Novel Modified Carboxy Terminal Fragments of Neuropeptide Y with High Affinity for Y_2-Type Receptors and Potent Functional Antagonism at a Y_1-Type Receptor

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Peptide analogs of neuropeptide Y (NPY) with a Tyr-32 and Leu-34 replacement resulted in the decapeptide TyrIleAsnLeuIleTyrArgLeuArgTyr-NH_2 (**9**; Table 1) and a 3700-fold improvement in affinity at Y_2 (rat brain; $IC_{50} = 8.2 \pm 3$ nM) receptors when compared to the native NPY(27–36) C-terminal fragment. In addition, compound **9** was an agonist at Y_1 (human erythroleukemia (HEL) cell; $ED_{50} = 8.8 \pm 0.5$ nM) receptors with potency comparable to that of NPY(1–36) ($ED_{50} = 5$ nM). Molecular dynamics and ^{1}H-NMR were used to propose a solution structure of decapeptide **9** and for subsequent analog design. The replacement of Leu with Pro at position 4 of decapeptide **9** afforded an *antagonist* of NPY in HEL cells (**18**, TyrIleAsnProIleTyrArgLeuArgTyr-NH_2; $IC_{50} = 100 \pm 5$ nM). Deletion of the N-terminal tyrosine of **18** resulted in a 10-fold improvement in antagonistic activity with a parallel 4-fold decrease in Y_2 affinity. This potent antagonist may provide further insight into the physiological role(s) for NPY in the mammalian and peripheral nervous system.

Introduction

Neuropeptide Y (NPY), a 36-residue amidated peptide, was first isolated from porcine brain tissue in 1982 by Tatemoto et al.[1] The peptide is widely distributed throughout the mammalian central and peripheral sympathetic nervous systems. In the latter, it is colocalized with norepinephrine and released upon nervous stimulation causing marked vasoconstriction.[2] When administered directly into the central nervous system (CNS), NPY functions as a potent appetite stimulant[3] and anxiolytic.[4] In addition, it promotes the release of prolactin, growth hormone, and luteinizing hormone.[5] NPY elicits effects by binding to specific G-protein-coupled receptors.[6,7] Two receptor subtypes, Y_1 and Y_2, have been characterized pharmacologically;[8,9] each mediates different activities and is located in different tissues. The Y_1 receptor apparently requires the intact peptide for optimal affinity and bioactivity, whereas the C-terminal fragment of NPY(13–36) is sufficient to bind strongly to the Y_2 receptor.[9,10]

Amino Acid Sequence of Porcine NPY.

1 5 10 15 20

Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-

25 30 35

Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2

The crystal structure of avian pancreatic polypeptide (aPP) was solved by Blundell et al., and the evidence reported suggests a similar secondary structural motif for NPY.[11] More recently, structural information obtained by solution ^{1}H-NMR was described for NPY itself.[12,13] In general, these studies support the folded structure predicted for NPY, although some disparities with the aPP crystal structure were observed. Specifically, the highly homologous C-terminal fragment of NPY(32–36) (unstructured in crystalline aPP) is incorporated into the extended α-helix initiated at residue 13 of NPY.

Several groups have used a computer-generated model of NPY, based on the coordinates of the crystal structure of aPP, to help develop shorter NPY peptide analogs. Krstenansky[14] and Beck-Sickinger[15,16] designed truncated NPY agonists in which residues in the N- and C-terminal regions are retained. In addition, Rivier et al.[17,18] developed deletion analogs with and without lactam bridges. From these studies, models were proposed both to better understand the mode of binding of NPY to its receptors and to design receptor subtype specific analogs.

No selective, potent antagonists of NPY have been reported. Such antagonists allow better understanding of the physiological and pathophysiological roles of NPY. Reports describing antagonists based on modified C-terminal fragments of NPY[19] and on analogs of the nonpeptide benextramine[20] are examples of efforts to discover useful NPY antagonists. However, these compounds have relatively weak receptor affinity and therefore are of limited value. Of potentially greater interest is a recent report by Balasubramanian et al. describing the antagonist activity of [DTrp-32]NPY in a feeding behavior model.[21]

In an effort to develop potent antagonists of NPY, we initiated a program to find small peptides with high affinity for NPY receptors. Such small peptide analogs may be a first step toward our goal of developing small peptidomimetic or nonpeptide antagonists. We describe here decapeptide NPY analogs with nanomolar affinity to rat brain NPY receptors and potent agonistic activity in human erythroleukemia (HEL) cells. We also report the development of the first potent NPY antagonist in a Y_1-type receptor functional assay.

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Table 1. Comparative Potencies of NPY, NPY Fragments, and Novel C-Terminally Modified NPY Fragments in Displacing [^{3}H]NPY Binding from Rat Brain Membranes and in Inducing the Release of Intracellular Calcium ($(Ca^{2+})_i$) or Inhibiting the NPY-Induced $(Ca^{2+})_i$ Release in HEL Cells[a]

