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Centrally truncated and stabilized porcine neuropeptide Y analogs: Design, synthesis, and mouse brain receptor binding

(conformational restriction)

JOHN L. KRSTENANSKY*, THOMAS J. OWEN, STEPHEN H. BUCK, KAREN A. HAGAMAN, AND LARRY R. MCLEAN

Merrell Dow Research Institute, 2110 East Galbraith Road, Cincinnati, OH 45215

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ABSTRACT **Porcine neuropeptide Y (pNPY) has been proposed to form an intramolecularly stabilized structure characterized by N- and C-terminal helical regions arranged antiparallel due to a central turn region. Analogs based on this structural model that have the central turn region and various amounts of the helical regions removed, yet retain the N and C termini in a similar spatial orientation were designed. The gap formed by removal of the central residues (residues 8–17 or 7–20) was spanned with a single 8-aminooctanoic acid residue (Aoc) and the structure was further stabilized by the introduction of a disulfide bridge. [D-Cys^{7},Aoc$^{8-17}$,Cys^{20}]pNPY and [Cys^{5},Aoc$^{7-20}$,D-Cys^{24}]pNPY were synthesized and found to have receptor binding affinities of 2.3 nM and 150 nM, respectively, in mouse brain membranes (pNPY affinity is 3.6 nM in this assay). It is proposed that the central region (residues 7–17) of pNPY serves a structural role in the peptide and is not involved in direct receptor interaction.**

Porcine neuropeptide Y (pNPY; Table 1, compound **1**) is a 36-amino acid residue peptide that belongs to a unique family of peptides (Table 2) having a wide distribution throughout the central and peripheral nervous systems (9). In high-salt buffer the circular dichroic (CD) spectrum of pNPY exhibits a large degree of α-helical character. Based on spectral data and peptide sequence, it has been proposed (10) that pNPY can assume an intramolecularly stabilized structure in solution similar to that of avian pancreatic polypeptide (APP, compound **6**). A similar conclusion was reached by Allen *et al.* (1) using molecular modeling techniques. The crystal structure of APP has been reported (8, 11, 12) and consists of an N-terminal polyproline helix, a central turn region, an amphipathic α-helical region, and a C-terminal turn structure. The two helical regions are arranged antiparallel with their lipophilic faces involved in van der Waals interactions allowing intramolecular stabilization of the helices.

Several studies have stressed the importance of both N and C termini of NPY for potent NPY receptor binding and the C-terminal region for activity in various bioassays (13, 14). Since few NPY analogs have been synthesized, the importance of the central region of NPY is unknown. If the purpose of the central residues is structural and not important for direct interaction with the NPY receptor, then it should be possible to remove these residues, replace them with other moieties that maintain the same structure for the remaining portion of the molecule and produce a molecule that retains full receptor binding characteristics. Since this type of modification is typically conformationally restrictive, activation of the receptor may be affected. In addition, if the binding requires conformational flexibility on the part of the ligand, the binding may be prevented. Some examples of this approach of truncation for the structural assessment of peptides have been reported for somatostatin (15), enkephalin (16, 17), and jaspamide (18). Therefore, the design of pNPY analogs was undertaken based on the crystallographic structure of APP having the central residues excised from the sequence and conformational restraints introduced that keep the N and C termini in a similar spatial relationship to one another as in the native peptide.

