Neuropeptide Y N-Terminal Deletion Fragments: Correlation between Solution Structure and Receptor Binding Activity at Y_1 Receptors in Rat Brain Cortex[†,‡]

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Received August 4, 1994[⊗]

N^{α}-Acetyl (Ac), N-terminal deletion fragments of porcine neuropeptide Y (NPY) have been synthesized and characterized for solution conformation properties by circular dichroism and for receptor binding activity at benextramine-sensitive Y_1 binding sites in rat brain cortex. Sequential deletion of Tyr^1, Pro^2, and Ser^3 had no effect on the structural (α-helical content of 32.5, 30.6, and 30.7%, respectively, at 1×10^{-5} M) or aggregation (monomer to dimer transition for N^{α}-Ac-NPY_{3-36} and N^{α}-Ac-NPY_{4-36}) properties of NPY. In contrast, deletion of Tyr^1 decreased receptor binding activity in rat brain cortex by 4-fold (IC_{50} = 13.0 nM versus 3.75 nM for NPY), but further deletion of Pro^2-Ser^3 had no additional detrimental effect on receptor binding activity relative to the desTyr^1 analog. Thus, Pro^2 and Ser^3 do not contribute either to the stability of the NPY tertiary structure nor directly to the receptor–ligand interactions. Additional removal of N-terminal amino acids Lys^4-Pro^5 decreased the helical content and abolished aggregation to a dimeric form of the resultant analog, results suggesting that the residues around Pro^5 are important for formation of NPY's compact, pancreatic polypeptide (PP)-fold structure. This loss in structure also correlated with a further 2–3-fold drop in receptor binding activity. These structure–activity correlations provide evidence for the importance of the PP-fold structure in the activity of NPY at Y_1 receptors in rat brain cortex.

Neuropeptide Y (NPY), a 36-amino acid peptide neurotransmitter isolated by Tatemoto in 1982[1] and subsequently localized in both the peripheral and central nervous systems of animals ranging from cartilaginous fish to mammals, controls or modulates feeding behavior, cardiovascular regulation, anxiety, learning, memory, and hormone release.[2] Allen et al.[3] and MacKerell[4] proposed an NPY tertiary structure using computer-assisted molecular modeling and dynamics based on the X-ray crystal structure of the homologous avian pancreatic polypeptide. In the model, NPY forms a compact tertiary structure, often referred to as the PP-fold, consisting of four secondary structure segments: an N-terminal polyproline type II helix in residues 1–9, a β-turn in residues 9–12, an amphiphilic α-helix in residues 14–31, and a C-terminal, structurally undefined tail (Figure 1). The polyproline and α-helices are antiparallel and associate through hydrophobic interactions between Pro^2, Pro^5, and Pro^8 in the proline stretch and a hydrophobic surface of the amphiphilic α-helix. This structural feature brings the N-terminal and C-terminal residues into close proximity. In addition to its role in presenting the functional groups of N-terminal residue Tyr^1 for receptor–ligand interaction, the N-terminal polyproline helix and β-turn of NPY play an important role in stabilizing the amphiphilic α-helix.[5]

NPY: Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2

Wahlestedt and co-workers first proposed a classification for NPY receptors based on the differential potencies of NPY and the C-terminal fragment NPY_{13-36} in various bioassays; the Y_1 but not the Y_2 receptor requires the peptide's full length for significant activity.[6] Fuhlendorf et al.[7] further extended this classification by the design and characterization of the Y_1-selective agonists [Leu^{31},Pro^{34}]NPY and [Pro^{34}]NPY. The rat central nervous system was reported to contain predominantly the Y_2 subtype using whole brain membrane preparations. However, studies using iodinated NPY and its homolog, polypeptide YY (PYY), detected the presence of two affinity sites in rat brain.[8,9] Autoradiographic studies using the selective ligands [Leu^{31},-Pro^{34}]NPY and NPY_{13-36} demonstrated the presence of multiple binding sites for NPY in the CNS; [Leu^{31},Pro^{34}]-NPY completely inhibited [^{125}I]PYY binding in cerebral cortex slices with little effect on the binding in the hypothalamus, while NPY_{13-36} inhibited binding in the hypothalamus at low concentrations but required higher concentrations to inhibit binding in the cerebral cortex.[10] Thus, it appears that rat cerebral cortex membrane NPY receptors are of the Y_1 subtype.

Studies in our laboratory also indicated possible NPY receptor heterogeneity in rat brain. Benextramine, a nonselective NPY receptor antagonist discovered in our laboratory, displaces a maximum of 61% of [^{3}H]NPY specifically bound to rat brain membrane binding sites.[11,12] We initially identified the benextramine-sensitive binding site as a Y_1 population on the basis of

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† Taken in part from the Ph.D. Dissertation of Longqin Hu, The University of Kansas, 1993.

‡ Abbreviations: NPY, porcine neuropeptide Y; PP, pancreatic polypeptide; PYY, polypeptide YY; FAB$^+$MS, positive ion fast-atom bombardment mass spectrometry; COD, coefficient of determination; MSC, model selection criterion; [^{3}H]NPY, *N*-[^{3}H]propionyl-NPY; CD, circular dichroism.

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⊗ Abstract published in *Advance ACS Abstracts*, September 1, 1994.

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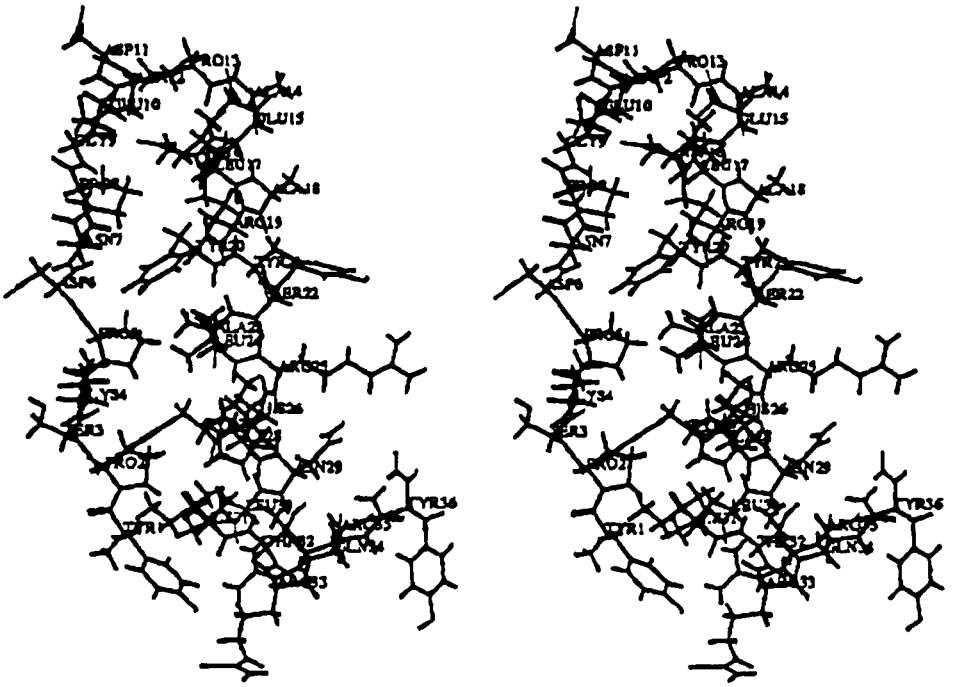