		HEL cell (Y_1)	
compound	rat brain (Y_2) IC_{50} (μM)	% activity at 100 nM or (ED_{50}, nM)	IC_{50} (nM)
1, NPY	0.00045 ± 0.00005	(3.9 ± 0.56)	
2, NPY(13-36)	0.015 ± 0.002	(333 ± 0.08)	
3, PheMetArgPhe-NH_2	40 ± 1.4	NT	
4, ThrArgGlnArgTyr-NH_2	>100	NT	
5, IleTyrArgLeuArgTyr-NH_2	2.95 ± 1.7	NT	
6, LeuIleTyrArgLeuArgTyr-NH_2	0.4 ± 0.01	NT	
7, AsnLeuIleTyrArgLeuArgTyr-NH_2	0.31 ± 0.01	NT	
8, IleAsnLeuIleTyrArgLeuArgTyr-NH_2	0.037 ± 0.006	33 ± 8	
9, TyrIleAsnLeuIleTyrArgLeuArgTyr-NH_2	0.008 ± 0.003	(8.8 ± 0.5)	
10, HisTyrIleAsnLeuIleTyrArgLeuArgTyr-NH_2	0.012 ± 0.002	100	
11, ArgHisTyrIleAsnLeuIleTyrArgLeuArgTyr-NH_2	0.012 ± 0.007	100	
12, TyrIleAsnLeuIleThrArgGlnArgTyr-NH_2	30 ± 12	0	
13, ArgHisTyrIleAsnLeuIleThrArgGlnArgTyr-NH_2	1.3 ± 0.4	0	
14, TyrIleAsnLeuIleThrArgLeuTyr-NH_2	2.1 ± 0.04	0	
15, TyrIleAsnLeuLeuTyrArgGlnArgTyr-NH_2	0.37 ± 0.16	8 ± 5	
16, TyrProSerLys-Aha-TyrIleAsnLeuIleTyrArgLeuArgTyr-NH_2	0.015 ± 0.004	40 ± 9.6	
17, Tyr-Aha-Aha-TyrIleAsnLeuIleTyrArgLeuArgTyr-NH_2	0.055 ± 0.015	23 ± 13	
18, TyrIleAsnProIleTyrArgLeuArgTyr-NH_2	0.044 ± 0.005		100 ± 5
19, IleAsnProIleTyrArgLeuArgTyr-NH_2	0.174 ± 0.02		9 ± 1.2
20, TyrIleAsnLeuLeuTyrArgProArgTyr-NH_2	2.7 ± 2		>100
21, NPY[Tyr^{32},Leu^{34}]	0.083 ± 0.038	(50)	

[a] For binding experiments, results are presented as the concentration of peptide that produces 50% displacement (IC_{50}) of specifically bound [^{3}H]NPY. Compounds tested in the HEL cell assay were initially tested at a concentration of 100 nM. Any agonistic (increase in cytosolic calcium) activity observed is expressed as percent of the response to a maximal dose of NPY (100 nM) or as ED_{50} (concentration that produces half-maximal response). Inhibitory activity was expressed as the IC_{50} (concentration that produces 50% inhibition of the response to a half-maximal dose of NPY (5 nM)). Compounds were added to the medium 30 s prior to NPY. NT indicates that these compounds were not tested in the HEL cell assay.

Chemical Synthesis and Characterization

Peptides were prepared by a modified version of the Merrifield[22] solid-phase method on a *p*-methylbenzhydrylamine (MBHA) resin. Peptide synthesis was carried out using the *tert*-butyloxycarbonyl (Boc)-protection strategy. Boc-protected amino acids were attached to the resin using a (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) coupling procedure[23] in dimethylformamide (DMF) in the presence of 1-methylimidazole. This procedure was performed automatically using an Applied Biosystems Model 430A peptide synthesizer as previously described.[24] All peptides were characterized by fast atom bombardment mass spectrometry (FAB-MS) or ionspray mass spectrometry (IS-MS) and amino acid analysis (AAA); their purity was established (>95%) by analytical HPLC and capillary zone electrophoresis (CZE).

Results and Discussion

Our initial strategy in the search for small peptide agonist/antagonists of NPY was to screen short C-terminal-amidated peptides containing aromatic and positively charged amino acids using a radiolabeled NPY receptor binding assay (Daniels, A. J., et al. Manuscript in preparation). These structural criteria were based, in part, on previously reported structure–affinity/activity studies[25] of NPY and large NPY-derived fragments that revealed the importance of specific amino acids located in the C- and N-termini. The residues Tyr-1, Arg-33, Arg-35, and Tyr-36 were essential for full affinity and bioactivity. As shown in Table 1, we found that the cardioactive tetrapeptide PheMetArgPhe-NH_2 (FMRF, **3**) displaced [^{3}H]NPY from rat brain membranes more effectively than the C-terminal pentapeptide NPY(32–36) (**4**, ThrArgGlnArgTyr-NH_2).[26]

There were several possible interpretations to account for the interaction of FMRF with rat brain NPY receptors. Inspection of a computer-generated model of NPY (Figure 1) suggested that FMRF may mimic a discontinuous epitope of NPY, specifically Tyr-1, Arg-33 or Arg-35, and Tyr-36. Alternatively, **3** may represent an active conformation of ThrArgGlnArgTyr-NH_2 (**4**), suggesting that the C-terminal region alone is sufficient for binding to Y_2-type NPY receptors; large NPY fragments, which lack the N-terminal region, such as NPY(13–36) (**2**), show high affinity for the Y_2-type receptors.[8] Finally, the possibility that FMRF interacted with a site, different from the NPY binding site, could not be ruled out.

