off}}\)). Assays were terminated by filtration and washing (3 × 200 μl) with ice-cold PBS using the MicroBeta Filtermat-96 Cell Harvester (PerkinElmer). Membranes were pretreated with 0.1% polyethyleneimine (w/v) in PBS.
Radioactivity was determined by scintillation counting (MicroBeta2; PerkinElmer). Binding assays were performed at least three times independently and were measured in duplicate.

**Inositol Phosphate Accumulation Assay (via Chimeric GαD\textsubscript{Gi/o}(myr)).** For easy and robust readout of receptor activity, a chimeric Gα\textsubscript{Gi/o}(myr) protein was cotransfected to redirect the endogenous G\textsubscript{i/o} signaling of Y receptors to the phospholipase C pathway (Kostenis et al., 2005). A detailed protocol has been described previously (Els et al., 2010; Witte et al., 2013). Briefly, COS7 cells transiently transfected with receptor and chimeric Gα\textsubscript{Gi/o}(myr) protein were labeled with 2 μCi/ml 3H-myo-inositol (PerkinElmer) in a 48-well plate format, stimulated with different concentrations of peptide, and 3H-inositol phosphates (IPs) were isolated from cell lysates by anion-exchange chromatography and measured by liquid scintillation counting. Experiments were performed in duplicate and repeated three times independently.

**cAMP Assays (via Endogenous G\textsubscript{i}).** Signal transduction was investigated in a reporter gene assay based on endogenous G\textsubscript{i}, (ONE-Glo Luciferase Reporter Gene Assay; Promega, Madison, WI). Plasmids encoding the receptor mutant (4 μg) and the luciferase reporter under the control of a cAMp response element (CRE) pGL4.29 [luc2P/CRE/Hygro] (4 μg) were cotransfected into 70% confluent HEK293 cells in six-well plates using Life Technologies Lipofectamine 2000 (Thermo Fisher Scientific, Carlsbad, CA) lipofection reagent according to manufacturer instructions. One day after transfection, cells were reseeded onto poly-D-lysine–coated 96-well plates (white, clear bottom; 125,000 cells/well) and grown for another day. Prior to stimulation, cells were serum deprived for 1 hour and then stimulated with varying NPY concentrations (10^{-13} to 10^{-6} M, each in triplicate) in the presence 1 μM forskolin (Sigma-Aldrich) in serum-free medium for 2 hours at 37°C. Cells were then washed once, 30 μl of serum-free medium/well was added, and cells were equilibrated to room temperature for 10 minutes. Subsequently, 30 μl of ONE-Glo reagent per well (room temperature) was added and incubated for 5 minutes in the dark before measuring luminescence in a plate reader (Tecan Infinite M 200; Tecan, Männedorf, Switzerland).

**Fluorescence Microscopy.** Membrane localization, arrestin3 recruitment, and internalization of Y\textsubscript{2}R variants was investigated in live HEK293 cells using an Axiovert Observers Z1 Microscope (with Apotome, Plan-Apochromat 63 x/1.40 Oil differential interference contrast objective; Carl Zeiss, Jena, Germany). HEK293 cells were seeded in μ-slides (Ibidi, Martinsried, Germany), and transiently transfected with Y\textsubscript{2}R-enhanced yellow fluorescent protein (eYFP) variants with or without addition of a vector encoding mCherry-arrestin3. One day after transfection, standard cell culture medium (containing 15% fetal calf serum) was changed to serum-reduced Gibco OptiMEM (Thermo Fisher Scientific), and nuclei were stained with Hoechst 33342 (5 μg/ml final concentration) for 30 minutes. Cellular localization of receptor and arrestin was assessed before (0 minute) and after stimulation with 1 or 10 μM NPY at 37°C for the time indicated by applying identical exposure time and image processing. Quantification of microscopy images was performed by using ImageJ (National Institutes of Health, Bethesda, MD; https://imagej.nih.gov/ij/). To demonstrate arrestin recruitment, line scans were carried out for stacked images from the mCherry (arrestin3)- and eYFP (Y\textsubscript{2}R) channel using the reslice function, and values were normalized. Receptor internalization was quantified by determining the mean fluorescence intensity at the plasma membrane with the segmented line function. Background fluorescence was subtracted. For each condition, at least 12 cells from four independent images were analyzed, and the experiment was repeated twice independently.

**BRET Assays.** BRET assays were performed with transiently transfected HEK293 cells. Cells were seeded in six-well plates and transfected with a gradient of Venus-tagged BRET acceptor at 70% confluence. For arrestin interaction studies, 4000 ng of DNA was transfected per well (100 ng of FluorX-arrestin3, 0–3900 ng of Y\textsubscript{2}R-eYFP), and the total DNA amount was balanced by empty pcDNA3 vector using 3 μl of MetafectenePro (Biontex, Munich, Germany) per microgram DNA. For the investigation of G protein interaction, the total DNA amount was reduced to 1800 ng (200 ng of Y\textsubscript{2}R-Rluc8, 0–1600 ng of Go-Venus). Cells were reseeded onto white and black poly-L-lysine–coated 96-well plates (125,000 cells/well) for measurement of BRET and the direct excitation of Venus to determine expression levels, respectively. Ligand concentration response and kinetic experiments were performed at maximal Venus/Rluc8 ratio (i.e., BRET donor saturation).

