18, 83665-55-8; **19**, 126257-65-6; **20**, 126257-66-7; **21**, 126257-67-8; **22**, 126257-68-9; **23**, 3279-81-0; **24** (diastereomer 1), 126451-71-6; **24** (diastereomer 2), 126373-60-2; **25** (diastereomer 1), 126257-70-3; **25** (diastereomer 2), 126373-66-8; **26** (diastereomer 1), 126373-64-6; **26** (diastereomer 2), 126373-65-7; ACE, 9015-82-1; H-Cys-OEt·HCl, 868-59-7; H-Hyp-OEt·HCl, 33996-30-4; 1-amino-3-chloro-4,6-benzenedisulfonamide, 121-30-2; (3-sulfamyl-4-chlorobenzoyl)-hydrazine, 5378-62-1; 2-[(methylamino)methyl]-6-chloro-7-sulfamyl-3,4-dihydrobenzo-1,2,4-thiadiazine 1,1-dioxide hydrochloride, 126257-71-4; 2-(2-bromoethyl)-1,3-dioxolane, 18742-02-4.

Structural Study of the N-Terminal Segment of Neuropeptide Tyrosine†

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A series of analogues of neuropeptide tyrosine (NPY) was synthesized by solid-phase peptide synthesis using BOP as a coupling reagent for the complete synthesis. A structure–activity study of the N-terminal portion of the molecule was performed with the analogues obtained by the successive replacement of the first 10 amino acids by the residue L-alanine. NPY and its analogues [Ala$^{1-10}$]hNPY were tested for their potency on rat vas deferens and for their affinity to central nervous system receptors on a rat brain membrane preparation. The results suggest that the hypothetical polyproline type II helix structure of the N-terminal segment is involved in both potency and affinity. Indeed, the substitution by L-Ala of proline residues in position 2, 5, or 8 showed important losses of activity and affinity. The more important losses were observed with the replacement of Pro-5 or Pro-8. A critical loss of potency of hNPY was also observed after the substitution of the Tyr-1 residue by L-Ala, thus confirming the important role played by this residue for the full expression of the biological activity of NPY.

Neuropeptide Y (NPY), a 36 amino acid peptide containing a N-terminal tyrosine and a C-terminal tyrosine amide, was isolated from porcine brain by Tatemoto et al.[1,2] in 1982. The elucidation of the primary structure showed that this peptide has a high degree of homology with peptide YY (70%) and pancreatic polypeptides (47–56%).[3] Several studies confirmed that NPY is largely distributed in peripheral anatomical structures, such as the heart,[4,5] the gastrointestinal tract,[6,7] the reproductive organs,[8] and the perivascular nerve fibers.[8] NPY has also been found in various areas of the central nervous system[9,10] (CNS) where it would be involved in the control of blood pressure and appetite.[11,12] In the peripheral nervous system, NPY exhibits a potent vasoconstrictor activity and participates to the tonus control of smooth muscle by playing a co-transmitter role with norepinephrine in noradrenergic neurons.[13]

Syntheses of NPY were reported by various groups using solution techniques,[14] the conventional DCC method,[15] or the symmetrical anhydride procedure.[3,16,17] Molecular analyses[17-21] and structure–activity studies[3,15,22-24] were also carried out with NPY itself and closely related peptides. These investigations allowed the elaboration of a structural model containing a N-terminal polyproline type II helix and a C-terminal α-helix, connected by a type II β-turn.

Table I. Protocol for a Synthetic Cycle Using BOP Reagent[a]

step	reagent	time
1	CH_2Cl_2	2 × 1 min
2	50% TFA/CH_2Cl_2[b]	1 × 5 min
3	50% TFA/CH_2Cl_2	1 × 20 min
4	CH_2Cl_2	3 × 1 min
5	DMF	3 × 1 min
6	3 equiv of Boc-AA-COOH/DMF + 3 equiv of BOP reagent/DMF + 5 equiv of DIEA	1 ×[c]
7	DMF	3 × 1 min
8	CH_2Cl_2	2 × 1 min

[a] Solvent for all washings and couplings were measured to volumes of 10–20 mL/g of resin. [b] 1% DMS was added after the incorporation of the methionine residue. [c] Couplings are usually achieved in less than 30 min.

