Mutational Analysis of Neuropeptide Y Reveals Unusual Thermal Stability Linked to Higher-Order Self-Association

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Supporting Information

ABSTRACT: Neuropeptide Y (NPY) is a 36-residue peptide, abundant in the central and peripheral nervous system. The peptide interacts with membrane-bound receptors to control processes such as food intake, vasoconstriction, and memory retention. The N-terminal polyproline sequence of NPY folds back onto a C-terminal α-helix to form a hairpin structure. The hairpin undergoes transient unfolding to allow the monomer to interact with its target membranes and receptors and to form reversible dimers in solution. Using computational, functional, and biophysical approaches, we characterized the role of two conserved tyrosines (Y20 and Y27) located within the hydrophobic core of the hairpin fold. Successive mutation of the tyrosines to more hydrophobic phenylalanines increased the thermal stability of NPY and reduced functional activity, consistent with computational studies predicting a more stable hairpin structure. However, mutant stability was high relative to wild-type: melting temperatures increased by approximately 20 °C for the single mutants (Y20F and Y27F) and by 30 °C for the double mutant (Y20F + Y27F). These findings suggested that the mutations were not just simply enhancing hairpin structure stability, but might also be driving self-association to dimer. Using analytical ultracentrifugation, we determined that the mutations indeed increased self-association, but shifted the equilibrium toward hexamer-like species. Notably, these latter species were not unique to the NPY mutants, but were found to preexist at low levels in the wild-type population. Collectively, the findings indicate that NPY self-association is more complex than previously recognized and that the ensemble of NPY quaternary states is tunable by modulating hairpin hydrophobicity.

INTRODUCTION

Neuropeptide Y (NPY) is a 36-residue, amidated peptide found in abundance in the central and peripheral nervous systems. NPY interacts with membrane-bound, G-protein-coupled receptors (GPCRs) to control regulation of food intake, vasoconstriction, memory retention, sleep regulation, and energy homeostasis. As such, GPCRs have been identified as potential drug targets for treating diseases such as epilepsy, obesity, neurodegenerative, and psychiatric disorders. Similarly, the NPY peptide has served as a platform for the development of various agonists and antagonists toward its receptors.

As shown in Figure 1, NPY is characterized by an N-terminal polyproline II helix (residues 1–10), a short β turn (residues 11–13), and a C-terminal α-helix (residues 14–31). Residues 32 through 36 are unstructured based on an inspection of all of the NMR and X-ray crystal structures available in the Protein Data Bank. Also seen is the pancreatic polypeptide fold found in NPY and its homologs, in which the N-terminal residues fold back onto the C-terminal α-helix to form a hairpin. Because of the dynamical properties of the N-terminal sequence, the hairpin undergoes transient unfolding, allowing NPY to bind to cell surface membranes and to its receptors. Transient unfolding is also coupled to reversible dimerization of NPY, which occurs with micromolar affinity and via the parallel or antiparallel orientation of α-helices from each protomer. Although the role of NPY dimerization is not entirely clear, it may serve to regulate the concentration of the functionally active monomer species. To better understand the role of individual residues in the structural stability, self-association, and function of NPY, we carried out a mutational analysis of two conserved tyrosines located within the hairpin core (Y20 and Y27; see Figure 1).

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The core is hydrophobic in character, in part via conserved prolines 2, 5, and 8.14 The tyrosines are known to contribute to stability of the hairpin,15 and inspection of the three-dimensional structure shows that they make contacts with prolines 5 and 8. However, their contribution to stability would seem to be modest relative to a more hydrophobic residue, such as phenylalanine. This could suggest that Y20 and Y27 play a regulatory role in the stability of the hairpin, resulting in modulation of NPY dimerization, membrane interactions, or signaling with GPCRs. Thus, we initially hypothesized that substitution of the two tyrosines with phenylalanine should lead to an increase in NPY hairpin stability and therefore a decrease in functional activity, specifically by increasing the hydrophobicity of the core to allow more optimal packing.

Using computational and experimental approaches, we examined the consequences of mutating Y20 and Y27 to phenylalanine. We found that NPY thermal stability was indeed increased and function was reduced, consistent with molecular dynamics simulations that predicted a more stable hairpin. However, the increase in thermal stability of the mutants was unusually high relative to wild-type: melting temperatures increased by roughly 20 °C for Y20F and Y27F, and by 30 °C for the double mutant, Y20F + Y27F. These findings suggested that the mutations were not just simply enhancing the stability of the hairpin, but might also be driving higher-order self-association. Using analytical ultracentrifugation, we determined that the mutations indeed increased self-association, but shifted the equilibrium toward hexamer-like species. Notably, these findings suggested that the local structure of the double mutant is calculated to be more stable than either of the single mutants, which in turn are more stable than wild-type peptide. In the region of PMFs between 30 and 70 Å, the clearest trend is the similarity between the Y20F and Y27F curves relative to WT and Y20F + Y27F, suggesting that the location of the individual mutations does not influence hairpin stability. Finally, from 70 to 80 Å, the PMFs begin to increase significantly, and this is the point at which the hydrophobic pocket of the hairpin begins to open. The wild-type pocket opens at approximately 70 Å, the single-mutant pockets open at 75 Å, and the double-mutant pocket opens at 80 Å. Representative structures associated with the PMF curves over these distances for each peptide are shown in Figure 2B.

