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Unwinding of the C-terminal Residues of Neuropeptide Y is critical for Y2 Receptor Binding and Activation

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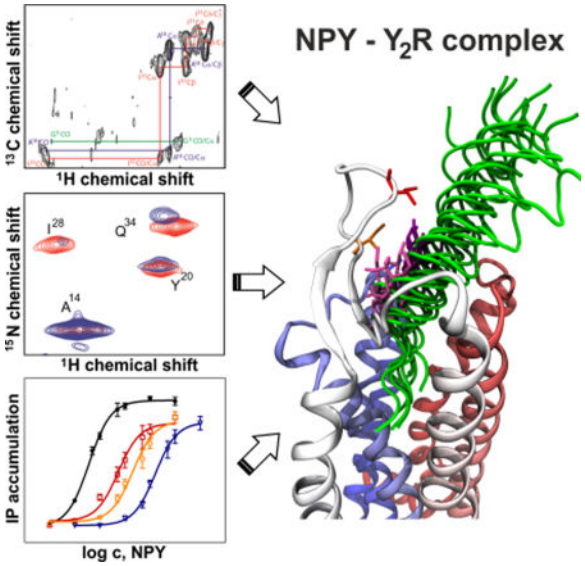
Abstract

Despite recent breakthroughs in G protein-coupled receptor (GPCR) structure characterization, there is only sparse data on how GPCRs recognize larger peptide ligands. Here, we integrate nuclear magnetic resonance (NMR) spectroscopy, molecular modeling, and double-cycle mutagenesis studies to obtain a structural model of the peptide hormone neuropeptide Y (NPY), bound to its human G protein-coupled Y2 receptor (Y_2R). Solid-state NMR measurements of specifically isotope-labeled NPY in complex with in vitro folded Y_2R reconstituted into phospholipid bicelles provide the bioactive structure of the peptide. Guided by solution NMR experiments, we find that the ligand is tethered to the second extracellular loop by hydrophobic contacts. The C-terminal α-helix of NPY, which is formed in membrane environment in the absence of the receptor, is unwound starting at T^{32} to provide optimal contacts in a deep binding pocket within the transmembrane bundle of the Y_2R.

Graphical abstract



Molecular actions of NPY at its Y_2 receptor subtype: The bioactive, receptor-bound structure of NPY is obtained from solid state NMR measurements at Y_2R reconstituted into lipid bicelles, and reveals unwinding of the C-terminal α-helix. Additional solution NMR data, double-cycle mutagenesis and molecular modeling allow a detailed view on peptide-receptor complex

Keywords

GPCR mutagenesis; NMR spectroscopy; NPY; peptide structure; receptors

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The interaction of GPCRs with their natural ligands plays a central role in numerous signal transduction pathways across the cell membrane. For small-molecule ligands including

peptides, a conserved binding cradle to class A (rhodopsin-like) GPCRs was recently proposed.[1] To date, structural models of the smaller GPCR-bound peptide ligands bradykinin,[2] a truncated six amino acid variant of neurotensin,[3] and the fatty acid leukotriene B_4[4] have been determined by NMR spectroscopy.

Here, we report a model of the structure and binding mode of the 36 amino acid, C-terminally amidated NPY bound to the Y_2R. This interaction plays an essential role in the control of food intake and memory retention, and is involved in mood disorders and epilepsy.[5] Receptor binding of NPY is suggested to be a two-step process.[6] According to this model, NPY first binds to the lipid membrane to increase its effective concentration, and is then recognized by the Y_2R.[6]