Figure 1. Stereoview of an NPY model. NPY was obtained in SYBYL (Tripos Associates, St. Louis, MO) by amino acid substitutions on the 1.4-Å crystal structure of APP followed by MaxMin2 energy minimization in the presence of one layer of water molecules.

Table 1. Analytical Characterization of Synthetic Peptides

peptide	t_R (min)[a]	MH$^+$ [found (calcd)][b]	analysis [amino acid, found (calcd)]
N$^\alpha$-Ac-NPY$_{2-36}$	30.4	4132.9 (4133.0)	A, 4.00 (4); B, 4.94 (5); G, 1.04 (1); H, 1.03 (1); I, 1.84 (2); K, 0.90 (1); L, 2.83 (3); P, 4.05 (4); R, 4.40 (4); S, 2.05 (2); T, 1.10 (1); Y, 3.73 (4); Z, 3.10 (3)
N$^\alpha$-Ac-NPY$_{3-36}$	30.6	4035.9 (4036.0)	A, 3.88 (4); B, 4.89 (5); G, 1.05 (1); H, 1.06 (1); I, 1.94 (2); K, 1.09 (1); L, 2.74 (3); P, 2.94 (3); R, 4.34 (4); S, 2.06 (2); T, 1.07 (1); Y, 3.68 (4); Z, 3.25 (3)
N$^\alpha$-Ac-NPY$_{4-36}$	30.5	3948.8 (3949.0)	A, 3.94 (4); B, 4.92 (5); G, 1.03 (1); H, 1.02 (1); I, 1.98 (2); K, 1.08 (1); L, 2.90 (3); P, 2.76 (3); R, 4.33 (4); S, 1.01 (1); T, 1.02 (1); Y, 3.90 (4); Z, 3.10 (3)
N$^\alpha$-Ac-NPY$_{5-36}$	33.2	3821.1 (3820.9)	A, 3.96 (4); B, 4.69 (5); G, 1.02, (1); H, 0.99 (1); I, 1.94 (2); L, 2.94 (3); P, 3.06 (3); R, 4.35 (4); S, 0.97 (1); T, 1.04 (1); Y, 4.00 (4); Z, 3.04 (3)
N$^\alpha$-Ac-NPY$_{6-36}$	33.15	3723.7 (3723.85)	A, 3.90 (4); B, 5.05 (5); G, 1.01 (1); H, 0.99 (1); I, 1.95 (2); L, 2.81 (3); P, 1.80 (2); R, 4.40 (4); S, 0.99 (1); T, 0.99 (1); Y, 3.82 (4); Z, 3.29 (3)
N$^\alpha$-Ac-NPY$_{8-36}$	33.22	3494.9 (3494.78)	A, 3.81 (4); B, 3.00 (3); G, 0.99 (1); H, 0.96 (1); I, 1.87 (2); L, 2.81 (3); P, 2.01 (2); R, 4.23 (4); S, 0.96 (1); T, 0.94 (1); Y, 3.77 (4); Z, 3.11 (3)
N$^\alpha$-Ac-NPY$_{9-36}$	33.13	3397.0 (3396.73)	A, 3.81 (4); B, 3.08 (3); G, 1.07 (1); H, 1.01 (1); I, 1.91 (2); L, 2.76 (3); P, 1.12 (1); R, 4.36 (4); S, 0.95 (1); T, 1.00 (1); Y, 3.50 (4); Z, 3.07 (3)

[a] Retention time for elution from Vydac C-4 reverse phase column with a 30-min gradient from 0 to 50% acetonitrile containing 0.1% trifluoroacetic acid. [b] Found (calcd) of the most abundant isotopic variant.

the observation that the same binding site selectivity was observed for both [Leu^{31},Pro^{34}]NPY and benextramine,[12] and we recently reported that the rat cerebral cortex is enriched in this benextramine-sensitive Y_1 site.[13] Interestingly, NPY$_{13-36}$ was only 28-fold less active than NPY at cerebral cortex membrane NPY binding sites with an IC_{50} of 106 nM, although NPY$_{13-36}$ is at least 2 orders of magnitude less active than NPY at Y_1 binding sites enriched in cell culture.[2,14] This high relative affinity of NPY$_{13-36}$ versus NPY at the binding sites in cerebral cortex membrane homogenates prompted us to investigate the structural requirements for NPY binding to the rat cerebral cortex binding sites. We have designed and synthesized a series of N^α-acetyl (Ac), N-terminal deletion fragments, studied their solution structure and aggregation properties using circular dichroism (CD) spectroscopy, and determined their receptor binding activity as the displacement of [^{3}H]-NPY from rat cerebral cortex membranes.

Results

Peptide Chemistry. A series of N-terminal deletion fragments of porcine NPY were synthesized using solid phase peptide synthesis methodology. Acetyl groups were introduced at the N-termini so as to avoid the introduction of additional positive charges at the N-termini; thus these deletion analogs contain the α-carbon equivalent but lack the side chain and α-amide of the preceding, deleted residue. Solid phase peptide synthesis of the N-terminal deletion fragments on the methylbenzhydrylamine resin gave crude peptides with one major peak as analyzed by reverse phase HPLC. A two-step purification using a combination of ion exchange FPLC and reverse phase HPLC was very efficient in obtaining peptides with greater than 97% purity. The peptides were synthesized in two batches; in the first, N$^\alpha$-Ac-NPY$_{4-36}$, N$^\alpha$-Ac-NPY$_{3-36}$, and N$^\alpha$-Ac-NPY$_{2-36}$ were synthsized, and in the second, N$^\alpha$-Ac-NPY$_{9-36}$, N$^\alpha$-Ac-NPY$_{8-36}$, N$^\alpha$-Ac-NPY$_{6-36}$, and N$^\alpha$-Ac-NPY$_{5-36}$ were synthesized. Amino acid analyses established the amino acid composition of all synthetic peptides (Table 1). In addition, the molecular weights (Table 1) of all synthesized peptides were confirmed by positive ion fast-atom bombardment mass spectroscopy (FAB$^+$MS).