The function of the N-terminal region of NPY is still not well understood, although one suggestion is that the N-terminal Tyr assists in stabilizing a bioactive conformation at the C-terminus while not interacting directly with the receptor.[30] We hypothesized that modification of NPY(32–36) by an "FMRF moiety" could induce a similar change in conformation, thus leading to improved binding affinity.

Irrespective of the identity of the specific binding pharmacophore or the role of the N-terminus of NPY, incorporating a larger segment of the C-terminus of NPY into an appropriately modified version of **3** might improve receptor affinity and selectivity. We decided to use the C-terminal decapeptide NPY(27–36) (**12**, TyrIleAsnLeuIleThrArgGlnArgTyr-NH_2) as a starting point and introduce modifications suggested by the FMRF sequence. This consideration, along with our awareness of known NPY structure–activity, resulted in the preparation of TyrIleAsnLeuIleTyrArgLeuArgTyr-NH_2 (**9**), which showed a 3700-fold gain in activity at Y_2 receptors with respect to the native C-terminal decapeptide **12**. In designing **9**, two Arg residues,

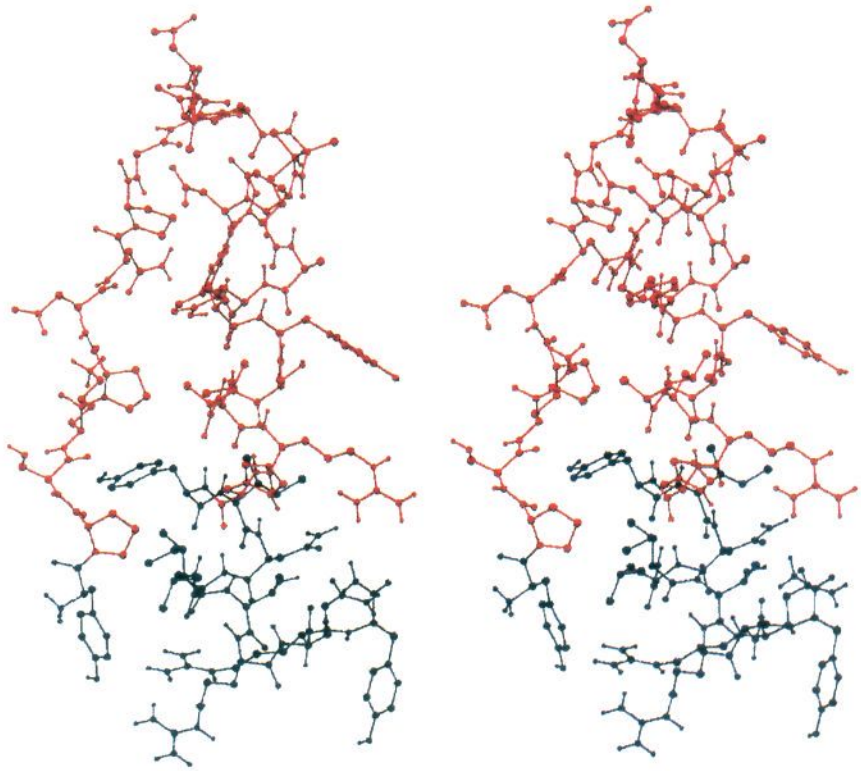

Figure 1. Stereoview of a computer-derived model of porcine neuropeptide Y (pNPY) based on the crystal structure of avian pancreatic polypeptide (aPP). Tyr-1 and residues 27−36 are highlighted in blue.

presumably corresponding to Arg-33 and Arg-35 of full-length NPY, were maintained. In addition, the Met residue of **3** was replaced by the common and chemically more stable equivalent Leu. Tyr was introduced in place of Thr-6 to accommodate the aromatic N-terminus of **3**. Neither individual replacement of Gln-8 by Leu nor Thr-6 by Tyr afforded high-affinity analogs; apparently both modifications are required (compare **9** to **14** and **15**, respectively; we have observed in a parallel series that substitution of Ile-5 by Leu had little effect on receptor binding).

In addition to showing strong affinity for brain NPY receptors, **9** also proved to be an agonist, one-half as potent as NPY, in the Y_1 receptor-mediated HEL cell Ca^{2+} assay (see Table 1). This result is noteworthy and suggests that, contrary to literature reports,[8,9] full-length NPY, particularly the N-terminus, is not required for high activity at Y_1 receptors.

The function of the critical Tyr-6 and Leu-8 residues of **9** (corresponding to residues 32 and 34 of NPY) in improving the affinity of the C-terminal decapeptide of NPY (**12**) has not been established. CD indicates that these residues increase helicity relative to the inactive native decapeptide sequence (Figure 3a). Several groups have proposed that the C-terminal α-helical domain of NPY provides an additional receptor recognition site and may play a role in receptor−subtype selectivity.[29] Furthermore, Tyr-6 may serve as a surrogate for Tyr−1 of NPY, either by directly interacting with the receptor[27] or, as suggested by Forest et al.,[30] by assisting in stabilizing a bioactive conformation at the C-terminus. The hypothesis that there is a functional relationship between Tyr-6 of compound **9** and Tyr-1 of NPY is appealing; however, additional structure−activity data need to be generated to further evaluate this proposal. We conclude that whatever the precise function of the N-terminus of NPY in inducing strong interactions with Y_1 and Y_2 receptors, these features appear to already be incorporated into the short, C-terminally modified NPY sequence of **9**.