We applied solid-state and solution NMR spectroscopy to derive a set of structural restraints for molecular modeling and targeted docking, which was complemented by double-cycle mutagenesis to verify NPY-Y_2R interactions. For the NMR measurements, ten NPY variants with $^{15}N/^{13}C$-labeled amino acids in different positions were synthesized, covering in total 30 of the 36 NPY residues (Table S1). Milligram amounts of a cysteine-deficient variant of the Y_2R[7] were prepared from recombinant *E. coli* inclusion body expression and purified in sodium dodecyl sulfate (SDS), as described before.[8] Receptor functionality was achieved in a two-step *in vitro* folding process (see SI for details). Briefly, in a first step the SDS concentration was reduced below its critical micelle concentration by dialysis,[7] and the native disulfide bridge between the two remaining cysteines was formed using glutathione. In the second step, the Y_2R was functionally reconstituted into 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/ 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC-c7) bicelles using heat cycling[9] at Y_2R/DMPC ratios of 1/200 or 1/600. The size of the bicelles was adjusted by varying the q-value (molar DMPC/DHPC-c7 ratio) from isotropically tumbling bicelles ($q < 0.25$) to large membrane structures with little residual detergent ($q > 20$) applicable to solution and solid-state NMR, respectively.[10] Finally, for NMR experiments the reconstituted Y_2R was concentrated by either pelleting (in case of large membrane structure with $q > 20$) or dialyzing against polyethylene glycol 20,000 (in case of small bicelles with $q < 0.25$) to remove water. Functionality of the Y_2R preparations was verified by NPY binding assays (Figure S1), yielding 89 ± 9% functional receptor molecules.

Solid-state NMR ^{13}C-double quantum/single quantum correlation spectra (shown in Figures S2a and S3) for all NPY variants in the Y_2R-bound state were recorded at −30°C to reduce the uniaxial rotational motion of the receptor about the membrane normal.[9] The assigned carbon chemical shifts of Y_2R-bound NPY (listed in Table S2) and the resulting chemical shift indices[11] (Figure 1a) were used to model peptide structures, by comparing predicted chemical shifts from an ensemble of 400,000 *de novo* folded[12] NPY molecules with the experimental data. The ten best scoring models, shown in Figure 1b, surprisingly revealed a C-terminal random coil structure from T^{32} to Y^{36}. This clearly deviates from the NPY structures in solution[13] and in the presence of micelles,[14] where the regular α-helix structure continues up to the amidated C-terminus. However, at very low, physiological concentration, NPY is putatively monomeric in solution and the C-terminus might not be entirely folded in this form.[13]

To reveal NPY residue-specific alterations upon Y_2R binding, two-dimensional ^{1}H-^{15}N HSQC spectra were recorded in the presence of bicelles containing the Y_2R and empty bicelles for all NPY variants (Figure S2b). The weighted chemical shift differences and signal broadening, caused by local altered exchange processes (e.g. exchange processes within the binding pocket) or reduced overall tumbling, are displayed in Figure 2a. Line broadening thresholds of >100 Hz and >300 Hz were arbitrarily chosen to illustrate this effect (line widths are given in Table S2). Significant alterations were observed for the six C-terminal residues, which have been identified to be critical for NPY binding to the Y_2R.[15] Also at the hydrophobic face of NPY's α-helix (L^{17}/A^{18}, Y^{20}, L^{24}, Y^{27}/I^{28}, I^{31}), chemical shift changes and/or signal broadening were detected, suggesting an additional binding site based on hydrophobic contacts. From a Y_2R comparative model, we suspected hydrophobic residues in the extracellular loop 2 (ECL2) as interaction partners, and exchanged them to asparagine (similarly sized, hydrophilic). $I^{4.71}$ and $I^{4.77}$ (Y_2R nomenclature according to[16]) were susceptible to mutation, and double-cycle mutagenesis[17] with modified ligands confirmed direct contacts to L^{24} and I^{28}, as shown in Figures 2b–e. For more details, the reader is referred to the SI and Table S3.

Using these contacts together with the previously described salt bridge between R^{33} of NPY and $D^{6.59}$ on top of trans-membrane helix 6 (TM6)[17] as restraints, the C-terminal part ($NPY^{13–36}$) of the top-scoring NPY models was docked into a comparative model of the Y_2R using ROSETTA (see SI for details). The NPY/Y_2R model with the best agreement to experimental data and NPY structure-activity relationship is depicted in Figure 3. Semi-quantitative energetic analysis of this complex (Figure 4) underlines increasing binding contributions towards the C-terminus of NPY, and is in good qualitative agreement with important receptor positions identified by mutagenesis.[15;17]

The hydrophobic contacts to the ECL2 constrain NPY at an angle of approximately 45° relative to the membrane normal. Taking into account the highly dynamic features of the Y_2R,[9] the position of NPY in the binding pocket is probably not static. Rather, the peptide ligand might follow the motions of the ECL2, constructed simultaneously with NPY docking to account for its high flexibility, resulting in a cone-like distribution with the C-terminal part as receptor-anchoring point and increasing amplitude motions towards the N-terminus (Figure 3a).