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† Abbreviations: The abbreviations for the amino acids are in accord with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9–37). L-isomers of amino acids were used. In addition: hNPY, human neuropeptide tyrosine; Boc, *tert*-butoxycarbonyl; BHA, benzhydrylamine, Tos, *p*-tolylsulfonyl; OcHx, cyclohexyl ester; ClZ, [(2-chlorobenzyl)oxy]carbonyl; Bzl, benzyl ester; Dcb, 2,6-dichlorobenzyl; DMF, dimethylformamide; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; DMS, dimethyl sulfide; DIEA, diisopropylethylamine; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; SAR, structure–activity relationship.

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Table II. Characterization of [Ala$^{1-10}$] hNPY Analogues by HPLC, TLC, and Amino Acid Analysis

no.	hNPY analogue	HPLC[a] ^tR, min	TLC[b] R_f	amino acid analysis[c]													
				Tyr	Pro	Ser	Lys	Asx	Gly	Glx	Ala	Met	Arg	Leu	His	Ile	Thr
1	hNPY	12.21	0.55	5.17	4.01	1.77	0.96	5.02	1.02	2.89	3.91	1.01	4.29	2.07	0.92	1.93	0.93
2	[Ala^1]hNPY	12.06	0.52	4.45	4.30	1.73	0.90	5.04	1.00	3.25	4.69	0.97	3.75	1.95	1.08	1.95	0.94
3	[Ala^2]hNPY	12.16	0.52	5.35	3.24	1.80	0.97	5.27	1.00	3.05	4.63	1.03	3.70	1.95	1.13	1.93	0.93
4	[Ala^3]hNPY	12.29	0.50	4.99	4.38	0.84	0.99	5.38	1.01	3.12	4.76	0.99	3.80	1.95	0.89	1.98	0.95
5	[Ala^4]hNPY	13.17	0.49	4.80	4.09	2.00	0.00	5.53	0.93	2.86	4.71	1.07	3.76	2.18	0.95	2.08	0.94
6	[Ala^5]hNPY	12.22	0.49	5.05	3.02	1.80	1.05	5.33	1.09	3.05	4.84	0.97	3.88	2.07	0.88	2.03	0.97
7	[Ala^6]hNPy	12.22	0.49	5.25	4.36	1.80	1.00	4.21	1.03	3.17	4.91	0.91	3.93	1.80	0.88	1.90	0.98
8	[Ala^7]hNPy	12.29	0.48	5.04	4.04	1.90	1.04	4.20	1.01	3.12	5.00	0.88	4.00	1.90	0.89	1.90	1.00
9	[Ala^8]hNPY	12.99	0.49	5.24	3.20	1.73	1.10	5.20	1.02	3.26	4.73	1.01	3.78	1.87	1.04	1.92	0.95
10	[Ala^9]hNPY	12.81	0.50	5.50	4.23	1.90	1.12	4.80	0.00	3.07	4.85	1.00	3.87	1.90	0.90	1.99	0.97
11	[Ala^{10}]hNPY	13.33	0.55	5.22	3.89	1.50	1.05	5.32	1.11	2.00	4.72	0.95	3.77	2.10	1.13	2.05	0.94

[a] Retention times in minutes following the analytical HPLC conditions described in the Experimental Section. [b] Thin-layer chromatography in the solvent system 1-butanol–acetic acid–pyridine–water (30:6:24:20, v/v). [c] Amino acid analyses from acid hydrolysates (distilled 6 N HCl at 110 °C in evacuated sealed tubes for 24 h).