BRET was measured at 37°C in Hanks’ balanced salt solution buffer supplemented with 25 mM HEPES (pH 7.4; 100 μl). Cells were preincubated with fresh Coelenterazine h (addition of 50 μl of 16.7 μM stock solution; final solution, 4.2 μM; NanoLight Technology, Pinetop, AZ) for 5 minutes, and stimulated (t = 0 minute) with 50 μl of 4× ligand solution (final volume, 200 μl/well). Fluorescence and luminescence were measured in a plate reader (Infinite M 200; Tecan) at the indicated times by using the filter sets Blue1 (luminescence, 370–480 nm) and Green1 (fluorescence, 520–570 nm). The BRET ratio was calculated as the ratio of fluorescence and luminescence subtracted by signals of donor-only (Rluc8) transfected cells. The netBRET ratio was determined by subtracting BRET signals of unstimulated cells from ligand-stimulated samples. To determine the expression levels of the BRET acceptor (Venus) in saturation BRET assays, fluorescence was measured by direct excitation [Exc 488(9), Em 530(20)] in black plates and divided by basal luminescence of donor-only transfected cells to calculate the acceptor to donor ratio (F/L ratio; x-axis).

Fig. 1. Model of NPY bound to Y\textsubscript{2}R (Kaiser et al., 2015). The peptide ligand (cyan) is accommodated deeply into the transmembrane bundle, with the side chain of Y\textsubscript{2}R (sand) in close proximity to W\textsuperscript{6.48}. The retameric state of W\textsuperscript{6.48} in the overlaid crystal structures of rhodopsin (pdb 1U19, pale green) and neurotensin receptor 1 (4GRV, sand) is given for comparison, and parts of TM4 are shown as a ribbon for clarity.
Data Analysis. Nonlinear regression analysis was performed with GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA). For saturation-binding radioligand experiments, specific binding was fit using the one-site binding isotherm (rectangular hyperbola) for the determination of $K_D$ and $B_{max}$. In displacement binding experiments, each receptor variant was normalized to its own specific binding, and data were analyzed using logistic functions to test for one affinity state (one site $k_{on}$ fit IC$_{50}$) or two affinity states (two sites $k_{on}$ fit IC$_{50}$). Kinetic radioligand-binding data ($k_{off}$) were normalized to specific binding at $t=5$ minute (100%), and fit using an exponential function for a two-phase decay with shared values for both time constants in the presence/absence of GTP$_{gS}$, but individually for each receptor mutant.

Concentration-response curves of signal transduction (second messenger and BRET) experiments were fit with a three-parameter logistic function with fixed Hill slope ($n_H = 1$) to determine EC$_{50}$ and maximal effect ($E_{max}$) values. In addition, concentration-response curves were fit using the operational model of agonism (Black and Leff, 1983) with the built-in function of GraphPad Prism, fixing the $E_{max}$ to the maximal system stimulation and allow for the fit of both the functional affinity constant ($K_A$) and $\tau$, as well as direct calculation of the ratio $\log (\tau/K_A)$. For second messenger assays, $\tau$ also incorporates the receptor number; thus, values were corrected for relative surface expression (Supplemental Fig. 1) to obtain $\tau_c$. Errors of surface expression determination were propagated. Saturation BRET experiments for basal and agonist-stimulated states were fit for one-site total binding to account for a nonspecific component by random collision (bystander BRET), and half-maximal BRET (BRET$_{50}$) as well as maximum BRET values are reported.

Results

Affinity of NPY Binding Is Not Affected by W6.48. Our recent model of NPY bound to the Y2R (Kaiser et al., 2015) has identified a hydrophobic patch in extracellular loop 2 guiding the binding path of the peptide through its amphipathic helix (Fig. 1), and explained the significance of several previously known mutations of peptide and receptor for binding. Most interestingly, we were able to characterize a deep transmembrane binding pocket for the C terminus of the peptide. Central to this pocket are contacts of the C-terminal peptide amide and the side chain of Q34 to Q3.32 in TM3 of the receptor. Furthermore, the positioning of Y36 in a long but narrow binding pocket between TM3, TM6, and TM7 was found. This brings Y36 in a T-shaped configuration relative to W6.48, a highly conserved residue within rhodopsin-like GPCRs that has been suggested to be part of a toggle switch (Schwartz et al., 2006; Holst et al., 2010). The side chain conformation of W6.48 in the Y2R model was fixed to the available