It is also tempting to speculate that hydrophobic contacts to the extracellular domains of the receptor might pick up the ligand from the membrane-bound or soluble state, and pre-orient it into the binding pocket. As a consequence of the increasing angle between the NPY α-helix and the membrane surface, important membrane binding residues (L^{17}/Y^{20}/Y^{21})[6] now become exposed to a rather polar environment, supported by the solution NMR data (Figure 2a).

Concomitantly, L^{24}, I^{28}, and the unwound C-terminal pentapeptide change their membrane contacts[6] to form thermodynamically more favorable direct interactions with the Y_2R (Figure 4). Thus, receptor contacts in the binding pocket can be maximized (Figure 3b), as proposed before.[15] Given that the second high-affinity natural ligand of the Y_2R, PYY, shares the same sequence for the C-terminal pentapeptide and prefers a C-terminal extended

structure already in the unbound state,[18] the thermodynamic barrier for such a transition is supposedly rather small.

The first unwinding residue T^{32} is located in a narrow point on top of the binding pocket and could fulfil two important features in the binding process: *(i)* By accepting a hydrogen bond from $Y^{2.64}$, the binding pocket is closed up and NPY is locked into its final binding position, supported by the measured signal broadening of >300 Hz for T^{32} and the neighboring residues. *(ii)* In addition, T^{32} could reduce the thermodynamic cost of helix unwinding in this rather apolar environment by donating a hydrogen bond to the exposed carbonyl-oxygen of N^{29}, thus capping the helix. A similar phenomenon is seen for example in the C-terminal helix of $G\alpha_{i/o}$-proteins, where a cysteine (C^{351} in human $G\alpha_{i1}$) 'catches' the unwinding of the α5 helix upon binding to activated receptor.[19]

The critical importance of R^{33} and R^{35} for NPY activity[5] is also well reflected in our model. While R^{33} makes narrow ionic contacts to $D^{6.59}$,[17] R^{35} is positioned in a mixed acidic-aromatic pocket of $W^{5.26}$ and $Y^{5.38}$ coordinated by $E^{5.24}$, in agreement with earlier studies that highlighted the requirement of aromatic properties at R^{35}[20] and explaining difficulties to identify its interaction partner within the binding pocket.[15]

A particularly important position for NPY activity at the Y_2R is Q^{34}.[5] Due to the deep binding mode of NPY, the side chain of Q^{34} is fairly restricted, and is oriented towards a small polar patch within TM2/3. Our model suggests a prominent interaction with $Q^{3.32}$, which also participates in an extensive hydrogen bond network involving the amidated C-terminus ($CONH_2$). To validate the latter interaction, we created a (slightly) basic interaction partner for the otherwise low affine free acid of NPY (NPY-COOH). As shown in Figure 3c, stimulation with NPY-COOH largely abrogated the potency deficits of $Q^{3.32}H$ compared to wt Y_2R, which likewise occurred upon stimulation with NPY-tyramide,[21] a non-discriminating analog lacking the $CONH_2$ functionality (see SI results for details).

The interaction network involving Q^{34}, $Q^{3.32}$, and C-terminal amide also determines the position of the Y^{36} side chain. Mainly surrounded by the conserved hydrophobic amino acids of the receptor ($C^{2.47}$, $W^{6.48}$, $L^{6.51}$, $M^{7.43}$), it fills a long, narrow pocket in the model. Of special interest is the proximity of Y^{36} to $W^{6.48}$, which has been discussed to act as a toggle switch triggering GPCR activation.[22] More recent investigations also support the hypothesis of direct interactions between $W^{6.48}$ and the ligand,[23] suggesting this to be a more general mechanism of GPCR activation.