The SAR studies showed the importance of both N- and C-terminal segments of the NPY molecule for attaining potent biological activities. It appears that residues found in the N-terminal portion are essential for the affinity while amino acids of the C-terminus would contain the message for triggering the biological response.[22-25]

In order to better understand the contribution of the N-terminal residues of NPY to the receptor affinity and the expression of the biological activity, we synthesized a series of N-terminal analogues by solid-phase peptide synthesis, in which each of the first 10 amino acid residues were successively substituted by an L-alanine. The biological activities of these analogues were evaluated by using the rat deferens bioassay, and their affinity to CNS receptors was estimated by using the rat brain membrane binding preparation.

Results and Discussion

NPY and analogues were synthesized with the BOP reagent,[26] according to synthetic methods recently described by Fournier and Coll.[27,28] As shown in Figure 1, an excellent crude material of hNPY was obtained by solid-phase peptide synthesis using BOP as a coupling reagent. All couplings, including those of Boc-asparagine and Boc-histidine(tosyl), were carried out with BOP. Indeed, we recently showed that no apparent detosylation nor racemization occurs when Boc-His(Tos) is used in combination with BOP,[28] a reagent producing hydroxybenzotriazole in situ during the coupling reaction. The quality of the crude material (Figure 1) shows that a complete synthesis of NPY can be performed with only BOP as a coupling reagent, instead of the troublesome dicyclohexylcarbodiimide (which forms dicyclohexylurea requiring filtration, necessitates long coupling times, and causes allergic reaction), as used by other groups.[3,15-17] As described in Table I, the concomittant neutralization–coupling step, initially suggested by Le-Nguyen et al.[29] was used for the syntheses. This technique which is made possible by the use of BOP, since the coupling is performed

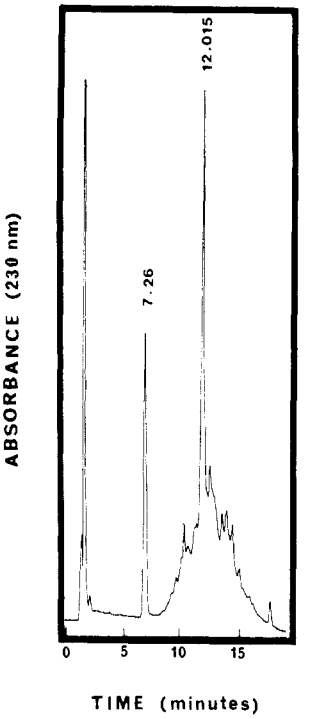

Figure 1. Analytical HPLC of crude hNPY after solid-phase synthesis with BOP reagent. Conditions: Vydac C_{18} column (10 μm; 30 × 0.39 cm); eluant, (A) 0.06% TFA and (B) CH_3CN; gradient, linear 25% to 65% B in 20 min; flow rate, 1.5 mL/min; detection, 230 nm.

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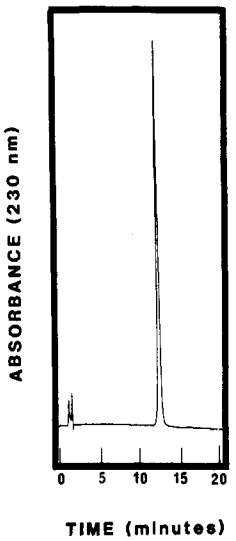

Figure 2. Analytical HPLC of purified hNPY. See legend of Figure 1 for conditions.

in the presence of base, contributing to a decrease in the time needed for a complete coupling cycle.

Hydrogen fluoride (HF) was used to cleave NPY and analogues from the benzhydrylamine resin support, and the peptide preparations were extracted with pure TFA. The use of TFA for the extraction probably explains the presence of the strongly absorbing side product found in the crude preparation and eluting at 7.26 min. Indeed, as we recently described,[28] residual *p*-toluenesulfonic acid, coming from side-chain deprotection and still present in the polymeric support even after numerous washings with diethyl ether, is extracted from the resin with TFA. All peptides were purified with preparative HPLC. Highly purified (≥98%) preparations were obtained as confirmed by analytical HPLC (Figure 2) and yields, although they were not optimized, were satisfactory (5%–15%). The characterization of the peptides, achieved by analytical HPLC, TLC, and amino acid analysis using the Waters Pico Tag method (Table II), assessed the excellent quality of the synthetic peptide preparations.