![Fig. 2. Binding properties of WT Y2R and W6.48 mutants. Both saturation (A) and displacement binding (B) displayed WT-like binding properties of W6.48 variants. (C) The receptor displayed two affinity states. High-affinity ligand binding was stabilized by the G protein: upon addition of GTP$_{gS}$ to disrupt the high-affinity R*G(empty) complex, maximal binding was reduced by increasing the portion of the fast phase of $k_{on}$, whereas there was only one $k_{on}$ in the concentration range of the radioligand tested. Accordingly, displacement experiments may be approximated using a two-site model (gray line). (D) This two-phase behavior in the dissociation kinetics indicative of two affinity states was also present in the W6.48 mutants and could be modulated by the addition of GTP$_{gS}$ in an analogous manner. Data represent the mean ± S.E.M. of at least three independent experiments, each performed in duplicate. For clarity, in (B–D) each receptor variant was normalized to its own total (100%) and unspecific (0%) binding. One hundred percent specific binding typically corresponded to about 1 (WT Y2R), 0.6 (W6.48Y), and 0.8 fmol (W6.48H, W6.48T) $^{125}$I-PYY. Numerical data and statistical evaluation can be found in Table 1.](molpharm.aspetjournals.org)
TABLE 1
Binding properties of WT and mutant Y2R
Results of equilibrium as well as kinetic experiments are presented. W6.48 mutants displayed similar binding characteristics compared with WT. A large difference between K_D and i values in quasi-homologous binding experiments suggested the presence of two affinity states, which was confirmed by biphasic properties in k_off experiments. The presence of GTPγS shifted the population of these states, suggesting the high-affinity state to be G protein dependent. All values are given as the mean (95% CI) corrected for multiple comparisons. Significance levels of K_D and logIC50, respectively, relative to WT Y2R were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett’s post-test. Data for percentage of fast phase with or without GTPγS were analysed by two-way ANOVA and compared with the values in the absence of GTPγS by Bonferroni’s post-test.

<table>
<thead>
<tr>
<th></th>
<th>Saturation Binding</th>
<th>Displacement Binding (60 pM 125I-PYY/ NPY)</th>
<th>Dissociation Rate k_off</th>
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<tr>
<td></td>
<td>K_D/pM</td>
<td>Bmax/nmol mg^-1</td>
<td>logIC50</td>
</tr>
<tr>
<td>WT Y2R</td>
<td>50 (7–93)</td>
<td>4329 (2802–5856)</td>
<td>–8.59 (8.74–8.44)</td>
</tr>
<tr>
<td>W6.48T</td>
<td>9 (10–107)</td>
<td>3421 (2173–4669)</td>
<td>–8.62 (8.76–8.48)n.s.</td>
</tr>
</tbody>
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*P < 0.05; **P < 0.01; ***P < 0.001; n.s, not significant.

TABLE 2
Receptor signaling in second messenger assays conducted with either chimeric GαD6qi4myr protein or native G_i.
Mutation of W6.48 mainly altered potency but not efficacy (E_max). Curves were analyzed with the classic logistic function (columns 1 and 2) and the operational model of agonism (columns 3–5). The efficacy term τ was corrected to the surface expression of each construct to retrieve τ_c. The Hill slope was set to unity for both analyses. All values are given as the mean (95% CI) corrected for multiple comparisons. Significance levels of log(τ_c/K_A) relative to WT Y2R were evaluated by one-way analysis of variance followed by Dunnett’s post-test.

<table>
<thead>
<tr>
<th></th>
<th>3H-IP (via GαD6qi4myr)</th>
<th>cAMP (via G_i)</th>
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<tr>
<td></td>
<td>logEC50</td>
<td>E_max%</td>
</tr>
<tr>
<td>WT Y2R</td>
<td>–9.48 (9.22–9.74)</td>
<td>100</td>
</tr>
<tr>
<td>W6.48Y</td>
<td>–8.70 (8.39–9.01)</td>
<td>91 (80–100)</td>
</tr>
<tr>
<td>W6.48H</td>
<td>–7.94 (7.9–8.83)</td>
<td>89 (72–106)</td>
</tr>
<tr>
<td>W6.48T</td>
<td>–8.43 (8.01–8.85)</td>
<td>85 (70–100)</td>
</tr>
</tbody>
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*P < 0.05; **P < 0.01; ***P < 0.001; n.s, not significant.
crystallographic data (Katritch et al., 2013), which all displayed a very similar orientation with the imidazole ring roughly perpendicular to the membrane plane (Fig. 1). Depending on the exact positioning, this would allow for binding contributions of W6.48 by T-shaped π-π interactions and/or a “domino effect” in which the side chain of Y36 slightly displaces W6.48, and thus potentially initiates receptor activation, as for instance has been suggested by comparison of rhodopsin structures in different activity states (Scheerer et al., 2008) and molecular dynamics simulations of adenosine A2A receptor (Li et al., 2013). To define the role of W6.48 for Y2R ligand binding and activation, we mutated this residue and first tested binding affinities.

To our surprise, a conservative mutation of W6.48 to phenylalanine was not tolerated, and caused defective folding and retention in intracellular compartments, most likely in the endoplasmic reticulum, as demonstrated by microscopy of eYFP fusion proteins (Supplemental Fig. 1). Although purely hydrophobic exchanges to leucine, isoleucine, or alanine were also folding deficient, exchange to tyrosine, histidine, and also the small, nonaromatic threonine was accepted (Supplemental Fig. 1) and investigated in more detail.