Circular Dichroism. The solution structures of

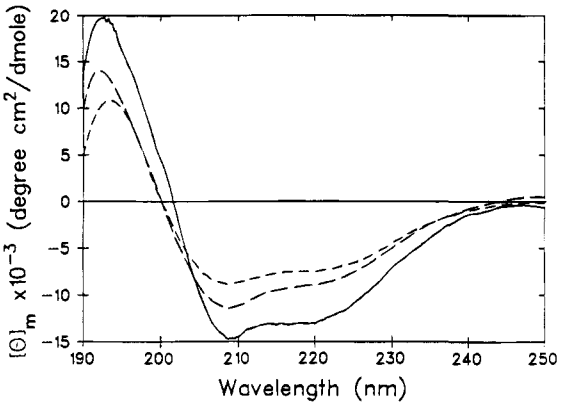

Figure 2. CD spectra of NPY (—), N$^\alpha$-Ac-NPY$_{6-36}$ (— — —), and N$^\alpha$-Ac-NPY$_{9-36}$ (— — —). Spectra were acquired at 1×10^{-5} M in 50 mM sodium phosphate buffer, pH 7.5 ($\mu = 0.15$) at 25 °C.

Table 2. Results of PROSEC Analysis of CD Spectra of NPY and Its N-Terminal Deletion Analogs[a]

compound	f_A	f_B	f_T	f_R
NPY	0.347	0.241	0.137	0.275
N$^\alpha$-Ac-NPY$_{2-36}$	0.325	0.275	0.105	0.295
N$^\alpha$-Ac-NPY$_{3-36}$	0.306	0.294	0.129	0.270
N$^\alpha$-Ac-NPY$_{4-36}$	0.307	0.237	0.167	0.289
N$^\alpha$-Ac-NPY$_{5-36}$	0.221	0.446	0.043	0.290
N$^\alpha$-Ac-NPY$_{6-36}$	0.236	0.382	0.064	0.318
N$^\alpha$-Ac-NPY$_{8-36}$	0.211	0.446	0.067	0.276
N$^\alpha$-Ac-NPY$_{9-36}$	0.184	0.492	0.058	0.266

[a] CD spectra were acquired at a peptide concentration of 1×10^{-5} M in phosphate buffer (pH 7.4, $\mu = 0.15$, 25 °C), the average of five scans was background corrected, and the CD data were analyzed from 190 to 240 nm in 1-nm increments: f_A = fraction of α-helix; f_B = fraction of β-structure; f_T = fraction of turn structure; f_R = fraction remaining (random structure).

NPY and the N-terminal deletion fragments were studied using CD spectrometry. Figure 2 shows the typical CD spectra of NPY, N$^\alpha$-Ac-NPY$_{6-36}$, and N$^\alpha$-Ac-NPY$_{9-36}$ at 1×10^{-5} M in 50 mM phosphate buffer (pH 7.5, $\mu = 0.15$, 25 °C). The CD spectra (and the CD spectra of all other deletion fragments) display distinct maxima at 194 nm, minima at 209 nm, and a shoulder or minima at 222 nm, characteristic of the $\pi-\pi^*$ and $n-\pi^*$ (222 nm) transitions of α-helical structures. Table 2 lists the results of PROSEC analysis of the CD spectra of peptides under these conditions; at this peptide concentration, there is a decrease in α-helical structure with deletion through residues 4 and 5 and a smaller drop with deletion through residue 8. The calculated α-helical contents are 34.7% (NPY), 32.5% (N$^\alpha$-Ac-NPY$_{2-36}$), 30.6% (N$^\alpha$-Ac-NPY$_{3-36}$), 30.7% (N$^\alpha$-Ac-NPY$_{4-36}$), 22.1% (N$^\alpha$-Ac-NPY$_{5-36}$), 23.6% (N$^\alpha$-Ac-NPY$_{6-36}$), 21.1% (N$^\alpha$-Ac-NPY$_{8-36}$), and 18.4% (N$^\alpha$-Ac-NPY$_{9-36}$).

The solution aggregation properties of N$^\alpha$-Ac-NPY$_{3-36}$, N$^\alpha$-Ac-NPY$_{4-36}$, N$^\alpha$-Ac-NPY$_{5-36}$, and N$^\alpha$-Ac-NPY$_{6-36}$ relative to that of NPY[5] were studied using CD spectrometry in 50 mM sodium phosphate buffer (pH 7.5, $\mu = 0.15$, 25 °C). For N$^\alpha$-Ac-NPY$_{3-36}$, N$^\alpha$-Ac-NPY$_{4-36}$, and N$^\alpha$-Ac-NPY$_{5-36}$, concentration-dependent CD data were collected in the range between 6.0×10^{-7} and 1.0×10^{-4} M, while for N$^\alpha$-Ac-NPY$_{6-36}$, data were collected in the concentration range between 6.0×10^{-7} and 8.0×10^{-5} M (Figure 3). The data were analyzed by nonlinear regression analysis of the negative ellipticity at 222 nm using eq 2 for a monomer to dimer ($n = 2$), monomer to trimer ($n = 3$), and monomer to tetramer ($n = 4$)

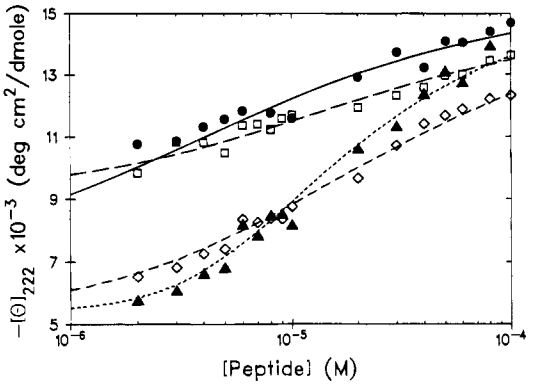

Figure 3. Concentration dependence of the N$^\alpha$-Ac-NPY$_{3-36}$ (●), N$^\alpha$-Ac-NPY$_{4-36}$ (□), N$^\alpha$-Ac-NPY$_{5-36}$ (◇), and N$^\alpha$-Ac-NPY$_{6-36}$ (▲) CD signals at 222 nm. CD spectra were acquired in 50 mM sodium phosphate buffer, pH 7.5 ($\mu = 0.15$) at 25 °C. The shown best fit lines were calculated using eq 2 for a monomer to dimer association ($n = 2$) for N$^\alpha$-Ac-NPY$_{3-36}$ (—) and N$^\alpha$-Ac-NPY$_{4-36}$ (— — —) and a monomer to trimer association ($n = 3$) for N$^\alpha$-Ac-NPY$_{5-36}$ (— — —) and N$^\alpha$-Ac-NPY$_{6-36}$ (• • •).