The rat vas deferens was chosen to evaluate the potency of each analogue. In this preparation, NPY is known to coexist wth norepinephrine in sympathetic neurons where it plays a role of a modulator to adrenergic neurotransmission. NPY inhibits in a concentration-dependent manner the electrically induced muscle twitches of the rat vas deferens[24] (Figure 3). The inhibition of muscle contraction obtained with hNPY served as a standard, representing 100% of inhibition. The relative potency of inhibition of the series [L-Ala^1]hNPY to [L-Ala^{10}]hNPY in the rat vas deferens (Table III) was as follows: hNPY > [Ala^6]hNPY > [Ala^3]hNPY > [Ala^7]hNPY > [Ala^4]-hNPY ≥ [Ala^2]hNPY ≥ [Ala^9]hNPY > [Ala^{10}]hNPY > [Ala^8]hNPY ≥ [Ala^5]hNPY > [Ala^1]hNPY. From these relative potencies, it clearly appears that positions 5 and 8, which are occupied by proline residues in the native molecule and are believed to contribute to the stability of the conformation by intramolecular hydrophobic interactions,[21] are extremely sensitive to a substitution by L-Ala. This observation strongly suggests the importance of the integrity of the polyproline type II helix for the full expression of the biological activity in the vas deferens preparation.

It was already known that a deletion of Tyr^1 (NPY$_{2-36}$) shows a reduction of potency.[22,24] This deletion is particularly detrimental in the vas deferens preparation, in which the fragment 2–36 is approximately 5-fold less potent than NPY itself.[30] When the N-terminal tyrosine is substituted by an L-alanine residue, the potency of this analogue is also considerably decreased, being approximately 40-fold less potent than hNPY (Table II). An explanation could be based on the hypothetical 3-D structure of NPY,[19–21] in which the position 1 and the terminal portion of the α-helix are in close spatial orientations. The substitution of the Tyr^1 by L-Ala would affect the conformation of NPY by decreasing the intramolecular stability. In fact, most representations of NPY show an extension of the tyrosine-27 side chain toward the N-terminal residues Tyr^1 and Pro^2. Therefore, a structure containing a stacking of the aromatic nuclei of Tyr^1 and Tyr^{27} is conceivable. This stacking would contribute to the stabilization of the hairpin shape of the molecule, thus

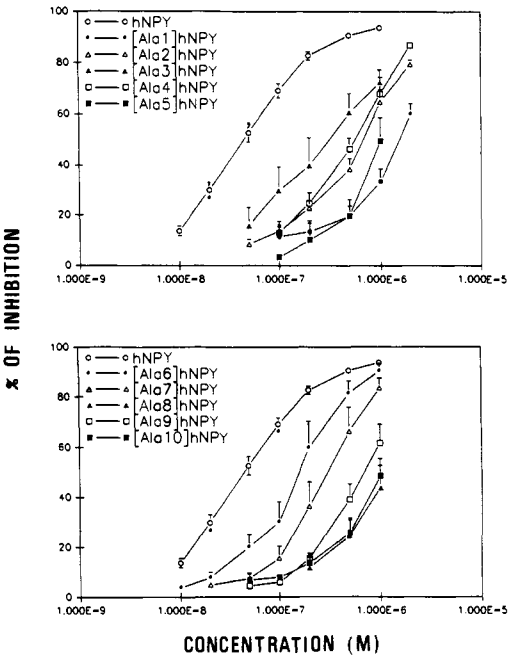

Figure 3. Dose–response curves of inhibition of the electrically stimulated twitch response of the rat vas deferens preparation for hNPY and synthetic analogues.