We performed saturation binding experiments of 125I-PYY at membrane preparations of transiently transfected HEK293 cells. These experiments demonstrated WT-like affinities of circa 50 pmol for all variants, and only a moderate loss of receptor expression level (Bmax: W6.48Y, 60% of WT; W6.48H, 65% of WT; W6.48T, 79% of WT) (Fig. 2A; Table 1). In addition, displacement binding experiments with NPY confirmed unchanged affinities compared with WT receptor (Fig. 2B). Remarkably, half-maximal displacement (i.e., IC50) occurred in the 2–3 nM range, equivalent to Kd values of the NPY competitor of ~1 nM. Since the displacement of 125I-PYY with NPY is quasi-homologous, Kd (NPY) should match Kd (125I-PYY) within experimental error. Alternatively, the two assay setups may reflect two different affinity states. We probed this hypothesis by recording the kinetics of radioligand dissociation, as shown in Fig. 2C. Indeed, radioligand dissociation clearly followed a two-phase behavior with a ratio of the two time constants (koff/slow/koff/fast) of ~50 for all variants (koff/slow = 0.007 min−1; koff/fast = 0.41 min−1 for WT Y2R). Taking into account the association rate constant (kass), which we determined to be kass = 6.4 × 10−4 M−1 min−1 (Fig. 2C), this yields a kinetic Kd value of 11 pM and 641 pM for the high- and low-affinity state of the WT receptor, respectively, matching the observed affinity differences reasonably well. Moreover, close examination of the dissociation experiments reveals some deviation of the curves from the standard steepness, and curves may be approximated better by assuming two affinity states at ~0.5 and ~11 nM (Fig. 2C). We speculated that the high-affinity state might be stabilized by Gβi binding, and thus repeated dissociation experiments in the presence of 100 μM GTPγS, a nonhydrolyzable GTP analog that should disrupt the high-affinity Y2R-Gi complex (no nucleotide bound). Indeed, the presence of GTPγS shifted the kinetics toward the fast phase representing the low-affinity state for the WT Y2 receptor as well as all W6.48 variants (Fig. 2, C and D). Hence, mutagenesis of W6.48 did not affect the affinity of the receptor for its ligand NPY, neither for the high-affinity state supposedly stabilized by G protein nor for the low-affinity state.

**W6.48 Is Critical to Activate Downstream Effectors.** Next, we were interested in how signal transduction of the receptor might be changed by the mutation of W6.48. We first measured the ability of the mutated receptors to elicit the accumulation of a second messenger by using a well-established chimeric G protein (Gα6q/4myr) redirecting the native Gi signaling to the phospholipase C pathway (Kostenis et al., 2005), which has also been applied in previous structure activity and complementary mutagenesis studies (Merten et al., 2007; Kaiser et al., 2015), and allows for robust readout with superb signal-to-noise ratio. The mutation of W6.48 mainly

**Fig. 3.** Signaling of W6.48 mutants in second-messenger assays. In (A), receptor response was redirected to the phospholipase C pathway using a chimeric Gα6q/4myr in transiently transfected COS7 cells. Assays were normalized to maximal NPY stimulation of WT Y2R (100%) and unstimulated cells (0%), and the signal amplitude was dependent on transient transfection and varied between 15,000 and 25,000 dpm (14-fold to 22-fold over basal). (B) Concentration-response curves obtained by a reporter gene assay downstream of the native Gαi in transiently transfected HEK293. Mutation of W6.48 shifted the EC50, but hardly affected the apparent maximal signal. Assays were normalized to the forskolin-induced signal (100%) and full NPY inhibition (0%) of WT Y2R, and the signal amplitude was dependent on transient transfection and varied between 200 and 500 RLU (2-fold to 5-fold over basal). Data represent the mean ± S.E.M. of three independent experiments, each performed in duplicate. Numerical data and statistical evaluation can be found in Table 2.
led to a shift in potency, with 5-fold to 10-fold right-shifted $EC_{50}$ in the order $Y < T = H$, and only slightly reduced $E_{\text{max}}$ (Fig. 3A). Next, the data were analyzed using the operational model of agonism (Black and Leff, 1983). For concentration-response curves with significant reduction of $E_{\text{max}}$ compared with the control curve, this analysis provides information about $\tau$ (efficacy term) and functional affinity $K_A$. However, any response that reaches close enough to the defined as the maximum response can be fit by some combinations of $\tau$ and $K_A$ (Kenakin et al., 2012; Kenakin and Christopoulos, 2013). Results may then depend on the individual errors of each data point and should therefore be taken with caution. Thus, the so-called “transduction coefficient term” $\tau/K_A$ was suggested as a more robust measure of activity (Kenakin et al., 2012; Kenakin and Christopoulos, 2013) for any shape of the concentration-response curve, which, however, leads to the loss of direct information on the proportion to which a decreased activity is split into its $\tau$ and $K_A$ components.

As expected, analysis of the IP accumulation of $Y_2R$ and its $W^{6.48}$ mutants using the operational model resulted in significantly reduced transduction coefficients $\log(\tau/K_A)$ (corrected for surface expression). Due to the excellent signal-to-noise ratio and reproducibility of the assay, the analysis also allowed for some insight into $K_A$ and the efficacy term $\tau$: Although the functional affinity remained largely unaltered, $\tau$ and also its surface expression—corrected equivalent $\tau_e$ (for details, see Materials and Methods) were strongly reduced in the order $Y < T = H$ (Table 2), supportive of a role for $W^{6.48}$ in the activation of the $Y_2R$. In an analogous manner, we analyzed the effects on the native $G_i$ pathway using a CRE reporter gene assay. Again, the mutation of $W^{6.48}$ mainly shifted $EC_{50}$ values (Fig. 3B) in the order $Y < T \leq H$, which translated to reduced $\tau_e$ and transduction coefficients log ($\tau/K_A$) (Table 2).