In conclusion, we present a detailed structural model of NPY bound to its Y_2 GPCR. NMR measurements revealed NPY to undergo remarkable structural changes within the C-terminus, and the C-terminal pentapeptide takes part in an extensive, but also fragile interaction network. Accordingly, changes in the C-terminal amino acids can easily disturb receptor binding or switch receptor selectivity as observed in numerous earlier structure-activity studies (reviewed in[5]). Moreover, our study indicates that also larger peptide ligands, even though not *a priori* expected to bind deep in the transmembrane bundle, share the proposed common ligand binding cradle of rhodopsin-like GPCRs,[1] thus having more general implications also for other peptide GPCR systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF, Babu MM. Nature. 2013; 494:185–194. [PubMed: 23407534]
2. Lopez JJ, Shukla AK, Reinhart C, Schwalbe H, Michel H, Glaubitz C. Angew.Chem.Int.Ed Engl. 2008; 47:1668–1671. *Angew.Chem.* 2008, *120* 1692. [PubMed: 18236494]
3. Luca S, White JF, Sohal AK, Filippov DV, van Boom JH, Grisshammer R, Baldus M. Proc.Natl.Acad.Sci.U.S.A. 2003; 100:10706–10711. [PubMed: 12960362]
4. Catoire LJ, Damian M, Giusti F, Martin A, van HC, Popot JL, Guittet E, Baneres JL. J.Am.Chem.Soc. 2010; 132:9049–9057. [PubMed: 20552979]
5. Pedragosa-Badia X, Stichel J, Beck-Sickinger AG. Front Endocrinol.(Lausanne.). 2013:4–5. [PubMed: 23420531]
6. Bader R, Zerbe O. Chembiochem. 2005; 6:1520–1534. [PubMed: 16038001]
7. Witte K, Kaiser A, Schmidt P, Splith V, Thomas L, Berndt S, Huster D, Beck-Sickinger AG. Biol.Chem. 2013; 394:1045–1056. [PubMed: 23732681]
8. Schmidt P, Berger C, Scheidt HA, Berndt S, Bunge A, Beck-Sickinger AG, Huster D. Biophys.Chem. 2010; 150:29–36. [PubMed: 20421142]
9. Schmidt P, Thomas L, Muller P, Scheidt HA, Huster D. Chemistry. 2014; 20:4986–4992. [PubMed: 24623336]
10. Son WS, Park SH, Nothnagel HJ, Lu GJ, Wang Y, Zhang H, Cook GA, Howell SC, Opella SJ. J.Magn Reson. 2012; 214:111–118. [PubMed: 22079194]
11. Spera S, Bax A. J.Am.Chem.Soc. 1991; 113:5490–5492.
12. Kim DE, Chivian D, Baker D. Nucleic Acids Res. 2004; 32:W526–W531. [PubMed: 15215442]
13. Monks SA, Karagianis G, Howlett GJ, Norton RS. J.Biomol.NMR. 1996; 8:379–390. [PubMed: 9008359]
14. Bader R, Bettio A, Beck-Sickinger AG, Zerbe O. J.Mol.Biol. 2001; 305:307–329. [PubMed: 11124908]
15. Xu B, Fallmar H, Boukharta L, Pruner J, Lundell I, Mohell N, Gutierrez-de-Teran H, Aqvist J, Larhammar D. Biochemistry. 2013; 52:7987–7998. [PubMed: 24111902]
16. Ballesteros, JA., Weinstein, H. Methods in Neurosciences. Vol. 25. Academic Press; 1995. p. 366-428.
17. Merten N, Lindner D, Rabe N, Rompler H, Morl K, Schoneberg T, Beck-Sickinger AG. J.Biol.Chem. 2007; 282:7543–7551. [PubMed: 17204471]
18. Lerch M, Mayrhofer M, Zerbe O. J.Mol.Biol. 2004; 339:1153–1168. [PubMed: 15178255]
19. Alexander NS, Preininger AM, Kaya AI, Stein RA, Hamm HE, Meiler J. Nat.Struct.Mol.Biol. 2014; 21:56–63. [PubMed: 24292645]
20. Albertsen L, Ostergaard S, Paulsson JF, Norrild JC, Stromgaard K. ChemMedChem. 2013; 8:1505–13. 1422. [PubMed: 23907926]
21. Hoffmann S, Rist B, Videnov G, Jung G, Beck-Sickinger AG. Regul.Pept. 1996; 65:61–70. [PubMed: 8876037]
22. Park JH, Scheerer P, Hofmann KP, Choe HW, Ernst OP. Nature. 2008; 454:183–187. [PubMed: 18563085]