MATERIALS AND METHODS

Molecular Modeling. The analogs were designed based on a model constructed from the crystallographically determined structure of APP in the Brookhaven Protein Data Bank (8). The manipulations were done on an Evans & Sutherland PS350 picture system using INSIGHT and DISCOVER software (Biosym). Nonhomologous residues were mutated to the pNPY sequence retaining as many of the side-chain torsional angles present in APP as possible. The model was then inspected for potential Cys-Cys disulfide crosslinking sites where minimal distortion of the helical backbone would occur and for sites where an 8-aminooctanoic acid residue (Aoc) could bridge between the N- and C-terminal helices without causing distortion or realignment of the helices. The two best combinations that were identified involved a disulfide bridge between a D-Cys-7 and a Cys-20 with a single Aoc residue bridging the gap left by removal of residues 8–17 (compound **2**) and a disulfide bridge between a Cys-5 and a D-Cys-24 with a single Aoc replacing residues 7–20 (compound **3**). The distance between the carbonyl carbon (C′) of residue 7 and the N of residue 18 (10.11 Å) and the distance between the C′ of residue 6 and the N of residue 21 (8.63 Å) correspond to the gaps that the Aoc residue must span in compounds **2** and **3**, respectively. A fully extended Aoc residue is capable of spanning 12.53 Å. The distance between the α-carbons of residues 7 and 20 (6.67 Å) and the distance between the α-carbons of residues 5 and 24 (6.07 Å) correspond to the distances that the disulfide bridge must span for compounds **2** and **3**, respectively. A disulfide bridge formed between two cysteine residues with a disulfide dihedral angle of 90° can span distances between two cysteine α-carbons that are less than 6.89 Å. The choice of D- or L-cysteine residues at the bridge locations was made after considering the four possible combinations of residues and observing in the model which would cause the least distortion in the orientation of the peptide backbone upon disulfide formation. Models of these analogs were constructed, minimized, and compared to a minimized structure of the pNPY model (Fig. 1). In all three cases, the modifications introduced virtually no change in the central backbone structure or N- and C-terminal orientations.

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Abbreviations: NPY, neuropeptide Y; p, porcine; Aoc, 8-aminooctanoic acid; APP, avian pancreatic peptide.

*To whom reprint requests should be addressed.

Table 1. Peptide sequences and mouse brain binding data

Compound	Sequence	IC_{50}, nM	Hill slope
	1 5 10 15 20 25 30 36		
1	YPSKPDNPGEDAPAEDLARYYSLARHYINLITRQRY#	3.6 ± 0.3	0.85
2	YPSKPDc----------ARCYSALRHYINLITRQRY#	2.3 ± 0.7	0.83
3	YPSKCD----------YSAcRHYINLITRQRY#	150 ± 28	0.33
4	YYSALRHYINLITRQRY#	266 ± 80	0.29
5	RHYINLITRQRY#	>10,000	—
6	GPSQPTYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY#	>10,000	—

In sequences **2** and **3**, a dashed line represents the residues replaced by a single 8-aminooctanoic acid residue (Aoc). Data for IC_{50} are mean ± SEM. #, C-terminal amide.

Peptide Synthesis. Peptides **4–6** (see Table 1) were purchased from Peninsula Laboratories. The peptides **1–3** were synthesized on a 0.5-mmol scale by solid-phase methods on *p*-methylbenzhydrylamine resin (0.40 mmol/g; Peptides International, Louisville, KY) using an Applied Biosystems model 430-A peptide synthesizer. All residues were double coupled using protocols supplied by the manufacturer as the symmetrical anhydrides of the N^{α}-*t*-butoxycarbonyl (boc)-protected amino acids with the exception of arginine, asparagine, and glutamine that were double coupled using *N*,*N*'-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. The side-chain protection was as follows: Arg(Tos), Asp(Chx), Cys(pMeBzl), Glu(Bzl), His(Tos), Ser(Bzl), and Tyr(2-BrZ), where Tos is tosyl, Chx is cyclohexyl, pMeBzl is *p*-methylbenzyl, Bzl is benzyl, and 2-BrZ is 2-bromobenzyloxycarbonyl. The peptides (0.25-mmol theory) were cleaved from the resin support and deprotected in liquid HF containing 5% (vol/vol) anisole at −5°C for 40 min. After removal of the HF *in vacuo*, the peptide was extracted from the resin with 30% (vol/vol) acetic acid and water. The extract was diluted to 1 liter; the pH was adjusted to between 8 and 9 with ammonium hydroxide and 0.01 M potassium ferricyanide was added until a yellow color persisted (≈25 ml). After stirring for 30 min, the pH was lowered to <5 with glacial acetic acid and the solution was stirred with 25 ml of settled AG 3-X4A resin (Bio-Rad) for 2 hr. The solution was filtered from the resin and lyophilized. The peptidic material that remained was purified by preparative HPLC on a Dynamax C_{18} column (41.4 × 250 mm; Rainin Instruments) using acetonitrile in 0.1% trifluoroacetic acid as an eluant. The purity and identity of the peptides were assessed by analytical HPLC [Vydac 218TP54 C_{18} column, 4.6 × 250 mm, 2.0 ml/min, t_o = 1.9 min, linear gradient of 15–40% or 25–50% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid over 25 min], by amino acid analysis [6 M HCl containing 8% (vol/vol) phenol; 106°C; 20 and 40 hr], and by fast atom bombardment–mass spectrometry (M-Scan). In addition, the cyclic analogs were shown to be negative when tested with Ellman reagent confirming the presence of the disulfide.

pNPY, compound **1**. The synthesis and characterization of this peptide have been reported (10).