A third relevant effector for the $Y_2R$ is arrestin3 (Berglund et al., 2003; Kilpatrick et al., 2010; Walther et al., 2010), which is generally suggested to mediate receptor desensitization, and might scaffold further signaling components such as MAP kinases (Lu et al., 2010). Arrestin3 is recruited only to the $Y_2R$ at relatively high agonist concentrations (Kilpatrick et al., 2010) and contributes to receptor internalization (Walther et al., 2010; Lundell et al., 2011). We probed arrestin recruitment by live cell fluorescence microscopy in cells cotransfected with mCherry-arrestin3 and $Y_2R$-eYFP fusion proteins (Fig. 4A). After stimulation with 1 $\mu$M NPY, arrestin3 was robustly relocated from the cytoplasm to the cell membrane for at least 30 minutes. The $W^{6.48S}$ variant also clearly recruited arrestin3 after ligand stimulation. This effect, however, appeared more transient as arrestin3 was distributed and first reading), and then remained stable for at least 15 minutes. Remarkably, none of the $W^{6.48}$ mutations significantly affected preassembly or reduced the maximal ligand-dependent netBRET (Fig. 5A, bars); thus, the mutants were still able to fully activate the $G_i$ pathway at 1 $\mu$M NPY. However, ligand concentration-response curves to evoke netBRET were right shifted in the order $W^{6.48S} < T \leq H$ (Fig. 5B), confirming a weakened propagation of agonist-induced activation.

We observed similar trends also for the chimeric $G_{q4i1}$ (maximum basal BRET: 0.08 vs. 0.19 for WT $Y_2R$ and $W^{6.48S}$ variants, respectively). Such a difference was not present for the

Interestingly, the effects on arrestin3 recruitment revealed differences between $W^{6.48S}$ and $W^{6.48H/T}$.
maximal ligand-induced netBRET (0.04 vs. 0.05), indicating a different initial conformation of the chimeric G protein, which becomes aligned during G protein activation (Table 3). In addition, ligand stimulation went along with improved BRET50 between Y2R and GαD6qi4myr (Table 3), corroborating differences in the interaction. Ligand-induced GαD6qi4myr activation was significantly affected by the mutation of W6.48. The netBRET in response to 1 μM NPY was reduced for all variants, whereas the amount of preassembly remained comparable (Fig. 5C). This can be followed in more detail in ligand concentration-response curves recorded at saturating F/L ratio (Fig. 5D), which were right shifted in the order W6.48Y < T < H. As judged from the EC50 values, GαD6qi4myr is about 10-fold less efficiently activated than WT Y2R and the native ligand NPY. Accordingly, W6.48 mutants shifted even further and were only fully activated at 10 μM NPY, with the exception of W6.48H, where saturation could not be reached even at this very high concentration.

Next, we probed arrestin3 recruitment by BRET. As expected from the microscopy data, arrestin3 was recruited to the receptor only in the presence of ligand (1 μM), and no basal interaction was observed (Fig. 6A; Table 4). The recruitment was slow and reached a stable plateau 10 minutes after stimulation with NPY for the WT, W6.48Y, and W6.48T Y2R. Interestingly, the W6.48Y variant displayed more transient kinetics with a maximum signal after 5 minutes which quickly declined again (Fig. 6C). At their respective signal maxima after 10 and 5 minutes of stimulation, respectively, the W6.48 mutants displayed significantly impaired arrestin3 recruitment with more than 50% decreased netBRET after NPY stimulation, with the W6.48H variant again being most deleterious. Notably, the BRET ratio (and thus possible maximum netBRET) is independent of the receptor expression level as long as measurements are performed at a saturating F/L ratio (Terrillon et al., 2003; Borroto-Escuela et al., 2013), which was assured (F/L > 0.012; i.e., ≥3× BRET50 for any construct). Accordingly, netBRET reductions indicate that the active state cannot be fully populated. This was also reflected in ligand concentration-response curves recorded at saturating F/L ratios (Fig. 6B). All W6.48 mutants displayed a reduced Emax/netBRET value, but differed in their EC50 values. Although W6.48Y displayed an EC50 identical to those of WT Y2R (WT, 76 nM; W6.48Y, 84 nM), W6.48T (EC50, 368 nM), and W6.48H (EC50, 970 nM) required significantly higher ligand concentrations for maximal arrestin3 recruitment.

Designed Ligands Can Restore Arrestin3 Recruitment. We speculated that the impaired signal transduction of the W6.48 variants is likely caused by a sterical component (i.e., the large tryptophan side chain might contribute to the opening of the intracellular effector binding site). Thus, we aimed to compensate the effects of W6.48 mutation by using
NPY ligands with modifications at position 36. To this end, we synthesized Nal36-NPY peptide analogs, which have previously been described to remain active in a G\textsubscript{i} second-messenger readout at WT Y2R (Albertsen et al., 2013). Two different configurations are possible: 1Nal resembles the indole ring conjunction of tryptophan (ortho-meta-substitution at the phenyl ring); whereas 2Nal is extended at the meta-para position (Fig. 7). We tested both peptides for receptor binding, activation of G\textsubscript{ai1}, and chimeric G\textsubscript{ai1}q\textsubscript{4},myr recruitment.