Table III. Relative Potencies in the Rat Vas Deferens Bioassay and Relative Affinities in the Rat Brain Membrane Binding Preparation of [Ala$^{1-10}$]hNPY Analogues

	(A) rat vas deferens		(B) rat brain membrane preparation	
NPY analogues	IC_{50}[a] ± SE,[b] nM	RP[c]	IC_{50} ± SE,[d] nM	RA[e]
hNPY	44 ± 2	100	4.1 ± 0.8	100
[Ala^1]hNPY	1698 ± 569	3	504 ± 152	0.8
[Ala^2]hNPY	651 ± 63	7	462 ± 93	0.9
[Ala^3]hNPY	227 ± 32	19	24 ± 6	17
[Ala^4]hNPY	554 ± 40	8	185 ± 21	2
[Ala^5]hNPY	1152 ± 13	4	1150 ± 266	0.4
[Ala^6]hNPY	155 ± 11	29	37 ± 8	11
[Ala^7]hNPY	303 ± 9	15	40 ± 8	10
[Ala^8]hNPY	1114 ± 231	4	2075 ± 408	0.2
[Ala^9]hNPY	681 ± 40	7	119 ± 13	4
[Ala^{10}]hNPY	981 ± 104	5	238 ± 83	2

[a] IC_{50} = the concentration of peptide producing a 50% inhibition of the maximum effect. Obtained from dose–response curves. [b] SE = standard error (vas deferens bioassay, $n \geq 6$; membrane preparation, $n \geq 3$). [c] RP = relative potency. [d] IC_{50} = the concentration of peptide producing a 50% inhibition of the maximum binding. Obtained from dose–response curves. [e] RA = relative affinity.

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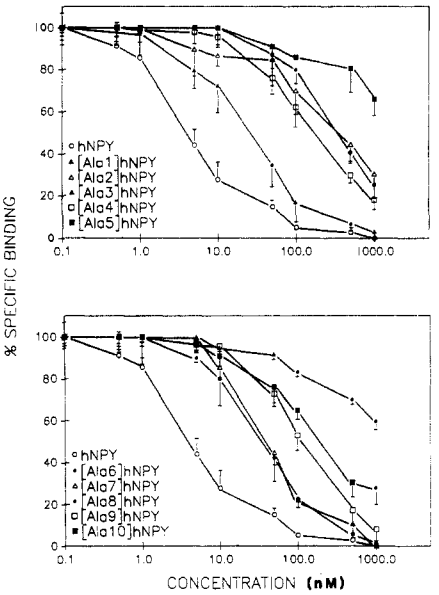

Figure 4. Competition curves of binding of [^{3}H]NPY to the rat brain membrane preparation, obtained with hNPY and synthetic analogues. Each point is the mean of at least three experiments performed in duplicate.

insuring a good recognition of the ligand by the receptor (see the binding results) and the expression of the activity. The presence of Tyr^1 would be a determinant factor, more precisely in the vas deferens preparation which allows the evaluation of the presynaptic activity of NPY. Concerning the other substitutions, it mainly appears that the tight central bend is also an important feature of the molecule since [Ala^{10}]hNPY is a weak agonist. Moreover, the contribution of 4-lysine will need further evaluations. Indeed, we observed for [Ala^4]hNPY a relative potency of only 8%. Similar results were reported by Boublik et al.,[15] who showed that the inversion of chirality of Lys^4 gives rise to an analogue being 5-fold less potent than NPY.