Previously, Beck-Sickinger et al.[15] demonstrated that appending an N-terminal segment of NPY (residues 1−4) linked by a flexible spacer onto NPY(25−36) produces potent Y_2 selective agonists. Our efforts to further improve the potency of **9** by employing this strategy (**16** and **17**) resulted in a marked reduction in Y_1 activity, although Y_2 affinity was only slightly affected. Moreover, replacement of the C-terminal decapeptide sequence in full-length NPY by **9** (NPY-[Tyr^{32},Leu^{34}], **21**) did not result in any further improvement in affinity for Y_2 receptors or activity at the Y_1 receptor. It is interesting to note that while **9** is much more active than NPY(27−36) (**12**) a corresponding enhancement in activity is not observed in the full-length analog of NPY (**21**).

Having achieved our initial goal of developing high-affinity small peptides for NPY receptors, we explored strategies for converting compounds, such as **9**, into antagonists. While no general principle for developing antagonists from agonists exists, modifications in peptide sequence, which have been successfully applied to other peptide hormones, suggested a reasonable starting point. For example, incorporation of unnatural or D-amino acids leads to the development of substance P antagonists.[31] In addition, introduction of amino acids with special conformational properties, such as proline, afforded potent gastrin-releasing peptide antagonists.[32] These strategies have been applied to full-length NPY analogs with little success, except for the recent report describing antagonistic activity of [DTrp-32]NPY.[21]

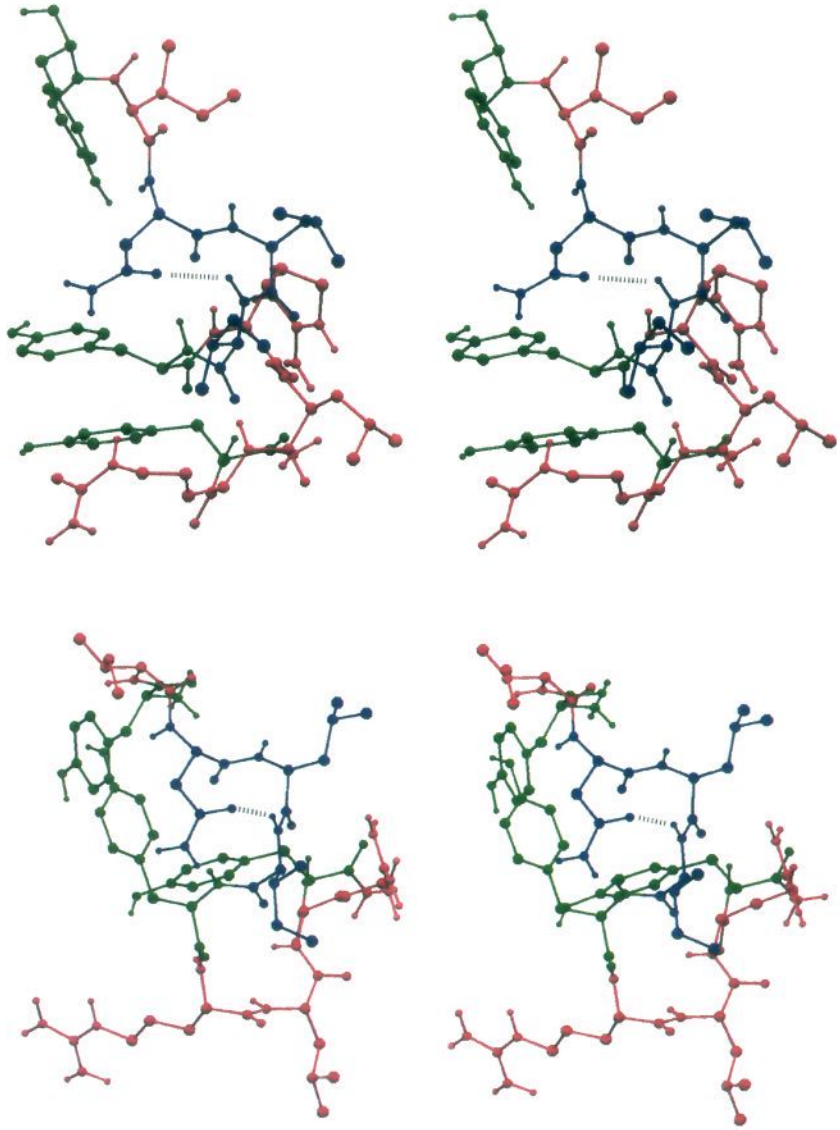

Figure 2. (top) ^{1}H-NMR-derived structure of **9**. This structure was obtained using 80 distance constraints including 32 interresidue *NOEs*, *33* intraresidue *NOEs*, *6 H*-bonds, and *9* torsional constraints. (bottom) Structure of ***9*** generated from an unconstrained molecular dynamics simulation. A representative, minimized structure (t = 600 ps) is shown and is typical of those observed from 160 ps until the end of the simulation. The Asn turn is highlighted by residues in blue (Asn-3, Leu-4, and Ile-5). The Tyr residues are shown in green. Dashed lines represent the hydrogen bond of the Asn turn from Asn-3 (CO) to Ile-5 (NH).