Despite its size, 2Nal36-NPY is accepted very well in the binding pocket and displayed only a very mild decrease in affinity at the WT Y2R and all W6.48 variants (Table 5), underlining the notion of a long binding pocket between TM2 and TM7 (Kaiser et al., 2015). Interestingly, the wider conformation of 1Nal36-NPY distinguished between the W6.48 variants (bar graph). This mechanism was preserved for W6.48 mutants (bar graph). (B and D) Ligand-dependent netBRET increase (recorded at saturating F/L ratio) was right shifted for W6.48 mutants, but E\textsubscript{max} was not reduced (except for W6.48H, where saturation could not be reached up to 10 \textmu M NPY). (E) Saturation BRET experiment for WT Y2R and the control construct G\textsubscript{ai1}q\textsubscript{4},myr-Venus lacking the C-terminal amino acids of G\textsubscript{ai1} (compare with G). Basal BRET was strongly reduced, and no changes in the BRET signal were seen upon agonist stimulation. (F) All Venus-tagged G proteins were localized comparably in the plasma membrane. (G) N- and C-terminal sequences of the G\textsubscript{i} proteins used and sites for post-translational modification. Data points represent the mean ± S.E.M. of at least three independent experiments performed in quadruplicate. Numerical data and statistical evaluation can be found in Table 3.
TABLE 3

Saturation BRET of Y2R-Rluc8 variants with Gαi1 and GαD6qi4myr-Venus complex. NPY stimulation increased total BRET, but a half-maximal signal occurred at similar F/L ratios (BRET50), indicative of structural reorganization rather than additional recruitment upon NPY activation. This mechanism was not sufficient to induce WT-like netBRET of W6.48 mutants with GαD6qi4myr-Venus (compare with EC50 values of activation in Table 5). All values are given as the mean (95% CI) corrected for multiple comparisons. Statistical significance of netBRET changes relative to the WT Y2R was assessed by one-way analysis of variance and Dunnett test.

<table>
<thead>
<tr>
<th></th>
<th>Gαi1</th>
<th>GαD6qi4myr</th>
<th>Unstimulated</th>
<th>Stimulated</th>
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<tbody>
<tr>
<td>W6.48</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W6.48H</td>
<td>0.189 (0.171–0.206)</td>
<td>0.008 (0.004–0.012)</td>
<td>0.245 (0.220–0.271)</td>
<td>0.369 (0.348–0.390)</td>
</tr>
<tr>
<td>W6.48T</td>
<td>0.192 (0.171–0.211)</td>
<td>0.010 (0.006–0.014)</td>
<td>0.156 (0.131–0.180)</td>
<td>0.138 (0.122–0.154)</td>
</tr>
<tr>
<td>W6.48Y</td>
<td>0.170 (0.154–0.186)</td>
<td>0.012 (0.008–0.016)</td>
<td>0.230 (0.204–0.256)</td>
<td>0.194 (0.178–0.210)</td>
</tr>
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</table>

Discussion

Several crystallographic structures have suggested a common binding crevice within the transmembrane core of GPCRs of up to 14 Å in depth (Venkatakrishnan et al., 2013). In many instances, this also involves W6.48 (Venkatakrishnan et al., 2013), suggesting that ligand contacts directly modulate the “transmission switch,” which consists of a cluster of hydrophobic residues (3.40, 5.51, 6.44, and 6.48) (Deupi and Standfuss, 2011; Venkatakrishnan et al., 2013; Tehan et al., 2014). Such a deep binding site is also partly seen in cocrystral instances, this also involves W6.48 (Venkatakrishnan et al., 2013). Based on our data, we present the following model of effector coupling to the Y2R (Fig. 8): the Y2R displays equal affinities to the inactive Gαi1-GDP-heterotrimer in the basal (R) and activated state (LR*), and forms preassembled complexes already in the absence of ligand. Ligand binding causes W6.48 to relocate and catalyzes GDP release accompanied by structural rearrangement of the complex (Alexander et al., 2014; Hamm et al., 2013), enabling GTP binding and activation of the Gα pathway. Arrestin3 recruitment, in contrast, occurs only after receptor activation, in agreement with the accepted models, and
is critically dependent on the opening of the intracellular crevice by the bulky side chain of W6.48. Mutation of this amino acid weakens the allosteric coupling between the ligand and effector binding sites, which attenuates arrestin recruitment. Preassembly of Gi to the receptor, however, is very robust and is not affected by such mutations, implying that the intracellular crevice is still wide enough to accommodate the C-terminal a5-helix of Ga. We suggest that this preassembly and “readiness” of the G protein ensures full activation of the pathway (E_max) despite the weakened allosteric coupling in W6.48 mutants. In this case, the lack of stabilization of the active state requires more frequent ligand-binding events (higher receptor occupancy) to catalyze GDP release, which is reflected in apparently reduced ligand potencies to Gi.