23. Stoddart LA, Kellam B, Briddon SJ, Hill SJ. Br.J.Pharmacol. 2014; 171:3827–3844. [PubMed: 24750014]

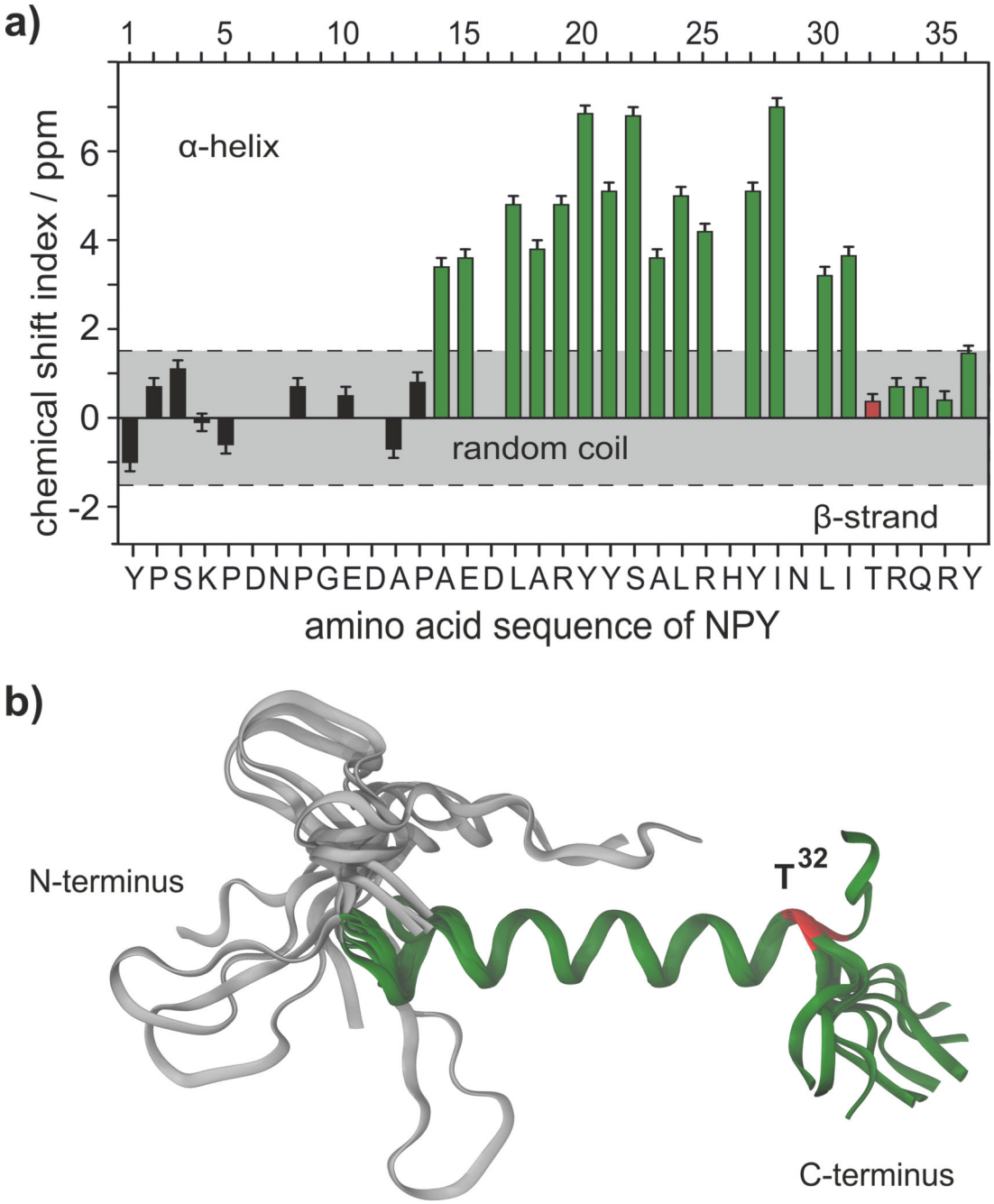

Figure 1.

a)^{13}C chemical shift index of Y_2R-bound NPY (measured ($C_{\pm}$-C?? - random coil ($C_{\pm}$-C?)). b) Superposition of the best ten scoring NPY models derived from solid-state NMR restraints. The N-terminus (gray) was excluded from scoring. The C-terminal α-helical structure of NPY (green) unwinds starting at T^{32} (highlighted in red) upon receptor binding.