To probe the origins of the stability differences observed among the peptides, we next carried out an analysis of interactions between hydrophobic core residues and solvent. Plotted in Figure 3A is the interaction energy between the hydrophobic residues of each peptide and water, over a range of pull distances. This was determined using the weighted averaging scheme for calculating the residue pair interaction energies and hydrogen-bonding patterns (see Materials and Methods). Each curve represents the results of the weighted average of all of the trajectories. The region marked by ree between 60 and 80 Å represents the range of end-to-end distances at which the hydrophobic pocket opens. In each case, after the pocket opens, the interaction of amino acid residues and backbone moieties in the pocket with water increases substantially: the more stable the peptide, the more shifted is the increase in interaction energy and hence opening of the pocket. For example, the wild-type pocket opens at ree approximately equal to 60 Å, whereas the single mutants open at approximately 75 Å. Finally, the double mutant exhibits the most shifted opening of the pocket at an ree of 80 Å. Taken together with the results of the PMF calculations (Figure 2A), the results reveal a clear peptide-specific trend for the opening of the hydrophobic pocket, and therefore peptide stability, as follows: wild-type < Y20F/Y27F < Y20F + Y27F.

In addition to hydrophobic interactions, differences in hydrogen-bonding patterns (either intrapeptide or with solvent) may also play a role in influencing stability among the peptides. Shown in Figure 3B is the average number of intrapeptide hydrogen bonds formed and broken over the course of mechanical unfolding for each peptide. Prior to unfolding, the peptides exhibit a similar trend in the number of hydrogen bond contacts, with the initial structures containing 5–10 bonds. Between the regions of 20 and 60 Å, the average number of intrapeptide bonds increases to a range of 20–25. The wild-type, Y20F, and Y27F peptides all have a maximum of approximately 20 bonds at an ree of 70 Å. Y20F + Y27F forms the most contacts at an ree of 80 Å, after which the number of bonds formed begins to decrease significantly. This ordering in the onset of the maximum number of hydrogen bond contacts coincides with the trend in the opening of the hydrophobic pocket presented in Figure 3A. Finally, we note that the reorganization of intrapeptide hydrogen bonds under the strain
Figure 2. PMF calculations and representative structures for wild-type NPY and mutants. (A) PMFs obtained for all 4 peptides using a sampling size of 100 trajectories per stage at a pulling velocity of 10 Å/ns; wild-type NPY (black), Y20F (blue), Y27F (green), and Y20F + Y27F (red). Only the first 70 Å of the pull are shown for clarity. (B) Representative structures for each peptide at 20, 40, and 60 Å.
is consistent with $i \rightarrow i + 4$ ($\alpha$-helical) bonding patterns observed for model helical peptides.\textsuperscript{16}

To further investigate the role of hydrogen-bonding patterns, hydrogen bonds formed between the peptide and the explicit water solvent were examined. As shown in Figure 3C, the wild-type and double mutant both begin with a slightly elevated number of hydrogen bond contacts with solvent at 85 contacts. The single mutants both begin with 80 contacts. For each peptide, the number of bonds formed to solvent remains constant over the first half of the unfolding process. The number of bonds begins to increase at an $r_{co}$ of 80 Å and peaks at 110 bonds at an $r_{co}$ of 110 Å. The number of bonds remains constant at 110 for the remainder of the reaction coordinate. Thus, the overall solvation of all of the mutants is similar to that of the wild-type at the maximum extension. This suggests that all four open chains have similar entropic contributions, which are consequently not the drivers of the peptide-specific trends observed experimentally.

**Spectroscopic Analyses Confirm That NPY Mutants Have Increased Thermal Stability.** To experimentally determine if mutations at Y20 and Y27 influenced overall peptide stability, we carried out melting studies using circular dichroism (CD) spectroscopy. Far-UV CD spectra are shown in Figure 4A. For all peptides, the spectrum is predominantly $\alpha$-helical in character, exhibiting minima at 208 and 222 nm. Helicity is presumably arising from C-terminal residues 14–31 and is consistent with the NMR structure in Figure 1. Interestingly, the Y27F peptide shows evidence of additional helicity, as evidenced by a larger mean molar ellipticity at 222 nm. This is possibly due to the induced structure of residues 32–34 at the C-terminus.\textsuperscript{17}

Shown in Figure 4B are thermal unfolding curves for wild-type NPY and the three mutants, collected at 222 nm. Wild-type unfolding is characterized by a broad transition from 15 to 65 °C and with a melting temperature ($T_m$) of 38.6 ± 0.4 °C. By contrast, Y20F and Y27F have the same $T_m$ value of 58 ± 0.3 °C and Y20F + Y27F has a $T_m$ of 68.9 ± 0.5 °C. Interestingly, the thermal transitions for at least the Y20F and Y20F + Y27F mutants show evidence of multiple transitions, suggesting that difference in their $T_m$ values is due to more than just differences in hairpin stability.

**NPY Mutants Have Reduced Affinity toward Membranes and GPCRs.** The above results indicate that both computationally and experimentally, mutations at Y20 and Y27 have a significant impact on hairpin and peptide stability. To determine the functional impact of these mutations, we measured the affinity of the peptides toward lipid membrane vesicles and the ability of the peptides to displace wild-type peptide from GPCRs. Surface plasmon resonance (SPR) was used for measuring NPY–membrane interactions, and a competition–displacement assay was used to measure NPY interactions with GPCRs.