[D-Cys^7,Aoc$^{8-17}$,Cys^{20}]pNPY, compound **2**. Analytical HPLC: retention time (t_r) = 10.7 min (25–50% gradient); amino acid analysis: $Asx_{2.03}(2)$,$Thr_{1.05}(1)$,$Ser_{1.83}(2)$,$Glx_{1.10}(1)$, $Pro_{1.98}(2)$,$Ala_{2.04}(2)$,$Ile_{1.93}(2)$,$Leu_{2.07}(2)$,$Tyr_{3.91}(4)$,$His_{0.98}(1)$, $Arg_{3.87}(4)$; fast atom bombardment–mass spectrometry: $(M + H)^+$ 3326.0 ± 1 mass units (calculated M_r, 3324.7).

[Cys^5,Aoc$^{7-20}$,D-Cys^{24}]pNPY, compound **3**. Analytical HPLC: t_r = 13.4 min (15–40% gradient); amino acid analysis: $Asx_{1.89}(2)$,$Thr_{0.99}(1)$,$Ser_{1.68}(2)$,$Glx_{1.13}(1)$,$Pro_{0.93}(1)$,$Ala_{1.03}(1)$, $Ile_{2.07}(2)$,$Leu_{1.09}(1)$,$Tyr_{3.88}(4)$,$His_{0.94}(1)$,$Arg_{3.06}(3)$; fast atom bombardment–mass spectrometry: $(M + H)^+$ 2886.1 ± 1 mass units (calculated M_r, 2887.4).

Table 2. Peptide sequences of the "Y" peptide and pancreatic polypeptide families

Peptide	Sequence	Ref(s).
	1 5 10 15 20 25 30 36	
Peptide Y		
Porcine NPY (**1**)	YPSKPDNPGEDA PAEDLARYYSALRHYINLITRQRY#	1
Human and rat NPY	YPSKPDNPGEDA PAEDMARYYSALRHYINLITRQRY#	1, 2
Porcine PYY	YPAKPEAPGEDASP EELSRYYASLRHYLNLVTRQRY#	1
Anglerfish PY	YPPKPETPGSNASP EDWASYQAAVRHYVNLITRQRYG	3
Sculpin PY	YPPQPESPGGNASP EDWAKYHAAVRHYVNLITRQRY#	4
Salmon PP	YPPKPENPGEDAPP EELAKYYTALRHYINLITRQRY#	5
Pancreatic polypeptide		
Avian		
Ostrich PP	GPAQPTYPGDDA PVEDLVRFYDNLQQYLNVVTRHRY#	6
Chicken PP (**6**)	GPSQPTYPGDDA PVEDLIRFYDNLQQYLNVVTRHRY#	6
Turkey PP	GPSQPTYPGDDA PVEDL?RFYNDLQQYLNVVTRHRY#	6
Goose PP	GPSQPTYPGNDA PVEDLIRFYDNLQQYRLVVFRHRY#	6
Reptilian		
Alligator PP	TPLQPKYPGDGA PVEDLIQFYNDLQQYLNVVTRPRF#	6
Mammalian		
Ovine PP	ASLEPEYPGDNATP EQMAQYAAELRRYINMLTRPRY#	6
Bovine PP	APLEPEYPGDNATP EQMAQYAAELRRYINMLTRPRY#	6
Rat PP	APLEPMYPGDYATH EQRAQYETQLRRYINTLTRPRY#	6
Guinea pig PP	APLEPVYPGDDATP QQMAQYAAEMRRYINMLTRPRY#	7
Porcine PP	APLEPVYPGDDATP EQMAQYAAELRRYINMLTRPRY#	6
Canine PP	APLEPVYPGDDATP EQMAQYAAELRRYINMLTRPRY#	6
Human PP	APLEPVYPGDNATP EQMAQYAADLRRYINMLTRPRY#	6
APP x-ray structure	pppppppptttttt*aaaaaaaaaaaaaaaaaaa*ttttt	

Information on the x-ray structure of APP is from Blundell *et al.* (8) and is encoded as polyproline-line helix (p), turn structure (t), and α-helical structure (α). The single-letter amino acid code is used. PP, pancreatic polypeptide; PY, peptide Y; PYY, peptide YY; #, C-terminal amide.