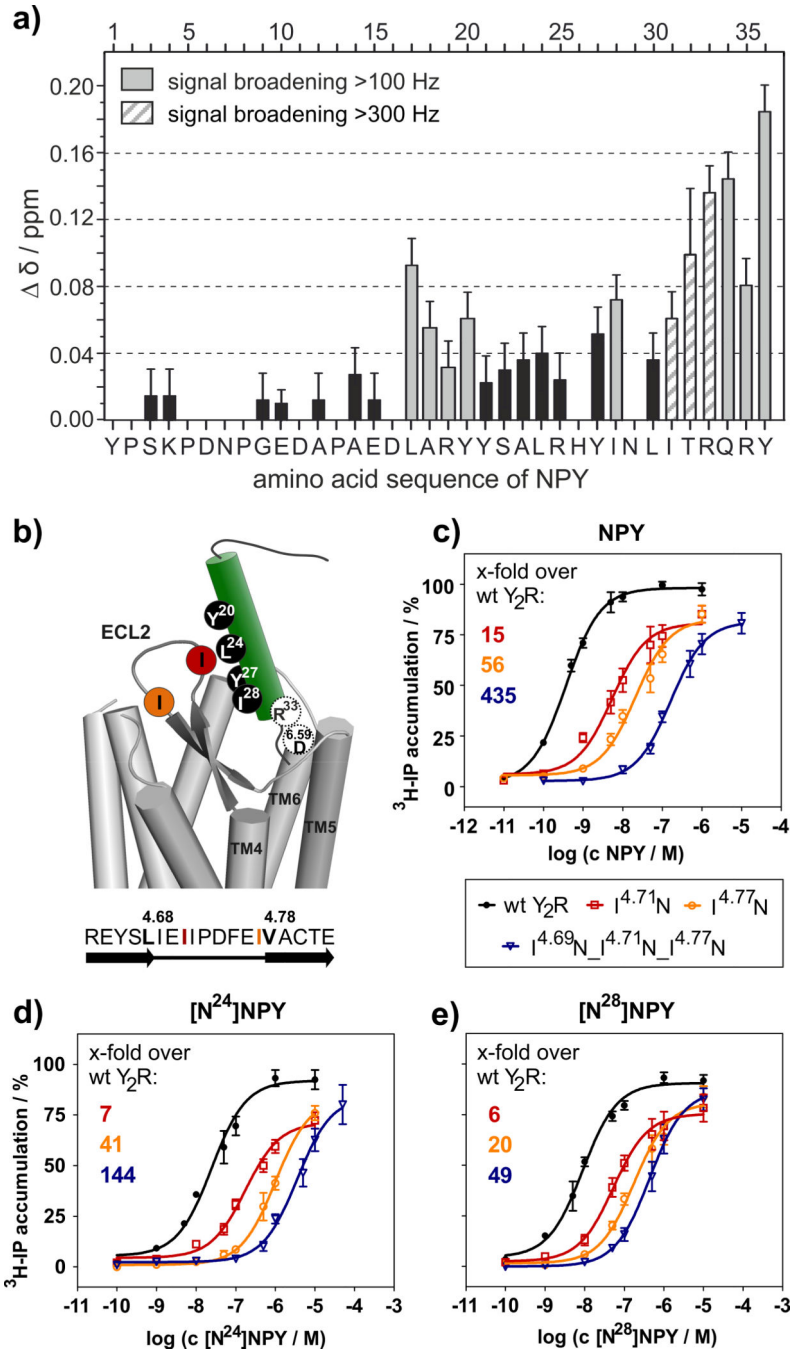

Figure 2.