Representative SPR data for interactions of each of the peptides with unilamellar vesicles are shown in Figure 5A, and the observed kinetics and apparent affinities of the peptides toward the vesicles are summarized in Table 1. Following the trends seen for the thermal stability measurements, all mutants show weaker affinities relative to wild-type, with the double mutant having the weakest affinity. With regard to the kinetics of the interactions, we find that the observed dissociation rate constants are similar, in the range of $(1.1–1.5) \times 10^{-2}$ s$^{-1}$, indicating that differences in NPY–membrane binding affinity
are driven by changes in the association rate constant (Table 1).

Shown in Figure 5B are competition–displacement curves for measuring NPY interactions with rat brain GPCRs. This assay measures the ability of each peptide to displace wild-type, fluorescently labeled NPY from its receptors. It is evident that all mutants displace bound peptide, but less effectively so compared to wild-type. Once again, the trend in peptide displacement mirrors the trend seen in the thermal unfolding studies (and the lipid binding experiments). The calculated IC\textsubscript{50} values for the displacement reaction are shown in Table 2. The IC\textsubscript{50} values in the nanomolar range are comparable to previously reported affinities for NPY–GPCR binding\textsuperscript{18,19} thus indicative of receptor-specific interactions.

NPY Mutants Perturb a Preexisting Equilibrium of Monomers, Dimers, and Higher-Order Species. The above results indicate that the mutations at Y20 and Y27 enhance NPY hairpin stability (as shown by the simulations) and overall peptide stability (that is, the unfolding of both the hairpin and the C-terminal α-helix, as shown by the CD thermal melt experiments). The thermal melt experiments measure overall peptide stability, but cannot partition that stability at the mechanistic level that the simulations reveal. Taken together, however, the simulations and melts indicate that the phenylalanine substitutions stabilize the hairpin (e.g., polyproline fold) and contribute to the stability of the α-helix. The changes in stability also reduce functional interactions with membranes (SPR experiments, Figure 5A) and receptors (receptor competition assay, Figure 5B). These findings are in line with our initial hypothesis that substitution of the tyrosines with more hydrophobic phenylalanines should lead to more efficient packing of the hydrophobic core. However, we found that the increases in thermal stability were unusually large, for example, model systems, such as lysozyme, reveal that single amino acid substitutions increase T\textsubscript{m} by no more than 5°C\textsuperscript{20}. Moreover, the melting curves for at least a subset of peptides showed multiple transitions, suggesting that other factors might be in play. Noting that NPY reversibly dimerizes

![Figure 4. CD analysis of NPY thermal stability. (A) Far-UV CD spectra of NPY and NPY mutants. All peptides were at 20 μM in 15 mM NaPO\textsubscript{4} (pH 7.0); wild-type NPY (closed circles), Y20F (closed squares), Y27F (open squares), and Y20F + Y27F (open circles). The data presented represent the average of three independent experiments, all of which were superimposable to within ±0.75 deg cm\textsuperscript{2}/dmol at a given wavelength. (B) Thermal denaturation of NPY and NPY mutants as measured by CD spectroscopy at 222 nm. Mean molar ellipticity values are converted to fraction unfolded peptide as described in Materials and Methods section; wild-type NPY (closed circles), Y20F (closed squares), Y27F (open squares), and Y20F + Y27F (open circles).]
via its hydrophobic core, we speculated that the unusual thermal stabilities might also be linked to higher-order self-association. The self-association properties of wild-type NPY and the three mutants were therefore assessed via sedimentation velocity and sedimentation equilibrium.

The sedimentation coefficient distribution of wild-type NPY collected over a 30-fold concentration range is shown in Figure 6A. At 10 and 30 μM, NPY sediments as a single, concentration-independent peak with a sedimentation coefficient of 4.3 (0.4) M−1 s−1.

Table 1. Average NPY−1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC)/1,2-Dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DMPG) (4:1 w/w) Membrane Interaction Rate Constants and Equilibrium Constants Determined by SPR

| peptide  | kₐ (M⁻¹ s⁻¹) | k₅ (s⁻¹) | Kₐ (M⁻¹) | χ²  |
|----------|---------------|-----------|-----------|-----|
| WT       | 1.2 × 10³ (110) | 1.1 × 10⁻² (0.003) | 1.1 × 10⁵ (2.1 × 10⁴) | 4.3 (0.4) |
| Y20F     | 8.5 × 10² (22) | 1.5 × 10⁻² (0.004) | 5.6 × 10⁴ (1.2 × 10⁴) | 6.7 (0.5) |
| Y27F     | 6.7 × 10² (31) | 1.4 × 10⁻² (0.002) | 4.8 × 10⁴ (1.3 × 10⁴) | 5.4 (0.5) |
| Y20F + Y27F | 2.7 × 10² (16) | 1.3 × 10⁻² (0.002) | 2.1 × 10⁴ (2.3 × 10³) | 8.2 (0.7) |

Values in parentheses represent the standard deviation. Average of three trials.