association. Table 3 lists the calculated parameters (±SD) and the statistical analyses for these peptides. For N$^\alpha$-Ac-NPY$_{3-36}$ and N$^\alpha$-Ac-NPY$_{4-36}$, the concentration-dependent ellipticity at 222 nm was best fit to a monomer/dimer equilibrim with $K_d = 1.07 \times 10^{-5}$ and 2.45×10^{-5} M, respectively. For N$^\alpha$-Ac-NPY$_{6-36}$, trimer formation could not be differentiated from tetramer formation on the basis of the coefficient of determination (COD) or the model selection criterion (MSC) but dimer formation could be excluded. For N$^\alpha$-Ac-NPY$_{5-36}$, the differences in COD and MSC between different equilibria were small, although there was a slight preference for the monomer to trimer association. The absence of a significant best fit to a dimer, trimer, or tetramer equilibrium suggests that this peptide may populate multiple aggregation states. Although the concentration-dependent studies could not exclude conclusively one equilibrium over the other, the concentration-dependent $[\theta]_{222}$ of N$^\alpha$-Ac-NPY$_{5-36}$ was shifted to higher concentration and the negative ellipticity at 222 nm of the monomeric form decreased significantly, similar to that observed for N$^\alpha$-Ac-NPY$_{6-36}$, results suggesting that deletion through residue 4 decreases the helical potential of NPY. Figure 3 presents the monomer to dimer association curves for N$^\alpha$-Ac-NPY$_{3-36}$ and N$^\alpha$-Ac-NPY$_{4-36}$ and the monomer to trimer association curves for N$^\alpha$-Ac-NPY$_{5-36}$ and N$^\alpha$-Ac-NPY$_{6-36}$.

Receptor Binding Activities. We assayed the activities of NPY and the N$^\alpha$-Ac N-terminal deletion analogs on cerebral cortex membrane, benextramine-sensitive NPY binding sites as the displacement of specifically-bound 1.0 nM [^{3}H]NPY. Figure 4 shows representative concentration-dependent displacement curves for selected peptides at the cerebral cortex NPY binding sites. An interesting result from the receptor binding assays is that N-terminal deletion fragments starting from N$^\alpha$-Ac-NPY$_{3-36}$ all failed to displace 100% of the [^{3}H]NPY from cerebral cortex membrane binding sites when using 1.0 μM NPY for determining nonspecific binding, indicating possible additional receptor heterogeneity in rat cerebral cortex. For example, N$^\alpha$-Ac-NPY$_{6-36}$ and N$^\alpha$-Ac-NPY$_{9-36}$ displaced only about 79 and 81%, respectively, of [^{3}H]NPY specifically bound to

Table 3. Calculated Best Fit Parameters for the Association of NPY N-Terminal Deletion Fragments[a]

peptide	n	K_d (M^{n-1})	$[\theta]_m \times 10^{-3}$	$[\theta]_{n\text{-mer}} \times 10^{-3}$ [b]	COD[c]	MSC[d]
N^{α}-Ac-NPY_{3-36}	2	$1.07 \pm 0.23 \times 10^{-5}$	-6.11 ± 0.96	-15.6 ± 0.3	0.972	3.325[e]
	3	$2.14 \pm 0.97 \times 10^{-10}$	-8.50 ± 0.40	-14.8 ± 0.3	0.959	2.817
N^{α}-Ac-NPY_{4-36}	2	$2.45 \pm 0.52 \times 10^{-5}$	-8.84 ± 0.33	-14.7 ± 0.3	0.962	3.025[e]
	3	$5.33 \pm 0.27 \times 10^{-10}$	-9.36 ± 0.33	-13.8 ± 0.2	0.950	2.637
N^{α}-Ac-NPY_{5-36}	2	$6.43 \pm 0.63 \times 10^{-5}$	-5.76 ± 0.25	-15.7 ± 0.5	0.984	3.927
	3	$3.09 \pm 0.39 \times 10^{-9}$	-6.52 ± 0.12	-13.9 ± 0.2	0.989	4.181
	4	$1.94 \pm 0.38 \times 10^{-13}$	-6.71 ± 0.13	-13.4 ± 0.2	0.985	3.950
N^{α}-Ac-NPY_{6-36}	2	$4.42 \pm 0.13 \times 10^{-5}$	-5.25 ± 0.60	-15.4 ± 1.6	0.906	2.144
	3	$3.36 \pm 0.48 \times 10^{-9}$	-5.81 ± 0.17	-16.9 ± 0.5	0.983	3.844[e]
	4	$2.07 \pm 0.41 \times 10^{-13}$	-6.01 ± 0.17	-15.2 ± 0.3	0.982	3.811[e]

[a] All parameters (±SD) were calculated by fitting the concentration-dependent CD data at 222 nm to eq 2. [b] $[\theta]_{n\text{-mer}}$ was constant to calculate the standard error of the other parameters. [c] COD, coefficient of determination. [d] MSC, model selection criterion. [e] Best fit equilibrium based on these statistical parameters.

Table 4. Receptor Binding Activity of N-Terminal Deletion Fragments in Rat Cerebral Cortex Membranes[a]

compound	IC_{50} (nM)[b]	$\%R_2$[c]	Hill number	r^2	RP[d]
NPY	3.75 ± 0.48[e]	0	0.95 ± 0.10	0.9883	1.00
[Leu^{31},Pro^{34}]NPY	5.52 ± 0.61[e]	0	0.75 ± 0.06	0.9915	0.68
N^{α}-Ac-NPY_{2-36}	13.0 ± 1.5[f]	0	0.82 ± 0.07	0.9972	0.29
N^{α}-Ac-NPY_{3-36}	12.1 ± 3.0[f]	17.1 ± 4.5	0.80 ± 0.12	0.9871	0.31
N^{α}-Ac-NPY_{4-36}	13.8 ± 4.6[f]	12.8 ± 6.1	0.70 ± 0.12	0.9829	0.27
N^{α}-Ac-NPY_{5-36}	26.0 ± 4.8[g]	14.9 ± 3.3	0.80 ± 0.09	0.9917	0.14
N^{α}-Ac-NPY_{6-36}	35.2 ± 5.5[g]	20.7 ± 2.9	0.92 ± 0.11	0.9929	0.11
N^{α}-Ac-NPY_{8-36}	30.3 ± 11.8[g]	14.4 ± 6.9	0.72 ± 0.15	0.9736	0.12
N^{α}-Ac-NPY_{9-36}	27.6 ± 6.1[g]	18.6 ± 3.9	0.86 ± 0.13	0.9872	0.14
NPY_{13-36}	106 ± 7.9	0	0.79 ± 0.1	0.9811	0.04
benextramine	$43.1 \pm 2.7 \times 10^3$	0	–	–	–[h]