a) Weighted chemical shift changes ($\Delta\delta$ $[(\Delta\delta^{1}H)^2 + (0.2\ \Delta\delta^{15}N)^2]^{1/2}$) for membrane and receptor-bound NPY, and ^{1}H NMR signal broadening upon binding of >100 Hz (gray) and >300 Hz (shaded) obtained from HSQC solution NMR is shown. b) Schematic representation of initial docking of NPY (green) into a hydrophobic groove of Y_2R. c–e) Double-cycle mutagenesis to identify the interacting residues between ECL2 and NPY. Y_2R mutants $I^{4.71}N$ (red), $I^{4.77}N$ (orange) and combination variant $I^{4.69}N\_^{4.71}N\_^{4.77}N$ (blue) were tested against $[N^{24/28}]$NPY variants to identify the interacting residues. Numbers in the

upper left represent EC_{50} shifts relative to wt Y_2R curve, reduced shifts indicate direct interaction of receptor and peptide at the modified positions.

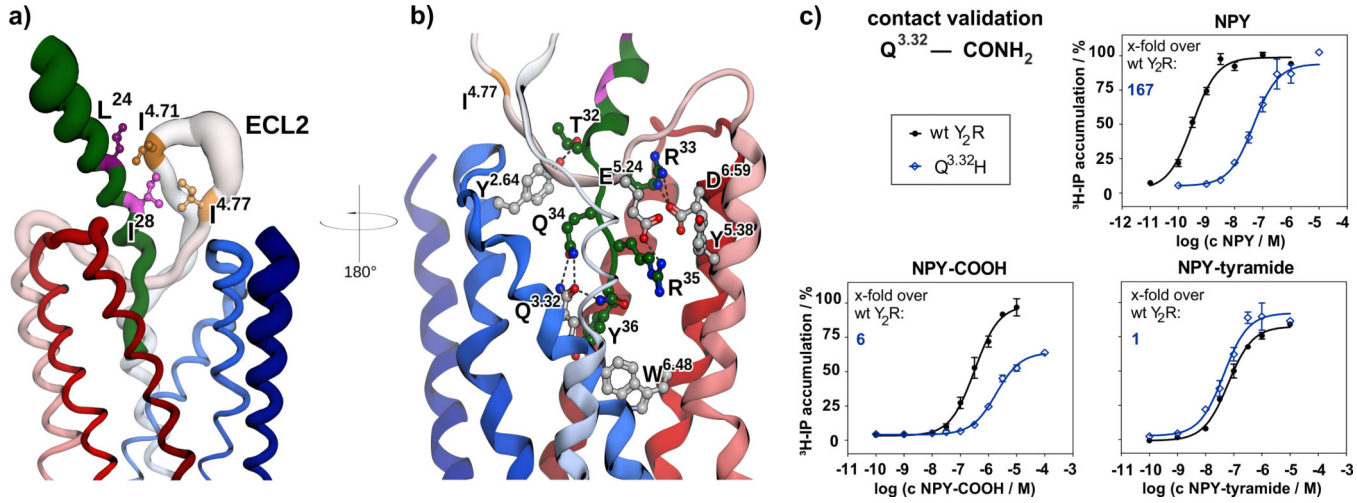

Figure 3.

a) Model of NPY (green) docked into Y_2R (N- to C-terminus in blue to red). Structural diversity of the ensemble is indicated by thickness of ribbons. NPY is tethered to ECL2 via interaction of L^{24} (purple) and I^{28} (pink) to $I^{4.71}$ (red) and $I^{4.77}$ (orange). b) Representative view of NPY's C-terminus. Polar interactions are indicated by dashed lines. c) The interaction of $Q^{3.32}$ (light blue) to NPY's C-terminal amidation was verified by double-cycle mutagenesis by creating a slightly basic interaction partner for the free acid form of NPY, or a non-discriminating analog completely lacking the C-terminal $CONH_2$ (NPY-tyramide).