Table 2. Half-Maximal Inhibitory Concentration (IC₅₀) for Wild-Type NPY Receptor Displacement

| peptide  | IC₅₀ (nM) |
|----------|-----------|
| WT       | 1.9 ± 0.5 |
| Y20F     | 4.6 ± 0.2 |
| Y27F     | 7.6 ± 0.3 |
| Y20F + Y27F | 14.2 ± 0.2 |

Values in parentheses represent the standard deviation. Average of three experiments ± standard deviation.
ficient of 0.80 S and an estimated molecular weight of 4.3 kDa. Noting that the calculated molecular weight of the NPY monomer is 4.27 kDa and that bead modeling predicts that the folded monomer in Figure 1A should have a sedimentation coefficient of 0.74 S, this peak is suggestive of monomeric NPY. As peptide concentration is increased to 100 μM, the distribution shifts rightward, with a second peak appearing at ~1.5 S. This concentration-dependent change in the distribution is indicative of a reversibly interacting system, although the second peak cannot be assigned a specific stoichiometry. Nonetheless, the results are qualitatively consistent with previous reports indicating that NPY reversibly dimerizes in the micromolar range. Unexpectedly, however, as peptide concentration is increased to 300 μM, the second peak continues to shift and broaden to ~2.5 S. Again, it is not possible to assign specific stoichiometries to this peak or within the distribution. However, for perspective, a spherical shape approximation for an NPY dimer predicts that it sediments at ca. 1.2–1.3 S. Thus, overall, the results suggest that NPY is self-
associating past dimer. We address this possibility in more detail using sedimentation equilibrium studies below.

To determine the affinities and stoichiometry of assembly for wild-type NPY, we carried out sedimentation equilibrium studies under buffer conditions identical to the sedimentation velocity experiments using multiple rotor speeds and a range of protein concentrations. The data were then globally fit to various self-association models, with the molecular weight of the monomer fixed at the known value of 4272.72 Da. On the basis of previous studies and the results in Figure 6A, we tested a variety of interaction models, including monomer–dimer, monomer–trimer, monomer–dimer–tetramer, etc. Only two models described the data to an acceptable level as determined by the RMSD of the fit: these were monomer–dimer–hexamer and monomer–trimer–hexamer. Shown in Figure 6B is a global fit to the monomer–dimer–hexamer model. This fit resolved a monomer–dimer association constant of 3620 (1740, 7576) M\(^{-1}\), corresponding to a \(K_d\) of \(\sim 123\) \(\mu\)M on a per dimer basis. The RMSD of the fit was 6.78 \(\times\) \(10^{-3}\) absorbance units. A global fit to a monomer–trimer–hexamer model resolved a comparable RMSD of 6.26 \(\times\) \(10^{-3}\) absorbance units (not shown).

By contrast, a simple monomer–dimer interaction model did not describe the data well, generating obvious systematic residuals and an increased RMSD of 1.59 \(\times\) \(10^{-2}\) (see Figure S1, Supporting Information). Other models generated similarly poor fits or resolved nonsensical interaction parameters. Although additional studies will be necessary to determine the precise stoichiometries and affinities of NPY self-association, both the sedimentation velocity and sedimentation equilibrium results indicate that wild-type NPY reversibly assembles to species greater than dimer.

To assess whether the Y20 and Y27 mutations influence the distribution seen for wild-type NPY, we carried out sedimentation velocity studies on each of the mutants (Figure 7). As seen for Y20F, a broad distribution of species, ranging from monomer to dimer to hexamer, was observed. The sedimentation velocity experiments for Y27F and Y20F + Y27F were consistent with these findings.
from ~0.8 to nearly 3 S, is already present even at the lowest peptide concentration. As concentration is increased, a 3 S species eventually dominates the distribution. This is again indicative of an interacting system, but based on our interpretation for wild-type NPY, one that also associates well past dimer and with greater affinity. A similar result is seen for Y27F, with differences in the distributions likely arising from differences in the kinetics and/or energetics of self-association.22 Finally, Y20F + Y27F exhibits the greatest enhancement in self-association, to the point that only a single, rapidly sedimenting peak at ~2.3 S is observed at nearly all NPY concentrations. This concentration-independent peak has an estimated molecular weight of 25.2 kD and is thus suggestive of hexamer. Therefore, the introduction of phenylalanines at Y20 and Y27 results in a redistribution of a complex and preexisting equilibrium to higher-order assembly states.

**DISCUSSION**

**Computational Insight into NPY Hairpin Stability.** Previous work by Hernandez and co-workers used ASMD to examine unfolding pathways for the hairpin structure of wild-type NPY.24 Here, we employ this simulation approach to determine how Y20F, Y27F, and Y20F + Y27F influence hairpin unfolding and stability. The results suggest that there may be two possible unfolding pathways that differ slightly but are discernable from each other. The single mutants result in PMFs that fall within the range of thermal fluctuation of one another and are different from the PMFs obtained for WT and Y20F + Y27F. Additionally, from our analysis, we gained insight into the stability of the hydrophobic core and helical region. Through the calculation of the interaction energy between the hydrophobic residues within the peptide and the water solvent, we can determine that mutations do effect the r_ea at which the hydrophobic core opens. As expected, the wild-type hydrophobic core opens at a relatively small r_ea of 60 Å, which indicates that it has the least stabilized core. This is followed by Y20F and Y27F, which both open at 75 Å, and finally by Y20F + Y27F opening at 80 Å. The analysis of the hydrogen bond patterns that emerge during the unfolding, either between peptide and solvent or between intrapeptide bonds, indicates that residues normally associated with a folded hydrophobic core do not participate in the formation of new hydrogen bonds, concomitant with core opening and exposure to solvent. Hence, the simulations presented here support the hypothesis that tyrosine to phenylalanine mutations serve to increase the hydrophobic character and packing of the core, and it is this optimization that leads to the computationally observed increases in peptide monomer stability. Additional computational and experimental studies probing the role of alanine, leucine, and tryptophan substitutions should provide further support for this hypothesis.