While these empirical approaches can lead to the successful design of antagonists, we decided to employ a strategy that focused on a rational modification of the three-dimensional structure of **9**. Toward this end, we examined the solution structure of **9** by ^{1}H-NMR and CD spectroscopy. In methanol, the number of resonances and their chemical shifts, coupling constant values, and nuclear Overhauser enhancements (NOEs) supported the presence of only one major conformer (Figure 2, top; detailed results to be published elsewhere). The most prominent feature of this structure was the presence of an α-helix from residues 3 to 10. (The CD spectrum of **9** yielded a type C spectrum (Figure 3a) indicative of a helical structure.) In addition, we observed an Asn turn,[33] highlighted by a hydrogen bond between the Asn-3 side chain carbonyl oxygen and the backbone amide N-H of Tyr-5 and/or Ile-6. Two features of the C-terminal Tyr are noteworthy. The aromatic ring is very closely associated with that of Tyr-6, and the primary carboxamide is incorporated into a reverse turn via a hydrogen bond to the carbonyl oxygen of Leu-8.

In addition to ^{1}H-NMR studies, we carried out an extended molecular dynamics simulation of **9**. The starting geometry for **9** was generated using coordinates taken from the partially helical, C-terminal decapeptide portion (residues 27−36) of an NPY model (Figure 1) derived from the crystal structure of aPP.[11] The residues corresponding to Thr-32 and Gln-34 were replaced with Tyr and Leu, respectively, and then minimized and subjected to an unconstrained molecular dynamics simulation within a spherical water shell at 310 K for 600 ps. The structures obtained from this simulation (a representative structure is shown in Figure 2, bottom) differ from those observed by ^{1}H-NMR in MeOH-d_4; however, they share important general features, specifically the formation of an Asn turn and close association of the Tyr aromatic rings at positions 6 and 10. Not

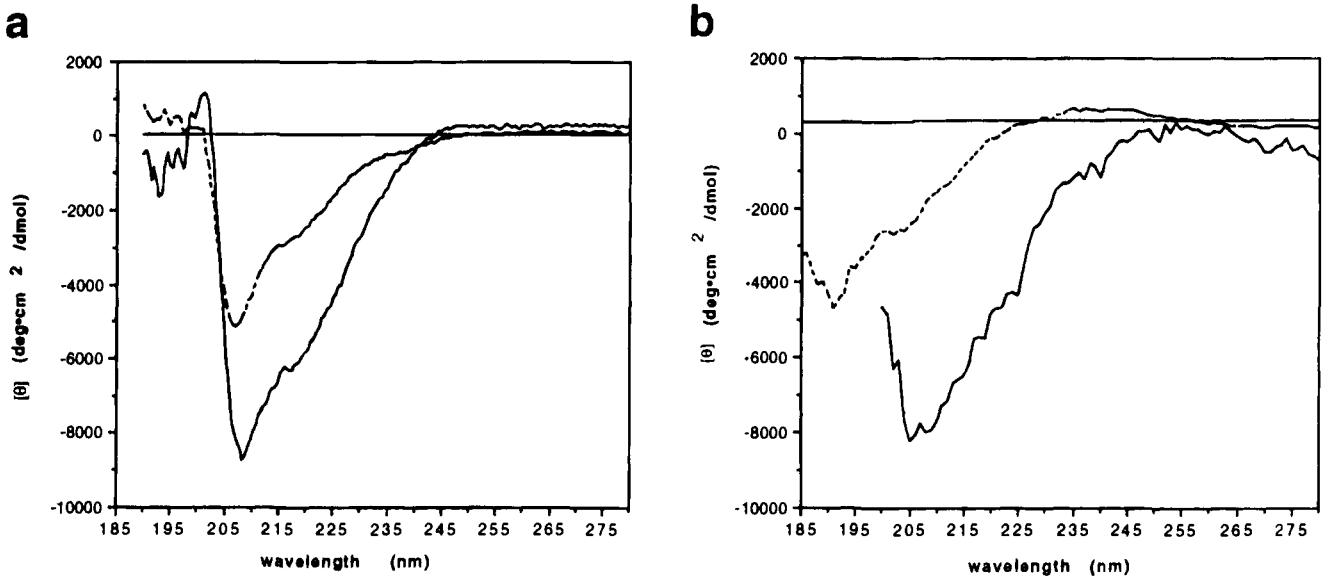

Figure 3. Far-UV region of the CD spectra of (a) compounds **9** (solid line) and **12** (dashed line) in MeOH and (b) compound **18** as a function of solvent. Solid lines correspond to 10 mM peptide in 10 mM sodium phosphate buffer, pH 7.5, and dashed lines correspond to 10 mM peptide in methanol. The CD spectrum in aqueous buffer corresponds to a mixture of predominantly unfolded conformers. Type C spectra are observed in methanol, corresponding to the presence of predominantly helical conformers.

surprisingly, a stable α-helix was not observed during the water simulation, consistent with observations made by both ^{1}H-NMR and CD of **9** in D_2O (A. Aulabaugh, to be published later).