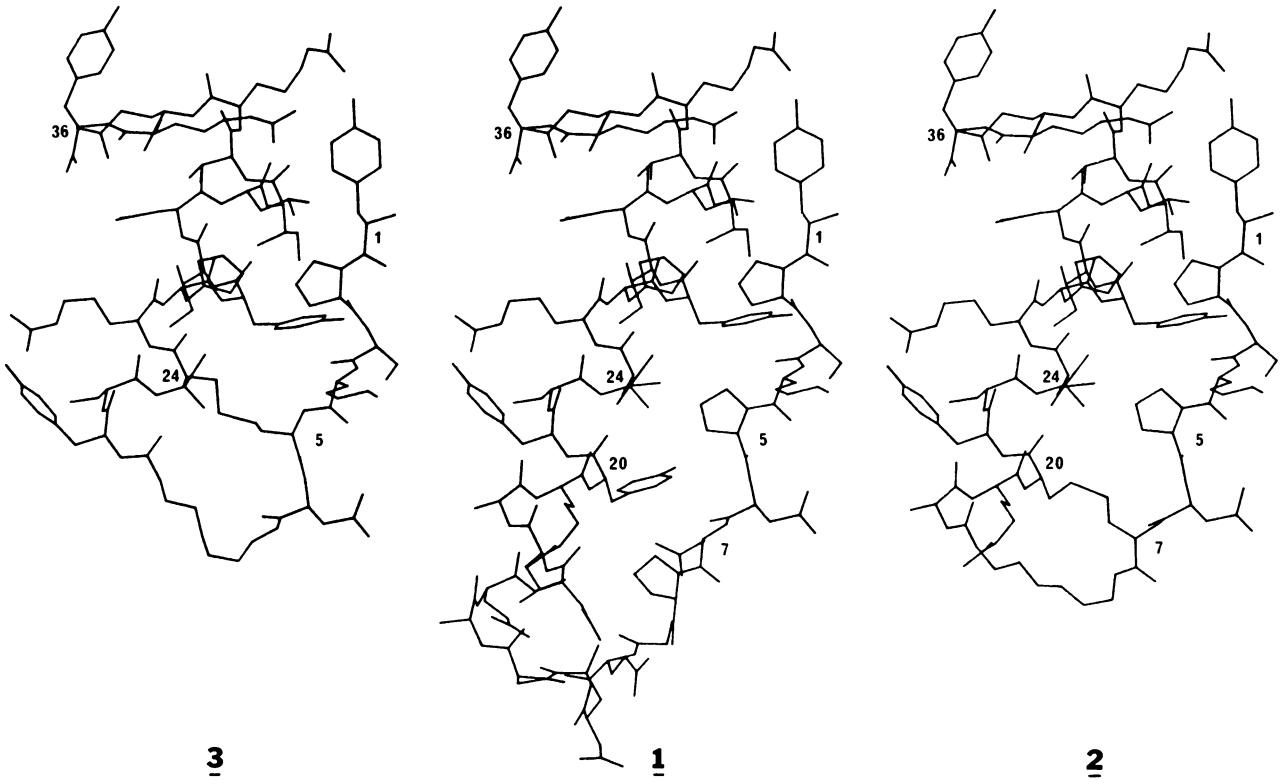

FIG. 1. Molecular models of compounds **1**, **2**, and **3** showing the retention of N- and C-terminal regions and the nondisruptive removal of the central turn region. Residue numbering is shown for selected positions to point out the N and C termini (Tyr-1 and Tyr-36) and the positions where the disulfide bridges were incorporated in the analogs.

CD. CD spectra of samples (0.05 mg/ml in 10 mM Tris·HCl, pH 7.40) in 1-mm circular cuvettes (Hellma) were obtained at 25°C on a Jasco J-500A spectro-polarimeter with a 2-nm slit width. The CD of buffer alone was subtracted from the CD of the sample after each scan. A total of nine scans were averaged. The data were transferred to a computer and the data were fit by a linear regression analysis with the reference spectra of Greenfield and Fasman (19) to obtain estimates of the secondary structure of the peptides in solution.