The ASMD simulation results are completely consistent with the experimental results discussed below, but do not account for the magnitude of the observed increase in thermal stability. They also cannot shed light on the contributions of higher-order structure to the observed increase in thermal stability. However, taken together with the analytical ultracentrifugation results, they show that thermal stability increases are due in part to monomer stabilization and/or stabilization brought about by higher-order structure formation. Future studies will be necessary to address the relative contributions of these two effects.

Similarly, the thermal CD experiments cannot distinguish between monomer unfolding and subunit dissociation. This may be because both processes occur simultaneously at the melting temperature or because subunit dissociation that may occur prior to the melting temperature is spectroscopically silent. The unfolding curves are broad and may therefore be indicative of a more complex process than a simple dissociation/unfolding reaction. We are in the process of harnessing other biophysical methods that can help dissect out the contributions of monomers and quaternary structure in the observed increase in thermal stability.

**Functional Implications of Increased NPY Stability and Higher-Order Structure.** The membrane- and GPCR binding studies indicate that the Y20F and Y27F mutations reduce NPY biological activity. Although we report only apparent binding affinities for the peptide–lipid interactions, previous studies have shown that NPY binds DMPC or DMPC/DMPG bilayers in a multistep process.25 These studies found that there is an initial interaction between NPY and the lipid surface that is characterized by distinct sets of rate constants. In the first phase of binding, it is thought that NPY undergoes a structural transition while optimally orienting to the bilayer surface.10,25 This is then followed by two-dimensional translocation to the receptor.10,25 Receptor selection is thus more efficient, with NPY movement along the membrane rather than via three-dimensional (3D) diffusion. Interestingly, our SPR analysis indicates a good fit via a single set of rate constants and a rather poor fit to the more complex lipid binding model described above (data not shown). Moreover, porcine NPY had a relatively poor fit to the multistep model in the study relative to other homologues.25 This could suggest that the kinetic mechanisms for NPY–family interactions with lipid membranes (or micelles) are species-dependent. Although there is still debate regarding the exact nature of this process (e.g., whether hairpin unfolding is prerequisite for receptor binding10,26), current thinking suggests that the active binding species is the monomer27 and that Y20 and Y27 are critical for NPY–lipid interactions.10 In fact, these studies10,25 suggest that the monomeric NPY form is not in the PP-fold conformation at the moment of lipid binding, in contrast to other studies.26 Our results suggest that the higher-order quaternary structures of Y20F, Y27F, and Y20F + Y27F must dissociate to monomer sometime prior to either lipid or receptor binding. This interpretation may also explain why the apparent binding affinities of the mutants in this work are controlled by on-rate rather than off-rate: dissociation of higher-order species may be a kinetically limiting step in NPY function. We note that this interpretation does not preclude the possibility of hairpin unfolding as part of membrane and receptor binding.

**NPY Higher-Order Structure.** Previous studies have shown that wild-type NPY undergoes reversible self-association to dimer, with an affinity ranging from 0.2 to 3 μM (reviewed in ref 23). Here, we find that dimerization affinity is considerably weaker, occurring in the hundreds of micromolar range. Although the exact basis of this difference will require further study, we note that a subset of previous studies was carried out under considerably different solution conditions. These include differences in pH and temperature, both of which are known to influence NPY dimerization affinity.7 For those studies carried out under similar conditions, a key difference may have been in the use of porcine NPY, rather than the human NPY examined.
here. The two peptides differ by a single M (human) to L (porcine) substitution at position 17, potentially implicating this position as a regulator of NPY self-association. An additional difference between earlier studies and the current work is our observation of a hexamer-like assembly state. This species was seen at low levels for wild-type NPY and is likely to exist in the mutant peptide populations. Although such a species has not been reported previously, it is worth speculating that observations of “higher aggregates” in spectroscopic studies of NPY dimerization may be reflective of the hexamers seen here.

Regarding the mechanism of NPY self-association, it is well established that association is dependent on whether the N-terminal polyproline sequence is packed against the C-terminal α-helix. In fact, fully stabilized and folded, NPY is prevented from forming higher oligomeric structures. Conversely, if the polyproline sequence is removed, the remaining α-helix forms trimeric or tetrameric structures. Both of these interactions are driven by the hydrophobic character of the C-terminal and N-terminal sequences. In the current study, replacing one or both tyrosines increases the hydrophobic character of the α-helix and promotes the formation of distinct and stable higher-order quaternary states. The mechanism for this is presumably through better core packing among interpeptide C-terminal helices, even as the intrapeptide hairpin is also stabilized. From a thermodynamic perspective, the free energy of higher-order self-association must therefore be greater than the free energy of hairpin stabilization. In summary, these results suggest that observations of “higher aggregates” in spectroscopic studies of NPY dimerization may be reflective of the hexamers seen here.