The ^{1}H-NMR and molecular dynamics experiments suggested modifications of **9** that might increase potency through stabilization of specific structural features. For example, the sequence Asn-Pro-Xxx is especially prone to formation of an Asn turn.[34] Thus, substitution of Leu-4 by Pro afforded TyrIleAsnProIleTyrArgLeuArg-Tyr-NH_2 (**18**), which maintained good binding affinity to the Y_2 receptor. More important, however, **18** inhibited the NPY-induced mobilization of intracellular Ca^{2+} in HEL cells (IC_{50} approximately 100 nM) and, in contrast to **9**, induced no observable increase in intracellular Ca^{2+} by itself at concentrations up to 1 μM. Thus, substitution of Leu-4 by Pro effectively converted a potent agonist into a pure antagonist at the Y_1-type NPY receptor. Deletion of the N-terminal Tyr from **18** increased antagonistic activity an additional 10 times (**19**, IC_{50} = 8 ± 1.2 nM; Figure 4A), and there was a corresponding 4-fold reduction in affinity at the Y_2 receptor. The competitive nature of **19** in the HEL cell assay is shown in Figure 4B. In good correlation, **19** effectively displaced radiolabeled NPY from Y_1-type receptors in SK-N-MC cells (IC_{50} = 3 ± 0.7 nM; A. J. Daniels et al., to be published later). Figure 5 shows the inhibition isotherms for the displacement of [^{3}H]-NPY from rat brain membranes and compares the affinity of our most potent decapeptide agonists and antagonists with NPY and its C-terminal decapeptide, NPY(27–36).

A solution structure of **18** was also determined by ^{1}H-NMR in MeOH-d_4. A comparison with **9** revealed few differences apart from a proline-induced kink in the first turn of the helix defined by residues 3–10. This type of helical perturbation was previously reported by Yun et al.[35] While it is conceivable that subtle distortions of an α-helical recognition element may in part be responsible for the observed antagonism of **18** and the nonapeptide **19**, there is no direct evidence for this. A more detailed analysis of the solution structures of agonist **9**, antagonist **19**, and the C-terminal decapeptide NPY(27–36) (**12**) will be the subject of a future publication.

The judicious introduction of a proline into the backbone of **9** to afford the antagonist **18**, and ultimately **19**, led us to examine alternative positions for Pro substitution. Of special interest were decapeptide analogs of [Leu-31,Pro-34]NPY, which is a well-known potent and remarkably selective Y_1 receptor agonist.[36] As shown in Table 1, the Leu-4,Pro-7 analog of **19** (**20**), demonstrated only modest antagonistic activity (30% inhibition at 100 nM) in the Y_1 receptor-mediated HEL cell Ca^{2+} assay and also poor affinity for rat brain Y_2 receptors.

Conclusions

We have described a structure-based approach to developing the first, small, potent antagonists of NPY. These compounds should lead to a better understanding of the physiological and pathophysiological roles of NPY. The information obtained by ^{1}H-NMR, CD, and molecular dynamics indicates secondary structural features of these small peptides that may be useful in the design of more potent and selective compounds along with small molecule/nonpeptide antagonists.

Experimental Section

Materials. Porcine neuropeptide Y (pNPY, **1**) was supplied by D. Klapper of the University of North Carolina at Chapel Hill, NC, and purified in house as described below. The MBHA resin was obtained from Advanced Chemtech of Louisville, KY. The BOP reagent was received from Richelieu Biotechnologies in St-Hyacinthe, Quebec, Canada. The majority of the natural Boc- and Fmoc-protected amino acids utilized were obtained from the Bachem Chemical Co. of Torrance, CA, including Boc-His(CBZ), Boc-Ile·H_2O, Boc-Leu·H_2O, Boc-Asn(Xan), Boc-Nle, Boc-Pro, Boc-Arg(Tos), Boc-Trp, and Boc-6-aminocaproic acid (Aha). The Aldrich Chemical Co. of Milwaukee, WS, supplied 1-methylimidazole.

Peptide Synthesis and Purification. New analogs of NPY were synthesized by a modified version of the Merrifield[22]

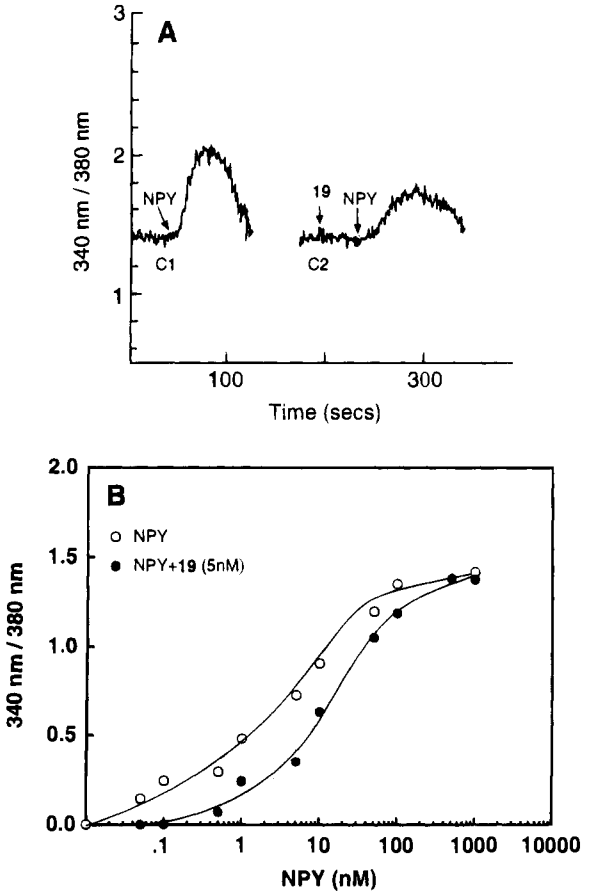

Figure 4. Characterization of the antagonistic effect of IleAsnProIleTyrArgLeuArgTyr-NH_2 (**19**) on the NPY-induced increase in cytosolic calcium in HEL cells. (A) C_1, cytosolic calcium increase in response to a half-maximal concentration of NPY (5 nM). C_2, cytosolic calcium response to 5 nM NPY, 30 s after the addition of 5 nM of compound **19** to the medium. (B) Dose–response curves for the NPY-induced increase in cytosolic calcium in the presence (●) and absence (○) of 5 nM inhibitor. The antagonist was added to the medium 30 s prior to the stimulation with NPY. Results are presented as the 340/380 nm fluorescence ratio and represent typical experiments repeated with different cell preparations with similar results.