[a] Receptor binding activities were determined using rat cerebral cortex membranes and [^{3}H]NPY. Data were fit to a two-site binding isotherm with affinity for only one site. [b] IC_{50} (±SD) is the concentration required to displace 50% of the specifically-bound [^{3}H]NPY from the first binding site. [c] $\%R_2$ (±SD) is the percent of the second site for which the ligand did not displace [^{3}H]NPY. [d] RP, relative potency, refers to the relative affinity of the peptide analog to that of NPY. [e] IC_{50} is significantly different from IC_{50}'s of all other peptides (paired t-test); $p < 0.05$. [f] IC_{50}'s are significantly different from IC_{50}'s of all other peptides except those in the same group (paired t-test); $p < 0.05$. [g] IC_{50}'s are significantly different from IC_{50}'s of all other peptides except those in the same group (paired t-test); $p < 0.05$. [h] Benextramine is an irreversible ligand; thus its IC_{50}, which contains a kinetic term, cannot be compared directly with the IC_{50} of NPY.

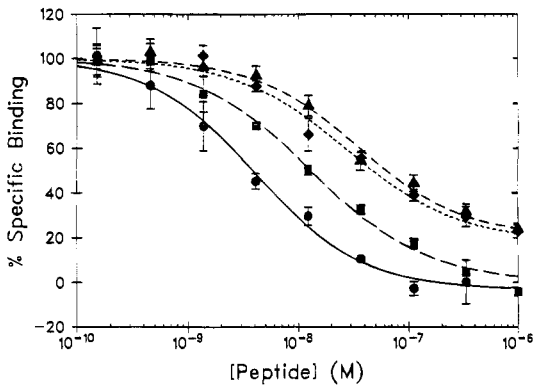

Figure 4. [^{3}H]NPY displacement curves for NPY (●—●), N^{α}-Ac-NPY_{2-36} (■– – –■), N^{α}-Ac-NPY_{6-36} (▲– – –▲), and N^{α}-Ac-NPY_{9-36} (◆···◆) at cerebral cortex receptor binding sites. Error bars represent the standard error of the mean (n = 4 or 6).

cerebral cortex membranes (Figure 4). A similar selectivity has also been observed for NPY_{15-36}.[5] The IC_{50} (±SD), Hill number, and the correlation coefficient (r^2) for each peptide obtained from nonlinear curve fitting of the competitive displacement data to a two-site model with one-site binding affinity are compiled in Table 4. NPY has an IC_{50} of 3.75 ± 0.48 nM at cerebral cortex NPY binding sites, an activity about 2.4-fold lower than that observed in whole brain preparations.[5] Removal of Tyr^1 reduced the receptor binding activity by about 4-fold, while deletion of Pro^2 and Ser^3 had no further effect on binding affinity. However, deletion through Lys^4 and Pro^5 dropped the activity by an additional 2–3-fold. Successive removal of Asp^6, Asn^7, and Pro^8 did not have further effects on *in vitro* receptor binding activity in the rat cerebral cortex.

We also include in Table 4 (for reference) the activities of the Y_1-selective ligand [Leu^{31},Pro^{34}]NPY, the Y_2-selective ligand NPY_{13-36}, and benextramine in displacing 1.0 nM [^{3}H]NPY from rat brain cortex binding sites. [Leu^{31},Pro^{34}]NPY displaced specifically-bound [^{3}H]NPY from cortex membrane binding sites with an IC_{50} of 5.5 nM, an activity about 60% that of NPY, whereas NPY_{13-36} has 0.04 times the potency of NPY in the rat cortex [^{3}H]NPY displacement assay. These results demonstrate that this receptor population is of the Y_1 subtype.[12] In addition, [Leu^{31},Pro^{34}]NPY, NPY_{13-36}, and benextramine each displaced 100% of specifically-bound [^{3}H]NPY.

Discussion

Krstenansky and Buck[15] and Minakata et al.,[16] using equilibrium centrifugation, demonstrated that neuropeptide Y forms dimers at high concentration in neutral, aqueous solution. These aggregation properties of NPY are identical to the behavior of APP both in the crystal and in solution.[17,18] In an earlier CD study, we demonstrated that in contrast to NPY, which aggregates from a mostly disordered monomer to an ordered dimer with increasing concentration, NPY_{15-36} and N^{α}-succinyl-NPY_{15-36} form higher-ordered oligomers at high concentration.[5] This difference between the solution properties of NPY and NPY_{15-36} fragments results from the lack of the polyproline helix and β-turn in the latter analogs; lack of the polyproline helix in NPY_{15-36} exposes the entire hydrophobic surface of the amphiphilic α-helix for intermolecular association. How-

ever, part of the hydrophobic surface of the NPY α-helix is occupied by the intramolecular association between the α- and polyproline helices, thus preventing the association to higher-ordered oligomers.[16] The entropy difference between the intra- and intermolecular associations in stabilizing the α-helix contributes to the different α-helical contents of the peptides at a given concentration.[5] In the current study, we have used the oligomerization of NPY analogs to dimers or higher-ordered oligomers as a measure of the potential of these peptides to form compact, PP-fold structures.

The solution aggregation properties of peptide fragments synthesized in this study indicate that deletion of Tyr^1-Pro^2 or Tyr^1-Pro^2-Ser^3 has no effect on the peptides' α-helical content at 1×10^{-5} M or on their ability to aggregate to a dimeric form. However, further deletion of Lys^4-Pro^5 resulted in a peptide (i.e., N^{α}-Ac-NPY_{6-36}) that clearly forms higher-ordered oligomers at high concentration. The best fit equilibrium for N^{α}-Ac-NPY_{6-36} is a monomer/trimer association. This change of solution aggregation properties after deletion of residues 4 and 5 indicates the importance of these two residues in maintaining the compact tertiary structure observed in the dimeric form of NPY.