**Binding Characteristics of the Y2R.** Notably, W6.48 functioned as an allosteric connector for receptor activation, but did not significantly contribute to ligand affinity. Investigation of the binding properties of WT and mutant Y2R moreover revealed two affinity states. Ligand binding to the activated R*-G protein complex (nucleotide free) displayed a very high affinity with picomolar dissociation constant, and was confirmed by the sensitivity to GTPγS treatment (Fig. 2C). Ligand binding to the receptor alone or with preassembled G protein (GDP bound), however, displayed a moderate affinity of about 1 nM and represents the relevant state for ligand-induced activation of the receptor in the biologic context. The recognition of two affinity sites might also bring together apparently contradictory findings on Y2R K_D values in the past using 3H-radioligands (0.5–0.7 nM) (Höfliger et al., 2003; Ziemek et al., 2006), and the more sensitive 125I-radionuclides (K_D around 20 pM) (Salaneck et al., 2000; Xu et al., 2013), respectively, and rationalize the discrepancies reported between K_D and K_i values in homologous displacement experiments (Xu et al., 2013), very much analogous to our results.

Intriguingly, the strong positive cooperativity of ligand binding and G protein activation for the Y2R also leads to some deviation from the proposed allosteric mechanism linking ligand-binding and effector-binding pockets (Freissmuth et al., 1991; DeVree et al., 2016). For the β2AR and selected other GPCRs, bound G protein or nanobody reduces the total ligand binding to the complex by the occlusion of the binding pocket. Conversely, uncoupling of the complex by the addition of GTPγS increases k_on values at these receptors, which allows for a greater maximal ligand binding (Freissmuth et al., 1991; DeVree et al., 2016). For the Y2R, however, we (Fig. 2C) and others (Freitag et al., 1995) observed strongly decreased total agonist binding in the uncoupled state (+GTPγS). We attribute this to insufficient ligand affinity in the absence of G protein. However, we found a slowed k_off value of the radioligand from the Y2R-G protein complex that was similar to other GPCRs (DeVree et al., 2016), supporting the concept...
of a stabilized agonist-binding pocket in the GPCR-effector complex.

**Steric and Polar Requirements at Position 6.48 for Activation of the Y2R.** By analyzing different W6.48 mutants and complementary ligands, we were able to gain more insight into the molecular mechanism of activation. The role of W6.48 for the communication of ligand binding contains a steric component as the effects of smaller amino acids at this position (W6.48T/W6.48H) could largely be compensated for by ligands carrying a bulky 1Nal substituent at the C-terminal ligand position. Of note, stimulation with 1Nal36-NPY not only restored the ligand potency for the activation of G\textsubscript{i1} and chimeric G\textsubscript{a6q4myr}, but also increased the maximal netBRET of arrestin3 recruitment almost back to the WT level for the table below.

### Table 4: Saturation BRET of Y2R-eYFP with Rluc8-arrestin3 and kinetic parameters of recruitment

<table>
<thead>
<tr>
<th></th>
<th>BRET\textsubscript{50}</th>
<th>netBRET</th>
<th>K\textsubscript{obs}/min(^{-1})</th>
<th>K\textsubscript{obs}/min(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Y2R</td>
<td>0.0011 (0.0005–0.0017)</td>
<td>0.429 (0.384–0.474)</td>
<td>0.286 (0.210–0.362)</td>
<td>0.292 (0.234–0.351)</td>
</tr>
<tr>
<td>W6.48Y</td>
<td>0.0020 (0.0045–0.0045)</td>
<td>0.241 (0.137–0.345)***</td>
<td>0.618 (0.391–0.845)</td>
<td>0.518 (0.222–0.805)</td>
</tr>
<tr>
<td>W6.48H</td>
<td>0.0044 (0.0004–0.0084)</td>
<td>0.176 (0.100–0.251)***</td>
<td>0.357 (0.233–0.489)</td>
<td>0.323 (0.219–0.427)</td>
</tr>
<tr>
<td>W6.48T</td>
<td>0.0022 (0.007–0.0037)</td>
<td>0.222 (0.161–0.283)***</td>
<td>0.452 (0.258–0.645)</td>
<td>0.391 (0.271–0.511)</td>
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***P < 0.001.

**Fig. 7.** Effects of modifications at position 36 of NPY on the activation/recruitment of G\textsubscript{i1} (left), G\textsubscript{a6q4myr} (middle), and arrestin3 (right). Ligand structures are given on the left. Activation was measured by ligand-dependent BRET at a saturating F/L ratio, and the curve of WT Y2R/NPY for the respective pathway is indicated as a dotted line for comparison. Stimulation with 1Nal36-NPY, but not 2Nal36-NPY, largely compensated for signaling deficits of W6.48 mutants and was able to increase maximum arrestin3 recruitment. Data points represent the mean ± S.E.M. of at least three independent experiments performed in quadruplicate. Numerical data and statistical evaluation can be found in Table 5.
Effects of modifications at position 36 of NPY on the binding affinity and activation of different pathways (Table 5).