Receptor Binding. ^{125}I-labeled Bolton–Hunter-NPY (Amersham) binding was carried out in mouse crude membranes by a modification of the methods of Lundberg *et al.* (20). Membranes from frozen brain were prepared as described for tachykinin peptide binding studies (21). An aliquot of membrane preparation (≈10 mg of tissue) was incubated with peptide at room temperature for 2 hr in buffer (pH 7.4) containing the peptide analog, 130 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM Hepes, bovine serum albumin (4 mg/ml), bacitracin (40 μg/ml), leupeptin (4 μg/ml), and chymostatin (4 μg/ml). ^{125}I-labeled Bolton–Hunter-NPY was included in a concentration of 0.1 mM and nonspecific binding was determined by the inclusion of 1 μM pNPY in some samples. Samples were rapidly filtered over Whatman GF/C filters [soaked overnight in 0.5% histone (type II-AS; Sigma)] and washed two times with ice-cold 20 mM Hepes (pH 7.4). IC_{50} values for the peptides were calculated from six- to eight-point competition curves.

RESULTS

Molecular Modeling. A model of pNPY, compound **1**, constructed from information available in the form of a crystal structure of APP, was the basis for the design of centrally truncated pNPY analogs. They incorporate constraints intended to keep the remaining N and C termini stable relative to one another upon removal of a central region of the peptide. This was done to compensate for the loss of intramolecular stabilization that results from removal of significant portions of the helical regions. The truncation was accomplished by bridging the gap formed by the removal of central amino acid residues with a single Aoc. The choice of residues that were removed was based on the ability of the Aoc to easily span the gap and for the functional groups to which the Aoc was bonded to be oriented toward each other so that severe contortions of the Aoc would not be required. In the native sequence of pNPY, no β-carbons of residues in the N- and C-terminal helical regions were close enough to allow for replacement with cysteine and disulfide bridging without introducing distortion in the structure. However, introducing D-cysteine at certain points allowed disulfide bridges to be formed without altering the overall structure of the peptide in the model (Fig. 1). Utilizing these design considerations, [D-Cys^{7},Aoc$^{8-17}$,Cys^{20}]pNPY (compound **2**) and [Cys^{5},Aoc$^{7-20}$,D-Cys^{24}]pNPY (compound **3**) were synthesized by solid-phase techniques (Table 1).

Receptor Binding. In a mouse brain receptor binding assay the inhibition constants (IC_{50}) for the peptides ability to compete against ^{125}I-labeled Bolton–Hunter-NPY receptor binding are given in Table 1. The CD spectra of analogs **1–3** are presented in Fig. 2. In accordance with the expected structure of the peptides, α-helical character [determined by the method of Greenfield & Fasman (19)] decreases progressively as the truncation entails more residues of the helical regions. Under these conditions the α-helical contents for compounds **1**, **2**, and **3** were 21, 11, and 0%, respectively.

DISCUSSION

Lundberg *et al.* (20) have reported the NPY–receptor binding affinity in porcine spleen membranes of the pNPY peptides pNPY-(13–36) and pNPY-(26–36) to be 14 and 4700 times less potent than pNPY, respectively. The vasoconstrictor effects

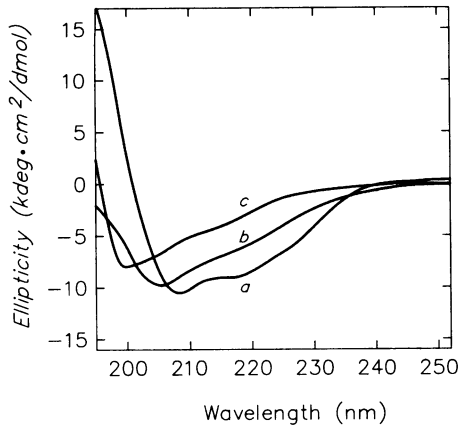

FIG. 2. CD spectra of compounds **1** (trace a), **2** (trace b), and **3** (trace c). There is a progressive loss of helical structure that correlates to the degree of truncation.

in isolated guinea pig heart for a series of pNPY C-terminal fragments have been reported by Rioux *et al.* (14). The loss of potency relative to pNPY was 12 times for pNPY-(2–36), 146 times for pNPY-(16–36), and 238 times for pNPY-(19–36). The fragment pNPY-(25–36) had no measurable activity at the concentrations tested (>4600 times less potent than pNPY). In frog pituitary, Danger *et al.* (22) demonstrated that the fragments NPY-(16–36) and NPY-(25–36) poorly inhibited the release of α-melanocyte-stimulating hormone (IC_{50} > 1000 nM) relative to NPY ($IC_{50} = 6 \times 10^{-8}$ M). Therefore, the literature indicates that although the N-terminal region of NPY is not essential for all of the actions of NPY, significant potency losses occur when this region is removed.