**Materials and Methods**

Peptide Synthesis. Human NPY sequences were synthesized using standard solid-phase synthetic techniques by New England Peptide, Inc. Each peptide had a standard N-terminal structure and contained either a carboxy or amidated C-terminus. For all solution studies presented here, the nature of the C-terminus had no impact on the results (data not shown). Molecular weight and purity were confirmed by mass spectroscopy and reversed-phase chromatography. Typical peptide purity was >95%. Sequences were as follows, with peptide purity was >95%. Sequences were as follows, with mutated residue(s) bold/underlined:

**WT:** NH₂-YPSKPDNPGEDAPAEDMARYYSALRHYIN-LITRQRY-COOH

**Y20F:** NH₂-YPSKPDNPGEDAPAEDMAREYSALRHYIN-LITRQRY-COOH

**Y27F:** NH₂-YPSKPDNPGEDAPAEDMARRYYSALRHÉIN-LITRQRY-COOH

**Y20F + Y27F:** NH₂-YPSKPDNPGEDAPAEDMAREYSALRHÉIN-LITRQRY-COOH

Circular Dichroism Studies and Thermal Melts.

Peptides were dissolved to a final concentration of 20 μM in 15 mM NaPO₄ (pH 7.0) for far-UV (190–250 nm) measurements. Spectra were obtained as a function of temperature on an Applied Photophysics Chirascan spectrophotometer utilizing a 1 mm path length quartz cuvette. Melting curves were obtained at 1 °C intervals after a 5 min incubation at the new temperature with an averaging time of 5 s. Thermal denaturation was fully reversible as evidenced by recovering ~99% of the CD signal upon cooling and by the observation that reverse and forward melting curves were superimposable. Raw CD data were converted to mean molar ellipticity according to

\[
\theta_m = \frac{mD}{PLnC}
\]

where \(mD\) is the raw CD reading in millidegrees, \(PL\) is the cell path length in millimeters, \(n\) is the number of amino acids in the peptide, and \(C\) is the micromolar concentration.

CD signal at 222 nm was fit to a two-state equilibrium unfolding model (after a linear fit of the folded and unfolded baselines) according to

\[
F_u = \theta_1 - \theta_u
\]

\[
\theta_u = \theta_1 - \theta_0
\]

where \(F_u\) is the fraction folded; \(\theta_1\), \(\theta_u\), and \(\theta_0\) are the CD signals of the native, unfolded, and 7th temperature state, respectively. Finally, the data presented represent the average of three independent experiments, all of which were superimposable to within 0.25 °C of \(T_m\).

Surface Plasmon Resonance Membrane Interaction Studies.

Small unilamellar vesicles (SUVs) were prepared essentially as described and consisted of a 4:1 w/w ratio of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) to 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DMPG) in 20 mM phosphate buffer (pH 7.0) and 125 mM NaCl (buffer I). Final SUV concentration was 60 nM. SPR experiments were conducted as described by Lerch et al. with several modifications. The L1 sensor chip was utilized in a BiaCore-X to produce a lipid bilayer sensor surface. The bare chip was washed with 10 μL of 20 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate at a flow rate of 5 mL/min. The SUV prepared was then injected over the surface at a flow rate of 2 μL/min for a total of 50 μL (100 μL of total SUV load). Next, 50 μL of 10 mM NaOH was passed over the surface at a flow rate of 50 μL/min to remove loosely bound vesicles from the chip surface. Finally, the surface was washed with 20 μL of buffer I at a flow rate of 15 μL/min. Peptide solutions in buffer I were flowed over the membrane chip surface at concentrations between 5 and 25 μM, to determine the concentration affording optimal signal (10 μM), at a flow rate of 15 μL/min at 25 °C for the 200 s association phase, during which, flow was switched to buffer I only for an additional 250 s for the dissociation phase.

Raw SPR curves were processed using Scrubber2 (BioLogic Software, Inc.). Final peptide–membrane SPR-binding isotherms were evaluated against multiple binding models using CLAMP, where it was ultimately determined that the NPY peptide–membrane binding data were best fit by simultaneously fitting the forward \((k_a)\) and reverse \((k_d)\) rate constants to

\[
\frac{dR}{dt} = k_a C R_{max} - (k_a C + k_d)R
\]
where $R$ is the SPR signal (in response units, RU) at time $t$, $R_{\text{max}}$ is the maximum binding capacity in RU, and $C$ is the peptide concentration. SPR-binding isotherms are presented in Results for peptide at 10 μM. Because the exact binding model is not known, we fit the data to obtain an apparent equilibrium affinity constant ($K_{\text{app}}$) that is defined as

$$K_{\text{app}} = \frac{k_{\text{on}}}{k_{\text{off}}}$$

**Receptor Binding Assays. Peptide Labeling.** A competition assay was developed to measure the relative affinities of the peptides toward receptors that were prebound with a fluorophore-modified wild-type NPY peptide. First, N-terminal acetylated wild-type NPY peptide was labeled with the fluorophore, Pacific Blue (Molecular Probes, Inc.), by reacting the succinimidyl ester derivative of the dye with the single NPY lysine residue. Briefly, lyophilized wild-type NPY peptide was brought to 1 mg/mL in 0.1 M Na bicarbonate, and 100 μL of this solution was added to a vial of Pacific Blue succinimidyl ester. Reactions were run in triplicate. Samples were gently agitated until the dye dissolved, and the reactions were incubated at 20 °C for 3 h with gentle agitation. The reaction mixtures were pooled and were loaded onto a Biogel P-2 column (10 cm × 0.78 cm2) in buffer I. Under these conditions, labeled NPY elutes in the void volume and dye/buffer components are in the latter included volume. Fractions containing peptide fluorescence (excitation wavelength = 410 nm, emission wavelength = 455 nm) were pooled and used for receptor binding without further modification.