The relative potency of each analogue in inhibiting [^{3}H]NPY specific binding (Figure 4) is in decreasing order as follows: hNPY > [Ala^3]hNPY > [Ala^6]hNPY ≥ [Ala^7]hNPY > [Ala^9]hNPY > [Ala^4]hNPY ≥ [Ala^{10}]hNPY > [Ala^2]hNPY ≥ [Ala^1]hNPY > [Ala^5]hNPY > [Ala^8]NPY (Table III). It clearly appears that the proline residues located in position 2, 5, and 8 are mainly involved in the recognition mechanism of NPY by its CNS receptor, by keeping the structural integrity of the polyproline type II helix found in the N-terminal segment of the molecule. In particular, positions 5 and 8 seem to be extremely critical for binding to receptors of the central nervous system since relative affinities of only 0.4% and 0.2% were obtained respectively for [Ala^5]- and [Ala^8]hNPY. As seen by using the rat deferens bioassay, the substitution of the N-terminal tyrosine also reveals an important decrease of affinity, this analogue giving a IC_{50} of 504 ± 152 in comparison to 4.1 ± 0.8 for hNPY (RA = 0.8%). This critical importance of Tyr^1 for the binding of NPY to CNS receptor was also observed by Martel et al.[30] with the fragment 2–36. However, porcine NPY_{2-36} retains a relative affinity of 20% (using pNPY as a standard) compared to only 0.8% for [Ala^1]hNPY (using hNPY as a standard). This suggests that, in addition to the above mentioned structural role proposed for Tyr^1, this amino acid would also participate in the recognition mechanism of the ligand by directly interacting with the CNS receptor.

As shown in Table III, there is a good correlation between the decrease of biological activity evaluated with the rat vas deferens bioassay and the decrease of affinity measured by using the rat brain membrane preparation. Even if few residues seem to be more important in one assay than in the other, a condition suggesting that the brain and vas deferens NPY receptors are different entities, although the brain might contain a heterogenous receptor population giving rise to a certain degree of overlap, it is clear that any changes involving the proline residues are responsible for important decreases of affinity and activity. The substitution of residues Lys, Gly, and Glu, at position 4, 9, and 10, respectively, with L-alanine also presents nonnegligeable reductions of potency and affinity. These observations are in agreement with results previously reported by Boublik et al.[15]

From this investigation, it can be concluded that proline residues and, more generally, the N-terminal polyproline type II helix are important structural features for a potent interaction of NPY with its receptor. Although the proline residues probably do not participate in the triggering of the biological response, they contribute to the full expression of the biological activity of NPY by stabilizing the conformation. This structural stabilization would occur by means of hydrophobic interactions with the antiparallel α-helix formed in the second segment of the molecule.[21] The importance of the N-terminal tyrosine residue was also demonstrated. According to our observations, this amino acid would play not only a structural role but would also directly participate in the interaction with the CNS receptor. This seems to be confirmed by the recent demonstrations that potent analogues of NPY are obtained by linking N-terminus amino acids to C-terminal residues[21,31] via spacers.

Experimental Section

Reagents and Solvents. Boc-protected amino acid derivatives and BOP reagent were purchased from Richelieu Biotechnologies (St-Hyacinthe, Québec, Canada). ACS-grade dimethylformamide and methylene chloride were obtained from Anachemia Canada Inc. (Ville St-Pierre, Québec, Canada) and Biograde trifluoroacetic acid was purchased from Halocarbon (Hackensack, NJ). Diisopropylethylamine was obtained from Pfaltz and Bauer (Waterbury, CT) and was distilled from ninhydrin before use. Finally, benzhydrylamine resin (copolystyrene–1% divinylbenzene, 0.27 mequiv/g) was from Bio-Méga Inc. (Laval, Québec, Canada).

1. **Peptide Synthesis and Purification.** NPY and its analogues were synthesized by using the solid-phase peptide synthesis (SPPS) method,[32] following the protocol described in Table I. Benzhydrylamine resin was used as a solid support and couplings were carried out with BOP reagent. The syntheses of the peptide analogues were simultaneously carried out on a manual homemade multireactor synthesizer. Side-chain protection of α-Boc-amino acids was as follows: Arg(Tos), Asp(OcHx), Glu(Bzl), His(Tos), Lys(ClZ), Ser(Bzl), Thr(Bzl), and Tyr(Dcb). Peptides were cleaved from the polymeric support with liquid hydrofluoric acid (HF) in presence of anisole (1 mL/g) and DMS (1 mL/g) at 0 °C for 90 min. After precipitation and washings with anhydrous diethyl ether, the crude peptides were extracted with pure trifluoroacetic acid followed by evaporation.