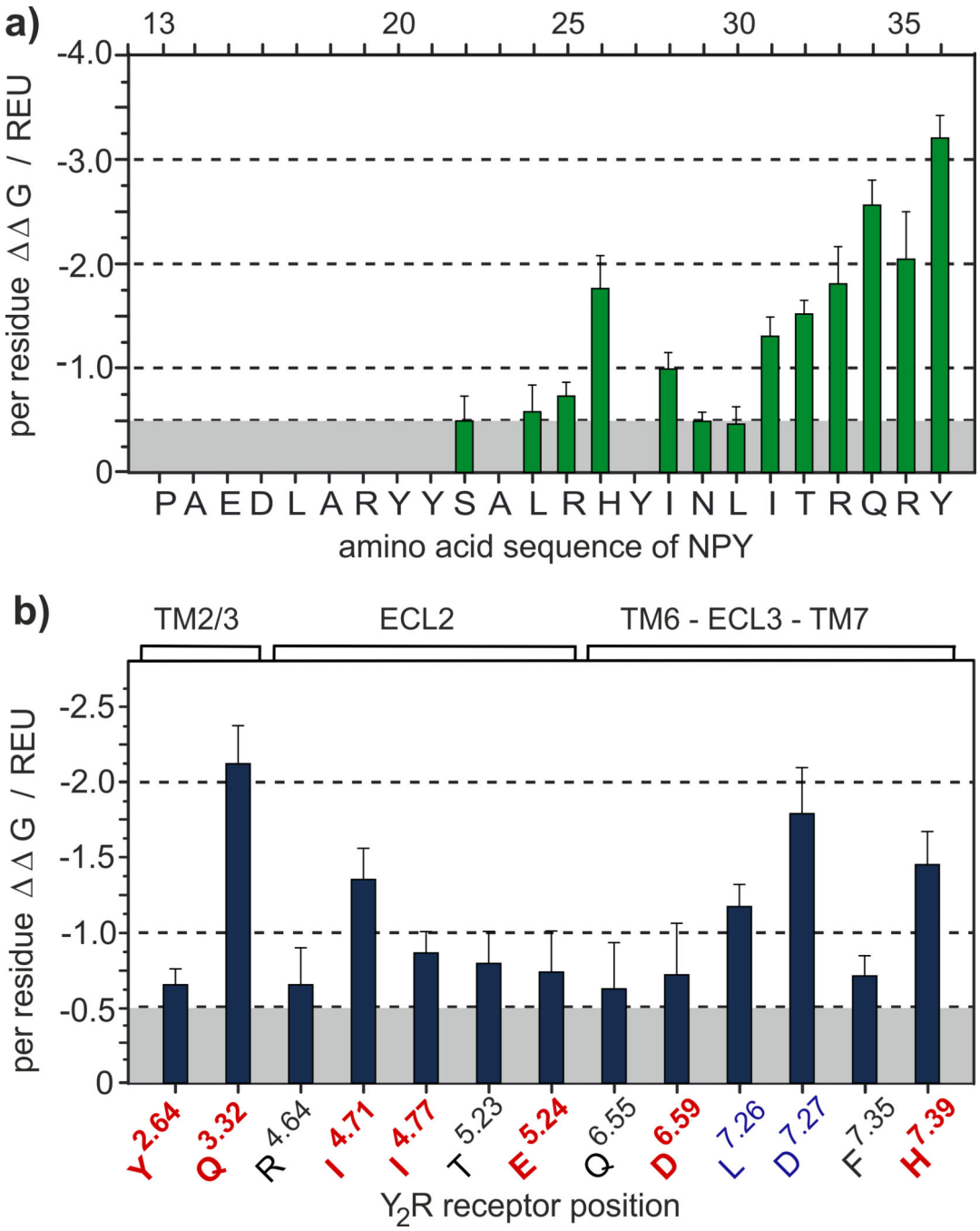

Figure 4.

Energetic analysis of $NPY^{13–36}$-Y_2R complex (see Figure 3b). a) Contribution of NPY residues to binding energy increases towards the C-terminus, in agreement with solution NMR (Figure 2a). b) For the Y_2R, significant binding energy is conveyed by 13 residues, mostly positions identified to be critical (red) in earlier[15;17] and the present study. $L^{7.26}$ and $D^{7.27}$ (blue) are false-positives triggered by supposed ionic contact of R^{25}/H^{26} with $D^{7.27}$ in

many low-energy models, but are not sensitive to mutagenesis. Significance threshold was 0.5 ROSETTA energy units, REU (gray background).