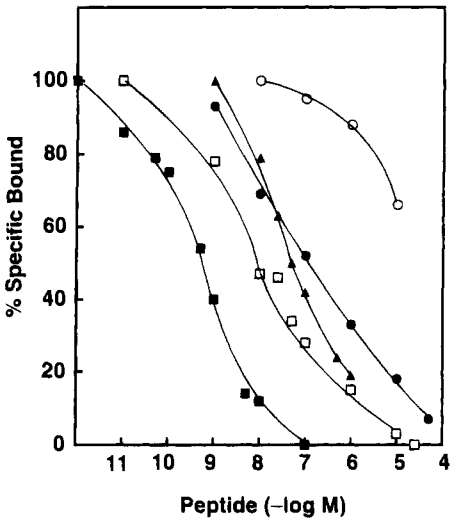

Figure 5. Inhibition isotherms for the specific binding of [^{3}H]-NPY to rat brain membranes as a function of competitor concentration. The amount of [^{3}H]NPY specifically bound at each concentration of competitor is expressed as a percentage of [^{3}H]NPY specifically bound in the absence of competitor: (■) NPY (1); (□) TyrIleAsnLeuIleTyrArgLeuArgTyr-NH_2 (**9**), (▲) TyrIleAsnProIleTyrArgLeuArgTyr-NH_2 (**18**), (●) IleAsnProIleTyrArgLeuArgTyr-NH_2 (**19**), and (○) TyrIleAsnLeuIleThrArgGlnArgTyr-NH_2 (**12**). Results represent a typical experiment repeated at least twice with identical results.

solid-phase method using the Boc-protection strategy on an Applied Biosystems Model 430A peptide synthesizer (Foster City, CA). Peptides were purified by high-performance liquid chromatography (HPLC), and their purity was established by analytical HPLC, capillary zone electrophoresis, amino acid analysis, and mass spectroscopy (fast atom bombardment or ionspray).

Peptides were synthesized by the Boc-protection procedure as follows: Boc-protected amino acids were coupled to the MBHA resin using a modified program to suit a BOP coupling procedure.[23] The coupling protocol involved dissolving Boc-protected amino acid (1 mmol), BOP (1 mmol), and 1 M 1-methylimidazole (1 mL, 1 mmol) in DMF (7 mL). The mixture was added to the resin (0.5 mmol), mixed for 1 h, and filtered. This procedure was performed automatically in the 430A peptide synthesizer with the Boc-protected amino acid and BOP filled into the amino acid cartridge using a short program routine to perform the above coupling steps. Afterward, a series of DMF and CH_2Cl_2 washes and TFA deprotection steps were performed using programs supplied by the manufacturer. This procedure was automatically repeated for each desired amino acid until the desired sequence was assembled in the synthesizer.

After the peptide was assembled on the resin, the peptide was deblocked and cleaved from the resin with liquid HF containing 10% anisole for 1 h at 0 °C, in a variation of the method described by Sakakibara et al.[37] The peptide and resin were washed with EtOAc. The peptide was then extracted with an aqueous 1% acetic acid solution and freeze-dried to obtain the dry, solid peptide.

The peptides were then purified by reverse-phase HPLC using a Vydac C_{18} column as previously described.[24]

Peptide Characterization. The composition of each analog was assessed using fast atom bombardment (FAB) mass spectroscopy (or ionspray mass spectroscopy) and amino acid analysis (see Table 1) as previously reported.[24] Capillary zone electrophoresis (CZE) was performed on a Dionex (Sunnyvale, CA) CES 1a instrument equipped with a variable wavelength detector. Fused silica capillaries (75 μm i.d., 375 μm o.d. with a length of 67 cm) were pretreated with 0.1 N sodium hydroxide for 10 min prior to use. All separations were effected with a solution of hexanesulfonic acid (50 mM) and phosphoric acid (20 mM) in Milli-Q purified water at a constant voltage of 23 kV with the capillary being rinsed with mobile phase prior to each run. The peptides were detected by UV absorbance at 215 nm. Analog data were acquired and processed on a Waters (Milford, MA) 845 data system. None of these characterization techniques showed major impurities (≥5%) in our analogs.

Circular Dichroism Spectra. Circular dichroism (CD) spectra were obtained with a Jasco J-600 spectropolarimeter operating at 22 °C. A cylindrical quartz cell of 0.5 cm was used, and spectra were recorded at 0.5 nm intervals over the wavelength range of 195–280 nm. Peptide concentrations were determined by measuring the tyrosine absorbance at 275 nm (water) or 278 nm (methanol) and using extinction coefficients of 1420 and 1790 $cm^{-1}\,M^{-1}$, respectively. Results are expressed in terms of mean residue ellipticity ($[\theta]$) in units of deg cm^2/dmol. All spectra were obtained by subtracting the buffer base line spectrum.