**Receptor Interactions.** The brain is highly enriched with membrane-bound NPY receptors. The membrane fraction was isolated as described in ref 34 from Brown Norway rats purchased from BioreclamationIVT. Brain membranes (20 μg) were placed in a siliconized Eppendorf tube, to which was added 1.0 μg of Pacific Blue-labeled NPY in buffer I plus 15 mM MgCl$_2$, 10 mM CaCl$_2$, 0.5% bovine serum albumin, 0.2% Tween-20, and 0.2 mM phenylmethylsulfonyl fluoride (buffer II, all chemicals from Sigma-Aldrich Chemical Co.) in a final assay volume of 200 μL. After incubation at 30 °C for 1 h, the membrane fraction was pelleted by centrifugation at 20 000g for 15 min, the supernatant was discarded, and the membranes were washed in buffer II, recentrifuged, and brought up into 200 μL of buffer II supplemented with various concentrations of unlabeled NPY peptides (from $10^{-4}$ to $10^{-10}$ M). The reaction was incubated and processed as above. However, at this last stage, the entire decanted supernatant was measured for fluorescence (excitation wavelength = 410 nm; emission wavelength = 455 nm). The fluorescence in the supernatant of a control reaction (labeled NPY bound to membrane receptors but without added peptide in step 2) was subtracted from each reaction to account for bound labeled NPY that dissociates from membrane receptors on its own during the assay. Fluorescence in the final supernatant represents receptor-bound Pacific Blue-labeled NPY that has been competed off brain NPY receptor-containing membranes by the addition of unlabeled peptide. Maximum fluorescence is arbitrarily defined as 100%. Data are presented as a plot of percent fluorescence in the supernatant against the log of the concentration of competing peptide.

**Sedimentation Velocity Analytical Ultracentrifugation.** Lyophilized peptide samples were reconstituted in phosphate-buffered saline (PBS) at pH 7.4 and to a concentration of ~800 μM. Samples were then prepared at concentrations ranging from 10 to 300 μM. Sedimentation velocity studies were carried out using a Beckman XL-A analytical ultracentrifuge equipped with absorbance optics and an An-50 Ti rotor. Two-channel, 12 or 3 mm path length centerpieces were used throughout, depending on peptide concentration. Data were collected at 20 °C and at 50 000 rpm, with scans taken as frequently as the instrument allowed. Data were collected at either 230 or 280 nm depending on peptide concentration. Scans were analyzed using Sedfit, version 14.81,22 to determine sedimentation coefficient distributions at each peptide concentration. Solvent density and peptide partial specific volumes were calculated using Sednterp (http://www.jphilo.mayway.com).

**Sedimentation Equilibrium Analytical Ultracentrifugation.** NPY concentrations ranging from 10 to 400 μM were allowed to reach sedimentation equilibrium at 30 500, 36 600, 42 900, and 52 800 rpm on a Beckman XL-A analytical ultracentrifuge. Rotor speeds were chosen on the basis of guidelines laid out by Laue,35 with the approach to equilibrium monitored by acquiring scans 30 min to 2 h apart and verified using the match algorithm in HeteroAnalysis (https://core.uconn.edu/resources/biophysics).36 Data were collected at 230 and 280 nm. Global fits were performed using SedAnal (http://www.sedanal.org), version 6.54.27 Buffer conditions were identical to the sedimentation velocity studies.

**Simulations. Mutations.** The solution NMR structure of the folded conformation of the wild-type NPY28 was used as the starting structure to make the mutants Y20F, Y27F, and the double mutant Y20F + Y27F in silico. Amino acid substitutions were made using the VMD Mutator plugin.38 Accommodation of the mutations did not alter the backbone structure as evidenced by a 3D least-squares superposition of the mutant peptide with the wild-type structure.

**Equilibration.** All of the simulations use nanoscale molecular dynamics (NAMD)39 to integrate the molecular dynamics equations of motion with respect to the c36 CHARMM all-atom force field.40,41 The following are the simulation parameters: the smooth switching function turns on at 8 Å, and 280 nm. Global fits were performed using SedAnal (http://www.sedanal.org), version 6.54.27 Buffer conditions were identical to the sedimentation velocity studies.

Peptides are solvated individually in a rectangular cuboid box with approximately 19 000 water molecules initialized using the NAMD solvation package. These explicit waters are represented by a pairwise TIP3P force field. The solvent box is built with two equal sides of length $L_x (=30$ Å) and a longer side of length $L_z (=150$ Å), along which the peptide ends will be steered apart through adaptive steered molecular dynamics (ASMD). After solvation, the overall charge of the peptide and solvent system is neutral. No ions are required to gain neutrality. The system then undergoes a three-step equilibration protocol:

1. The first step of the protocol is the equilibration of the solvent side chains and solvent while the backbone of the peptide is restrained. This step allows the water solvent to reach a density of approximately 0.9998 g/cm$^3$ while simultaneously allowing the peptide side chains to find favorable conformations. Energy minimization is performed for 10 000 steps to remove bad contacts using the conjugate gradient method. The system is then equilibrated for 1 ns at 300 K under NPT conditions. Pressure is maintained using the Nose–Hoover
Langevin piston method with a damping coefficient of 5 ps\(^{-1}\), a decay period of 100 fs, and a damping time constant of 50 fs. Temperature is maintained using a Langevin thermostat.