Binding affinities were determined in displacement binding experiments comparing different pathways (see Table 1). Activation was measured by ligand-dependent BRET (60 pM²[125I-PYY]) in CHO cell assays and fold increase in BRET (log(BRET/ATP)) over WT % WT log (EC₅₀) was determined. All experiments were performed in triplicate and data are presented as mean ± SD. Differences between means were assessed by one-way ANOVA followed by Tukey’s post-hoc test for each mutant relative to the WT Y₂R treated with the same peptide.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>WT Y₂R</th>
<th>W₆.₄₈H</th>
<th>W₆.₄₈Y</th>
<th>W₆.₄₈T</th>
</tr>
</thead>
<tbody>
<tr>
<td>log(BRET/ATP)</td>
<td>7.15</td>
<td>6.90</td>
<td>6.79</td>
<td>6.75</td>
</tr>
<tr>
<td>EC₅₀ (nM)</td>
<td>100</td>
<td>62.6</td>
<td>74.4</td>
<td>76.1</td>
</tr>
</tbody>
</table>

Conclusions. The present study demonstrates that W₆.₄₈Y and W₆.₄₈T variants (Fig. 7; Table 5). This implies reinforcement of the allostERIC coupling between the receptor and the a₅-helix of G₁, and leads to a wider opening of the intracellular crevice, thus regaining the ability to bind the arrestin finger-loop (referred to as "core conformation") (Shukla et al., 2014).

Our data also provide support for contributions of a specific polar interaction network involved in the regulation of Y₂R activation as suggested for other receptors (Valentin-Hansen et al., 2015; Yuan et al., 2015): 1) nonpolar amino acids were not accepted and led to misfolded receptors that were retained in the endoplasmic reticulum, and, thus, hydrogen-bonding capacities seem to be highly conserved and vital for Y₂R function; and 2) mutation of W₆.₄₈ to the relatively large, aromatic histidine was more deleterious compared with the small threonine. Most likely, these alterations arise from altered hydrogen bonding in the core of the receptor due to a different positioning of the hydrogen bond donor (NH/OH) compared with the original indole side chain of tryptophan.

Y₂R Displays a Strong Inherent Bias for Gᵢ over Arrestin3, Which Is Maintained in W₆.₄₈ Mutants. The preassembly of G₁ to the Y₂R appeared very robust even after mutagenesis of W₆.₄₈, and is an important hallmark of Y₂R-effector interactions. Interestingly, similar data have been obtained by in vitro (Alves et al., 2003, 2005) as well as in BRET studies (Gales et al., 2006; Audet et al., 2008) of other G₁-coupled GPCRs, suggesting that this will emerge as a more common mechanism at least for this class of G proteins that might be facilitated by the subtle shape of the G₁ C terminus (Rose et al., 2014). To evaluate whether preassembly is also reflected in signaling balance, we quantitatively compared the responses of WT and mutant receptors to different pathways. The WT receptor inherently activated G₁ about 1.24 orders of magnitude more efficiently than it recruited arrestin [log(κ²) for G₁ = 8.39; arrestin3 = 7.15] (Table 5). Interestingly, activation of the chimeric G₁₃₉ was also significantly less efficient [log(κ²) for G₁₃₉ = 7.33] compared with the native G₁₃, underlining the differences in the receptor interaction. Of note, preassembly did not per se reduce requirements for G₁ (or G₁₃) activation: comparison of transduction coefficients Δlog(κ²) between WT and mutant for a given pathway demonstrates that mutagenesis of W₆.₄₈ generally resulted in a similar loss of function for G₁ versus arrestin3 interaction (Table 5), i.e., mutation of W₆.₄₈ did not induce signaling bias.

Thus, in general terms, W₆.₄₈ controlled the activation of downstream effectors in a similar manner and did not contribute significantly to the preference for G₁ activation over arrestin3 recruitment. Similarly, introduction of sterically demanding side chains at the C-terminal residue of the peptide did not shift signaling of WT Y₂R to the benefit of arrestin3. Thus, alternative positions should be considered if aiming at the design of arrestin3-prefering ligands at this receptor. Given the highly robust interaction of the Y₂R with inhibitory G proteins and the similar requirements for activation/recruitment; however, the design of such ligands might prove difficult.

Conclusions. The present study demonstrates that W₆.₄₈ functions as an allostERIC connector between the ligand-binding pocket and effector activation at the neuropeptide Y₂ receptor. In agreement with our model of NPY-bound Y₂R (Kaiser et al., 2015), we confirmed that the C terminus of the peptide reaches deeply into the transmembrane core of the
Fig. 8. Suggested mechanism of effector coupling to the Y2R. Inhibitory G proteins have a high affinity to the receptor even in the basal state, and are preassembled. Ligand binding and activation of the receptor relocate W6.48 and stabilize conformations with an opened intracellular crevice, which enables G protein activation and recruitment of arrestin. Mutation of W6.48 to smaller amino acids interferes with this function as a bulky leaver, but also alternative hydrogen bonding in the core of the receptor stabilizes inactive conformations.

Acknowledgments
The authors thank R. Steinborn for help with cloning and initial characterization of tagged chimeric G proteins, and C. Hartig for cloning of the Gα15-β2δ1-αq34 heterotrimer. We also thank C. Damann, K. Löhr, R. Reppich-Sacher, and J. Schweisinger for skilled technical assistance.

Authorship Contributions
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Conducted experiments: Kaiser, Hempel, Wanka, Schubert.
Wrote or contributed to the writing of the manuscript: Kaiser, Hamm, Beck-Sicking er.

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