Compound **2**, which retains only residues 18, 19, and 21–36 of the pNPY C terminus, possesses NPY receptor binding affinity equal to that of NPY (Table 1). It is much more potent than would be expected for the C-terminal fragment itself. Fragment analogs **4** and **5** exhibit the lowered potency reported for C-terminal fragments of NPY. The good potency of compound **2** could result from the presence of an N-terminal binding region that has been restored in analog **2** or from favorable conformational stabilization of the C-terminal region by the modifications present in the molecule. The present information cannot distinguish between the two possible models. Further truncation of the molecule as in compound **3** led to considerable loss of potency at the receptor. Although data in the literature point to Tyr-1 being important for NPY potency (14), most evidence suggests that the C-terminal region is involved in receptor binding. The influence of the N-terminal region on the C-terminal receptor-bound conformation has not been studied. Since compound **2** retains high receptor binding potency, it is proposed that residues 7–17 and 20 are not important for direct NPY–receptor interaction and the pNPY region from residue 7 to residue 17 incorporates a reverse turn that places the N and C termini near each other. Neither the receptor-bound conformation nor the preferred conformation in solution for compound **2** is known; however, molecular modeling shows that compound **2** can assume a conformation analogous to that of the crystal structure of APP. Table 2 shows the sequences of the reported pancreatic polypeptides and various "Y" peptides that make up the family to which pNPY belongs. The region of residues 7–17 shows a high degree of homology throughout the family. With proteins, highly homologous regions are typically associated with structural roles or in the case of enzymes are involved in the catalytic center. This suggests, in conjunction with the data presented here, that the role of the pNPY region of residues 7–17 is structural. This region may promote and stabilize a conformation that is appropriate for favorable receptor binding. Residues 1, 4, 26, and 34, which have been suggested as being potentially important for NPY–receptor specificity (10), are retained in analogs **2** and **3**. APP (compound **6**), which differs from NPY at these positions, has a much less potency at these receptors than any of these truncated analogs (Table 1).

It should be noted that in the receptor binding assays pNPY (compound **1**) and [D-Cys^7,Aoc^{8-17},Cys^{20}]pNPY (compound **2**) had Hill slopes of 0.85 and 0.83, respectively. The remaining peptides that were tested have Hill slopes of 0.33 or less. This phenomenon is likely due to the presence of at least two receptor populations in the mouse brain, where pNPY binds to both with similar affinities and where C-terminal fragments of NPY have differing affinities on the receptors. More experiments will be required to conclusively demonstrate distinct multiple binding sites. However, Wahlestedt *et al.* (23) have given evidence for distinct pre- and postjunctional receptors and NPY C-terminal fragments are selective for the presynaptic receptor.

Lipid binding and conformational and receptor activation studies of these pNPY analogs should provide valuable information. The C-terminal pNPY region of residues 14–30 has the potential of forming an amphiphilic α-helix (10) as does the same region in APP (11). Such structures, which occur in a number of peptide hormones, often have the ability to disrupt lipid bilayers and this interaction is believed to be important for their respective receptor affinities (24). For the amphipathic α-helical region of pNPY to disrupt lipid bilayers, intramolecular van der Waals interactions with the N-terminal helical region would have to be disrupted and the molecule would have to unfold. Such an unfolding would result in the exposure of a much smaller lipophilic face in compound **2**. In fact, NPY will rapidly clear an emulsion of lipid, but compound **2** will clear the lipid only very slowly (unpublished results). Therefore, disruption of lipid bilayers is not expected to be correlated with receptor recognition by pNPY. Since intramolecular stabilization in pNPY appears to be an important aspect of the molecule for at least some NPY receptors, the description of APP as a small globular protein hormone (8) applies to pNPY as well.

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