In the second step of the equilibration protocol, the system undergoes a constant-volume equilibration under NVT conditions. The backbone is initially restrained with a harmonic potential of 10 kcal/(mol Å\(^2\)). The constraints on the backbone are gradually released during a series of three 100 ps intervals ending at 5.0, 1.0, and 0 kcal/(mol Å\(^2\)). The gradual release of the constraints on the peptide backbone ensures that its atoms do not shift too quickly in relation to the solvent motion. The entire system is then allowed to equilibrate for another 500 ps.

In the third step, the peptide is realigned back to the z axis. This is needed because the free motion of the peptide during the second step of the equilibration protocol can induce substantial drift away from the z axis. This is achieved by first extracting the peptide from solvent, that is, removing the TIP3P waters from the representation and then translating and extracting the peptide from solvent, that is, removing the same procedures as before. Following the solvation, the C\(_z\) atoms of the 1st and 36th residues are aligned with the z axis. The transformation of the peptide does not in any way change the equilibrated structure obtained in the second step. Instead of applying the transformation to the water molecules, for simplicity, the peptide is then resoluted using the same procedures as before. Following the solvation, the C\(_z\) atoms of the 1st and 36th residues are restrained, but all other motions unconstrained. A 1 ns equilibration of the water and peptide system is then performed to equilibrate the solvent and side chains. The equilibration is verified through analysis of the secondary structure and root-mean-square deviations using the NAMD plugin Timeline.

**Unfolding Simulation.** In this work, the PMFs for the unfolding pathway of the four NPY peptides are obtained computationally using the ASMD method. This technique was previously used to determine the mechanism by which wild-type NPY “unhinged” using a nonlinear pulling pathway.\(^{23}\) The efficiency of the algorithm has also been benchmarked for various solvent conditions, including vacuum,\(^{42}\) implicit solvent,\(^{45}\) and explicit solvent.\(^{54}\)

In all of the ASMD simulations, the peptides are stretched, mimicking unfolding by an external agent, along the long z axis, for which the box can accommodate the extended or stretched peptide structure. All simulations are carried out at a temperature of 300 K. The stretching coordinate \(r_{eq}\) is defined as the distance between the C\(_z\) of the 1st and 36th residues. At the start of each simulation, the distance between the stationary and pulled atom is compressed to 16 Å. This constraint is used to ensure that the peptide accesses the local minimum obtained from the equilibration protocol during the driven unfolding. The peptides are gradually stretched for 120 Å as the end-to-end distance is extended from 16 to 136 Å. The ASMD simulations are partitioned across stages with constant separation in the stretching coordinate at the endpoints \(r_{eqj}\) for \(j\) running from 0 to 40. The separations in each stage are 3 Å and resulted in changes in the free energy across the stage that never exceeded 3k\(_B\)T, which was seen in our earlier work to be an important criterion for satisfying convergence.\(^{42−44}\) At the end of each stage, the PMF is calculated using the Jarzynski equality\(^{45−48}\) as in a typical SMD simulation for computing the average work \(W\)

\[
\bar{W}(r_{eq}(t)) = \bar{W}(r_{eqj}) - \beta^{-1} \ln \left\{ \sum_{i=1}^{N} e^{-\beta W(i)} \right\} 
\]

where \(i\) is the \(i\)th trajectory in the nonequilibrium ensemble stretched from \(r_{eqj}\). A more rigorous description of the algorithm is presented in ref 49.

Once the Jarzynski average is calculated, the trajectory with the closest final work value to the average is selected for use in the next stage. In particular, the coordinates and velocities at the end of the selected trajectory are taken as the initial values for the beginning of the next stage. The PMFs of the peptides are evaluated at a stretching velocity of 10 Å/ns with a sampling size of 100 trajectories per stage. Once a PMF is obtained, it is shifted (vertically) to reset the zero of energy at its minimum. This shifting allows for a more direct comparison of the PMFs.

In addition to the PMFs, other observable quantities can be calculated and compared to further reveal the underlying causes of stability of a peptide. These include hydrogen-bonding patterns and interaction energies of specific pairs of residues. Expected values of observables along the unfolding (stretching) pathway are obtained using the weights for the work associated with the nonequilibrium paths. To calculate the number of hydrogen bonds between two sets of atoms along the unfolding pathways, we define the sets \(S_1\) and \(S_2\) containing the indices of the selected atoms and/or of collective variables in the overall configuration \(\xi\). The weighted average number of hydrogen bonds between the two sets can then be written as

\[
\langle N_H(S_1, S_2) \rangle = \frac{\sum_{i=1}^{N} \hat{N}_H(S_1, S_2) e^{-\beta W(i)}}{\sum_{i=1}^{N} e^{-\beta W(i)}} \tag{6}
\]

where the instantaneous number of hydrogen bonds between the sets is

\[
\hat{N}_H(S_1, S_2) = \sum_{i \in S_1, j \in S_2} \hat{N}_H(\xi^{(4)}, \xi^{(0)}) \tag{7}
\]

The average interaction energy between residues indexed in sets \(S_1\) and \(S_2\) can be obtained in an analogous form by replacing \(N_H\) with \(E\).

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01949.

Global fitting of wild-type NPY sedimentation equilibrium data using SedAnal software (Figure S1) (PDF)

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

NPY, neuropeptide Y; GPCR, G-protein-coupled receptor; PMP, potential of mean force; CD, circular dichroism; T_en, melting temperature; ASMD, assisted steered molecular dynamics; SPR, surface plasmon resonance; PBS, phosphate-buffered saline; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol); RMSD, root-mean-square deviation

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