NMR Experiments. Proton NMR spectra were recorded at an observation frequency of 499.843 MHz on a Varian VXR-500S spectrometer at 25 °C. Proton NMR samples contained 2 mM peptide in MeOH-d_4 or D_2O.

NOE cross-peaks were separated into four distance categories depending on the intensity of the contours. Strong NOEs were given an upper constraint of 2.5 Å, medium NOEs 2.5–3.2 Å, weak–medium 2.5–4.5 Å, and weak NOEs 5.5 Å. Torsional constraints were converted to distance constraints, and H-bonding distances were used for the slow-exchanging

protons in defined secondary structure segments. A total of 10 structures satisfying the distance constraints were calculated from 100 randomly generated starting structures using the program DGEOM[38] and then further energy minimized.

Molecular Modeling. A protocol for generating a structural model for NPY based on the crystal structure of aPP is described in the literature.[17,27] We employed an analogous procedure using MacroModel V 2.5.[28] The heavy atom aPP crystallographic coordinates at 1.4 Å resolution were extracted from the Brookhaven Protein Data Bank. Each nonhomologous residue was sequentially replaced with the corresponding residue for NPY and subjected to a localized energy minimization using the torsional search (TSrch) option. For molecular dynamics, the starting geometry for **9** was generated using coordinates taken from the partially helical, C-terminal decapeptide portion (residues 27–36) of the NPY model (Figure 1) derived from the crystal structure of aPP.[12] The residues corresponding to Thr–32 and Gln–34 were replaced with Tyr and Leu, respectively, and subjected to a localized energy minimization using the torsional search option in MacroModel V 2.5. A spherical water shell was then constructed around the peptide using the EDIT option of AMBER 3.0,[39] and the system was equilibrated at 10 K for 5 ps to eliminate bad interactions. The temperature was then slowly increased to 310 K followed by unconstrained molecular dynamics at 310 K for 600 ps. A 2 fs time step and a nonbonded cutoff of 7 Å was employed.

[^{3}H]NPY Rat Brain Membrane Binding Assay. Rat brain membranes were prepared following a modified procedure of Chang et al.[40] For binding assays, 200 μL of brain membranes (0.075 mg of protein) was incubated for 60 min at 37 °C in an Eppendorf tube in a final volume of 500 μL of 50 mM Tris buffer (pH 7.4) containing BSA (0.05%), $CaCl_2$ (5 mM), $MgCl_2$ (2 mM), bacitracin (0.22%), leupeptin (0.4%), and porcine [^{3}H]propionyl NPY (Amersham; specific activity 70 Ci/mmol) at concentrations ranging from 0.1 to 1.5 nM. Specific binding was defined as that displaced by excess (100 nM) unlabeled porcine NPY and represented 65% of total binding at a ligand concentration of 0.2 nM. All conditions were tested in triplicate. Scatchard analysis of the data indicated an apparent homogeneous population of receptors with a K_d of 0.36 ± 0.03 nM and a B_{max} of 306 ± 17 fmol/mg of protein (mean ± SE; $n = 19$). Displacement curves were obtained by incubation of various dilutions of the test peptides in the presence of 0.2 nM [^{3}H]NPY under the same experimental conditions as above. The IC_{50} for NPY and each compound, in the displacement of specifically bound [^{3}H]NPY, were calculated from nonlinear regression analysis (Hill plot) of the data. The Hill slope for the NPY displacement curve was consistent with a single binding site: $n_H = 1.02 \pm 0.05$ ($n = 19$). All peptides were dissolved in 50 mM Tris buffer containing 0.5% BSA. Incubations were terminated by addition of 500 μL of ice-cold 50 mM Tris buffer containing 0.02% BSA, 2.5 mM $CaCl_2$, and 2.5 mM $MgCl_2$ followed by centrifugation at 14000*g* for 10 min in a Sorvall refrigerated centrifuge (SH-MT rotor). After centrifugation, the supernatants were carefully aspirated, and the pellets were dissolved in 200 μL of 1 N NaOH containing 0.02% Triton X-100. Aliquots of the pellet suspension (in 5 mL of Optifluor) were analyzed for radioactivity content by liquid scintillation (Beckman, LS5801).

Intracellular Calcium Measurements. Cytosolic calcium was measured as described by Daniels et al.,[41] using fura-2-loaded HEL cells in suspension. Compounds were added to the media 30 s prior to stimulation with NPY, and results were recorded as the ratio of fluorescence at 340/380 nm. Compounds, prepared in the above buffer containing 0.5% BSA, were initially tested at a concentration of 100 nM. Any agonistic (increase in cytosolic calcium) activity observed was expressed as a percent of the response to a maximal dose of NPY (100 nM) or as ED_{50} (concentration that produces half-maximal response). Inhibitory activity was expressed as the IC_{50} (concentration that produces 50% inhibition of the response to a half-maximal dose of NPY).

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Note Added in Proof: Doods and co-workers have recently reported a potent, Y_1 selective, nonpeptide, neuropeptide Y antagonist, see: Klaus, R.; Eberlein, W.; Engel, E.; Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Beck-Sickinger, A. G.; Doods, H. N. The first potent and selective non-peptide neuropeptide Y Y_1 antagonist: BIBP3226. *Eur. J. Pharmacol.* **1994**, *271*, R11–R13.

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