The monomer to trimer association of N^{α}-Ac-NPY_{6-36} ($K_d = 3.36 \times 10^{-9}$ M^2), and the association curve of N^{α}-Ac-NPY_{5-36} as well, is also accompanied by a shift to higher concentration. We observed an even larger loss of helical potential with deletion of residues 1−14; the trimer to monomer dissociation constant for NPY_{15-36} is 1.90×10^{-7} M^2,[5] 50-fold higher than that of N^{α}-Ac-NPY_{6-36}. However, removal of the positive charge and introduction of an additional negative charge at the N-terminus partially compensate for this loss of α-helical potential in NPY_{15-36}. Thus, N^{α}-succinyl-NPY_{15-36} undergoes a trimer to monomer dissociation with a dissociation constant ($K_d = 4.62 \times 10^{-9}$ M^2) that is 41-fold lower than that observed for NPY_{15-36}. Interestingly, the dissociation constant of N^{α}-Ac-NPY_{6-36} is nearly equal to that of N^{α}-succinyl-NPY_{15-36}, suggesting that the former peptide retains the helix-stabilizing effect of the succinyl group of N^{α}-succinyl-NPY_{15-36}. We attributed the enhanced helical potential of N^{α}-succinyl-NPY_{15-36} relative to NPY_{15-36} to the favorable charge−dipole interaction between the N^{α}-succinyl group of the former peptide and the α-helix dipole.[5] N^{α}-Ac-NPY_{6-36} also contains such potential charge−dipole interactions since the two negatively-charged residues at positions 10 and 11 are near the N-terminal end of the α-helix. Thus, the increased α-helical potential of N^{α}-Ac-NPY_{6-36} relative to NPY_{15-36} can also be rationalized in terms of the α-helix dipole model.[19]

The loss of calculated α-helical content with successive deletion of N-terminal residues from NPY as determined by CD at 1×10^{-5} M also parallels the shift of the concentration-dependent curves to higher concentration and the shift from a dimer to a higher-ordered oligomer. Thus removal of residues through Ser^3 has no significant effect on the calculated α-helical content of the resultant peptides as compared to NPY, although deletion through Lys^4 and beyond drops the α-helical content by 45% relative to NPY. This drop in helical potential correlates with the shift from a dimer to a trimer association. We chose 10^{-5} M for these CD studies because this concentration is in the midpoint of the concentration-dependent curves for this peptide series. Thus, although these single-concentration CD studies do not define the origin of the loss in helical potential, they do give a static picture of the loss of helical potential in this NPY fragment series. Additionally, the α-helical content of the monomeric form of these peptides calculated by the method of Minakata et al.[16] drops from about 10% for fragments 3−36 and 4−36 to less than 5% for fragments 5−36 and 6−36, results that also correlate with a shift from a dimer to trimer association. Thus, even in the monomeric state, the proline helix has some effect on the stability of the NPY α-helix.[5]

Receptor binding assays indicate that N^{α}-Ac-NPY_{2-36}, a fragment only lacking the N^{α}-amino and the *p*-hydroxybenzyl groups of Tyr^1, has only about 25% binding affinity for the NPY rat cerebral cortex membrane receptors. The proposed PP-fold model places the Tyr^1 residue spatially close to the C-terminal residues 32−36, presumably the more important region involved in receptor interaction.[3,20] The model also shows a close contact between Tyr^1 and hydrophobic residues at positions 30 and 31, suggesting that Tyr^1 might also play a role in stabilizing the overall tertiary structure of NPY. In addition, a recent ^{1}H-NMR study of human NPY identified long-range nuclear Overhauser effects between the side chain protons of Tyr^1 in the polyproline helix and the side chain protons of residues 28 and 33 in a short segment of amphiphilic α-helix. However, our CD studies show that deletion of Tyr^1 has no effect on the α-helicity or aggregation properties of the molecule. Thus, given that deletion of Tyr^1 (and Tyr^1-Pro^2) has no effect on the helical content of NPY and that N^{α}-Ac-NPY_{3-36} and N^{α}-Ac-NPY_{4-36} maintain the same structural properties as NPY both in the monomeric and dimeric forms, we conclude that the main effect of deleting Tyr^1 is to remove functional groups involved in receptor−ligand interaction. Further deletion of Pro^2 and Ser^3 had no additional detrimental effect on receptor binding activity when compared with deletion of the Tyr^1 residue alone. Further decreases in receptor binding required deletion of the first four to five residues of NPY, and this drop in activity correlated with loss of the structural integrity of the NPY dimer. These results suggest that Pro^2 and Ser^3 do not contribute directly to the stabilization of the PP-fold structure nor to direct receptor−ligand interaction but, instead, contribute to the activity of NPY by presenting Tyr^1 in position for interaction with the receptor binding sites.

Further deletion of N-terminal residues through Lys^4 and Pro^5 decreased receptor binding activity by about 2- and 3-fold, respectively, and further deletion of residues 6−8 had no further deleterious effect on receptor binding. This effect of deletion on receptor binding activity correlates with the structural effects as observed in our CD studies. Once the tertiary structure is destabilized after deletion of residues 4 and 5, further deletion of the entire polyproline helix had no additional detrimental effect on either receptor binding activity or α-helical content. These results are similar to that observed by Forest and colleagues on NPY analogs with successive alanine substitution at the N-terminus.[21] In their studies, the greatest loss of activity in rat brain membrane binding assays occurred when alanine replaced proline residues at either posi-

tion 5 or 8. In the rat brain receptor binding assay, Pro to Ala substitution at position 5 or 8 reduced binding affinity by 250- or 500-fold, respectively. In contrast, the substitution by alanine of Tyr^1 or Pro^2 produced only 125- or 111-fold reduction, respectively, of rat brain receptor binding affinity.[21] Although they did not determine the effect of these Ala substitutions on NPY's solution structure, their data also suggest that Pro^5 and Pro^8 are relatively more important for maintaining the receptor binding activity of NPY.

Martel and co-workers also studied the effect of deletion of multiple N-terminal residues on rat brain receptor binding affinities. NPY_{2-36} was 8–15-fold less active than NPY in rat whole brain,[22,23] whereas in this study N^{α}-Ac-NPY_{2-36} was only 4-fold less active than NPY. However, the rat whole brain is a mixture of the benextramine-insensitive Y_2 and the benextramine-sensitive Y_1 binding site populations.[12] We reported that in whole brain N^{α}-Ac-NPY_{2-36} is 9.3-fold less potent than NPY, with the major difference relative to this study being an increase in the K_i for NPY in cortex.[5] Thus, the difference in our results in brain cortex relative to those reported by Martel and co-workers might relate to the relative proportions of the Y_2 receptor in the tissues screened. More recently, Kalra et al.[24] reported that NPY_{2-36} is only 3.8-fold less potent than NPY in displacing [^{125}I]PYY from Y_1 receptors on rat hypothalamic membranes, results consistent with the activity of N^{α}-Ac-NPY_{2-36} at Y_1 binding sites in rat brain cortex. It is also of interest that the activity of NPY_{5-36} in displacing [^{125}I]PYY from Y_1 receptors on rat hypothalamic membranes is only 5-fold lower than that of NPY_{2-36}, and further deletion through residue 12 was without effect on activity.[24] Nevertheless, although NPY_{2-36} was active *in vivo*, NPY_{5-36} and shorter deletion fragments were inactive.[24] A similar observation was made by Boublik and co-workers[25] who observed that NPY_{2-36} had 40% of the hypertensive activity of NPY, whereas NPY_{8-36} and shorter C-terminal fragments were inactive. Thus, the poor *in vivo* activity of NPY fragments shorter than NPY_{4-36} correlates with the structural instability we observed, an instability resulting from an inability of these short deletion fragments to form a PP-fold structure.

In summary, structure–activity relationship studies using a series of N-terminal deletion fragments at the benextramine-sensitive Y_1 NPY binding sites in rat cerebral cortex indicate that tyrosine at position 1 does not contribute to the stability of the NPY tertiary structure but is directly involved in receptor–ligand interaction and that residues around Pro^5 are important in maintaining the overall tertiary structure and receptor binding activity of NPY. Surprisingly, residues 2 and 3 have no role in stabilizing the overall tertiary structure nor do they contribute directly to receptor binding, but they do function to present the functional groups of Tyr^1 for receptor–ligand interaction. These results are entirely consistent with the proposed role for, and the importance of, the PP-fold in the structure and function of NPY in rat brain cortex.

Experimental Procedures

Materials. Protected amino acid derivatives, obtained from Fisher Scientific (Fair Lawn, NJ) or Peninsula Laboratories, Inc. (Belmont, CA), were of peptide synthesis grade and analyzed by TLC for purity prior to use. Peptide synthesis solvents and reagents were reagent grade, and solvents were further purified by distillation. Buffer, BSA, and bacitracin were purchased from Sigma (St. Louis, MO), *N*-[^{3}H]Propionyl-NPY ([^{3}H]NPY) was purchased from Amersham (Arlington Heights, IL), and Sprague–Dawley rats, 250–300 g, were purchased from Sasco (Omaha, NE).

Peptide Synthesis, Purification, and Characterization. Peptides were synthesized using Merrifield's solid phase synthesis methodology on the methylbenzhydrylamine resin (0.5 mequiv/g). The synthesis was performed on a Vega Coupler 1000 semiautomated peptide synthesizer using the following synthesis protocol: dichloromethane, 3 × 1 min; 50% trifluoroacetic acid in dichloromethane or 4 N HCl in dioxane, 1 × 2 min and 1 × 30 min; dichloromethane, 3 × 1 min; ethanol, 2 × 1 min; dichloromethane, 3 × 1 min; 5% diisopropylethylamine in dichloromethane, 1 × 2 min and 1 × 5 min; dichloromethane, 3 × 1 min; 3 equiv of symmetical anhydride or 5 equiv of HOBt-activated ester coupling, 1 × 30 min plus 10 min after the addition of 1 equiv diisopropylethylamine; dichloromethane, 3 × 1 min; 20% ethanol in dichloromethane, 1 × 1 min; ethanol, 1 × 1 min; dichloromethane, 2 × 1 min. During the synthesis of NPY, portions of protected peptide resin, i.e., Boc-NPY_{4-36}-resin or Boc-NPY_{9-36}-resin, were removed for the subsequent synthesis of N^{α}-Ac-NPY_{2-36}, N^{α}-Ac-NPY_{3-36}, and N^{α}-Ac-NPY_{4-36} or N^{α}-Ac-NPY_{5-36}, N^{α}-Ac-NPY_{6-36}, N^{α}-Ac-NPY_{8-36}, and N^{α}-Ac-NPY_{9-36}, respectively. After completion of each synthesis, the terminal N^{α}-*t*-Boc group was removed with 50% trifluoroacetic acid in dichloromethane and acetyl groups were incorporated at the N^{α}-position by acylation with acetic anhydride. The 2,4-dinitrophenyl protecting group was removed using 10% thiophenol in DMF at room temperature for 6 h. The protected peptide resin was then cleaved in liquid hydrogen fluoride (HF) and extracted through Multiple Peptide Systems (San Diego, CA) custom peptide cleavage service.

The crude peptides obtained from HF cleavage were then subjected to a two-step purificaion using ion exchange fast-protein liquid chromatography (FPLC) and reverse phase high-performance liquid chromatography (HPLC). Crude peptides were purified by FPLC (flow rate = 1 mL/min) using the following conditions: for fragments 2–36, 3–36, and 4–36, 100% A (1 mM NH_4OAc, 50% MeCN, pH 5.0) to 60% B (200 mM NH_4OAc, 50% MeCN, pH 5.0) in 30 min and 60–100% B in 1 min on MonoS; for fragment 8–36, 100% A (1 mM NH_4-OAc, 50% MeCN, pH 5.5) to 100% B (200 mM NH_4OAc, 50% MeCN, pH 5.5) in 15 min on MonoS; for fragments 5–36 and 6–36, 100% A (1 mM NH_4OAc, 50% MeCN, pH 7.4) to 70% B (200 mM NH_4OAc, 50% MeCN, pH 7.4) in 15 min and 70–100% B in 2 min on MonoQ; for fragment 9–36, 100% A (1 mM NH_4OAc, 50% MeCN, pH 7.5) to 60% B (200 mM NH_4-OAc, 50% MeCN, pH 7.5) in 20 min and 60–100% B in 2 min on MonoQ. Peptides were then purified by reverse phase HPLC at a flow rate of 2.5–3 mL/min in buffers A (0.1% TFA) and B (0.1% TFA in MeCN) using the following gradients: for fragments 2–36, 3–36, and 4–36, 25–50% B in 10 min and 50% B for 8 min; for fragments 5–36, 6–36, 8–36, and 9–36, 25–60% B in 10 min and 60% B for 9 min. The purified peptides were analyzed for purity by analytical HPLC and FPLC with detection at 275 nm and by amino acid analysis and FAB^+MS. The MS analysis was performed on an AUTOSPEC-Q (Fisions VG, Ltd., Manchester, U.K.) equipped with a 35-keV cesium gun; the analysis was performed at 5000 resolution by voltage scanning over the mass range of 4500–2500. Amino acid analyses were performed by the University of Kansas Biochemical Research Service Laboratory using PITC precolumn derivatization.

We determined the concentration of stock NPY solutions by UV spectroscopy in 0.1 M HCl using the absorptivity coefficient obtained by summing the individual contribution of each tyrosine residue,[26] i.e., for NPY, $\epsilon_{275} = 6.70 \times 10^{-3}$ M^{-1} cm^{-1} in 0.1 M HCl. This absorptivity coefficient is identical to that reported for the acidic form of NPY by Krstenansky and Buck.[15] We also confirmed the concentration of a stock solution of NPY (concentration determined in 0.1 M HCl) using the method of Brandts and Kaplan.[27] In 6 M guanidine hydrochloride, pH 6.4, the experimentally-determined absor-

tivity coefficient of an NPY stock solution was $\epsilon_{275} = 7.20 \times 10^{-3}$ M^{-1} cm^{-1}, an average of 1.44×10^{-3} M^{-1} cm^{-1} per tyrosine, a value within experimental error of the expected value. The concentrations of the NPY fragments were subsequently determined in 0.1 M HCl using the absorptivity coefficient of NPY normalized to total tyrosine content.

Circular Dichroism Studies. CD spectra were recorded on a AVIV 60DS spectrometer standardized with (1S)-(+)-10-camphorsulfonic acid. CD measurements were carried out at 25 °C using siliconized 0.2-, 1-, 10-, and 50-mm CD cells. Sodium phosphate buffer (50 mM, pH 7.5) was used for all CD experiments, and the ionic strength of the buffer was adjusted to 0.15 by the addition of NaCl. The results are expressed as mean residue molar ellipticity as calculated using eq 1,

$$[\theta] = \mathrm{mD} \cdot \frac{1}{\mathrm{PL} \cdot \# \cdot \mathrm{C}} \quad (1)$$

where $[\theta]$ is the mean residue molar ellipticity in deg·cm²/dmol, mD is the CD signal in mdeg, PL is the path length of the CD cell used in mm, # is the number of residues in the peptide, and C is the total peptide concentration in M. CD spectra of peptides were taken in wavelength mode from 190 to 250 nm with a scan step size of 0.25 nm, a bandwidth of 1 nm, and a time constant of 0.2 s. All CD spectra were base line corrected. α-Helicity of peptides under these conditions was calculated from the acquired spectra using PROSEC (Aviv associates), a protein secondary structure estimation program based on the algorithm and reference spectra of Chang et al.[28]

For concentration-dependent CD studies, signals at 222 nm at different concentrations were collected every second in the kinetic mode and averaged over 1 min (a total of 61 points) with a bandwidth of 1 nm and a time constant of 0.2 s. Concentration-dependent self-association of each peptide was analyzed with a nonlinear curve-fitting program, MINSQ (Micromath, Salt Lake City, UT), by fitting the concentration-dependent CD data to eq 2,

$$C^{n-1} = \frac{K_d([\theta]_{n\text{-mer}} - [\theta]_m)^{n-1}([\theta]_{ob} - [\theta]_m)}{n[([\theta]_{n\text{-mer}} - [\theta]_m) - ([\theta]_{ob} - [\theta]_m)]^n} \quad (2)$$

where K_d is the dissociation constant for the monomer/n-mer equilibrium (n-mer ⇌ n monomer; $K_d = [\text{monomer}]^n/[n\text{-mer}]$), C is the total peptide concentration; $[\theta]_{ob}$ is the experimentally-determined concentration-dependent mean residue molar ellipticity at 222 nm, and $[\theta]_m$ and $[\theta]_{n\text{-mer}}$ are the calculated values at 222 nm for the peptide in its monomeric and n-meric states, respectively. During the nonlinear curve fitting, n was set to 2, 3, or 4 for dimer, trimer, or tetramer association, respectively. Best fit n values were chosen on the basis of the coefficient of determination (a measure of the fraction of the total variance accounted for by the model) and the model selection criterion ("information content" of the model where the most appropriate model is that with the largest MSC).

Receptor Binding Assays. Receptor binding assays were performed as reported previously using Sprague–Dawley rat cerebral cortex membrane homogenate and [^{3}H]NPY.[5] Male Sprague–Dawley rats (250–300 g) were euthanized in a CO_2 chamber for 30 s and decapitated. The rat brain was removed, placed in cold buffer (Krebs–Ringer bicarbonate buffer containing 50 mM sodium phosphate at pH 7.4), and washed twice with buffer. The cerebral cortex was then dissected, weighed, and homogenized in buffer using a Potter–Elvehjem Teflon pestle glass homogenizer (Fisher Scientific) to give the first homogenate; this homogenate was centrifuged in a Beckman Model J2-21 centrifuge at 1000 rpm for 5 min to give the first supernatant and pellet. The pellet was rehomogenized, and the resulting homogenate was centrifuged at 1000 rpm for 5 min; the pellet was discarded. The first and second supernatants were combined and centrifuged at 10 000 rpm for 20 min. The pellet was washed with and centrifuged from buffer once and then suspended in 6 mL of assay buffer (Krebs–Ringer bicarbonate buffer containing 50 mM sodium phosphate, 0.2% BSA, and 0.05% bacitracin at pH 7.4). The homogenate was diluted five times to 1.5–2.0 mg of protein/mL in the final incubation mixture. For assays, the membrane suspensions containing different concentrations of tested peptides (10^{-11}–10^{-5} M) and 1 nM [^{3}H]NPY in a total volume of 0.5 mL of assay buffer were incubated in siliconized poly(ethylene) culture tubes at 25 °C for 2 h. Nonspecific binding was determined in the presence of 1 μM NPY. Membranes were then collected using a Cambridge PHD Model 280 cell harvester on siliconized, poly(ethylenimine)-pretreated Cambridge grade 934AH glass fiber filter strips (Cambridge Technology, Inc., Watertown, MA), washed four times with 3 mL each of cold assay buffer, and dried at 55 °C for 1.5 h. The radioactivity remaining on the filters was determined in 5 mL of Scintiverse II (Fisher Chemical) using a Packard Tri-Carb 1900 TR liquid scintillation analyzer. The assays were performed in duplicate and repeated in duplicate or triplicate. The displacement data were analyzed by MINSQ by fitting the concentration-dependent, percent specific binding data to either a one- or two-site competitive binding isotherm.[29]

Acknowledgment. The authors gratefully acknowledge the American Heart Association for providing financial support for this work and Dr. Todd Williams (KU Mass Spec Lab) for providing the peptide FAB$^+$-MS spectra.

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