2. **Peptide Purification.** All crude peptides were purified by preparative reverse-phase HPLC on a Waters Prep LC 3000 system equipped with a Waters 1000 Prep Pak Module and a Model 441 absorbance detector. Peptide solutions were injected on a DeltaPak C_{18} (15 μm, 300 Å) column (30 × 5.7 cm). The material was eluted with successive linear gradients of (A) H_2O (0.06% TFA) and (B) CH_3CN (40%) in H_2O containing 0.06% TFA, as follows: 0% B to 40% B in 10 min, 40% B to 60% B in 15 min and 60% B to 100% B in 40 min. The flow rate was maintained at 64 mL/min and detection was at 230 nm. Individual fractions were analyzed by HPLC on a 600 Multisolvent

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Delivery System equipped with a Lambda-Max Model 481 LC spectrophotometer. The analyses were carried out with a Vydac (10 μm) reverse-phase column (30 × 0.39 cm) and an eluant of (A) H_2O (0.06% TFA) and (B) CH_3CN in a linear gradient mode (25% B to 65% B in 20 min) at a flow rate of 1.5 mL/min and detection at 230 nm. The fractions corresponding to the purified peptide were pooled and lyophilized.

3. Peptide Characterization. Each preparation was characterized by analytical HPLC with the system described in the previous section and by TLC on 0.25 mm thick Merck chromatogram plates with fluorescent indicator (F-254) in the solvent system BAPW (1-butanol–acetic acid–pyridine–water, 30:6:24:20). The peptide preparations were also characterized by amino acid analysis following hydrolysis in constantly boiling HCl containing 0.1% (w/v) phenol at 110 °C for 24 h. The samples were analyzed after drying and derivatization with PITC, according to the method described by Waters Chromatography. The analyses were carried out with an HPLC system comprising two Waters 510 pumps, a Waters 715 Ultra Wisp sample processor, a Waters TCM temperature controller coupled to a column heater module, and a Waters PICO.TAG amino acid analysis column. During the analysis, the column was kept at 38 °C and the elution of the PTC amino acid derivatives was achieved with successive gradients of CH_3CN in a sodium acetate buffer, according to the operation table described by Waters. The system was controlled and the data were processed with the Waters Baseline 810 chromatography workstation software with the NEC-APC IV Power Mate computer. The results were printed with a NEC Pinwriter P5200.

Bioassay and Binding. 1. Rat Vas Deferens Preparation. Adult male Sprague–Dawley (175–225 g) from Charles River (St-Constant, Québec, Canada) were sacrificed by decapitation. The abdominal cavity was incised, and the vasa deferentia were dissected out and placed immediately in oxygenated (95% O_2–5% CO_2) Krebs–Ringer buffer solution [(mmol/L) NaCl (118), KCl (5.4) $CaCl_2$ (2.5), KH_2PO_4 (1.2), $NaHCO_3$ (23.8), and glucose (11.1)] at 37 °C. The vasa deferentia were carefully freed from connective tissue and mounted on platinum electrodes in a double-jacketed tissue bath containing oxygenated Krebs–Ringer buffer and maintained at 37 °C. The tissues were equilibrated for 1 h at a tension of 0.5–1 g. The tissues were stimulated with square electrical pulses (frequency, 0.15 Hz; duration, 0.5 ms; and amplitude 60–90 V), and the force of the twitch response was recorded on a Grass polygraph (Quincy, MA) with a force transducer. The effects of NPY and NPY analogues were measured by adding cumulative doses of the peptides in the concentration range from 1 nM to 1000 nM. A dose–response curve was measured for each analogue and their potency was evaluated with hNPY as 100%.

2. Rat Brain Membrane Binding Preparation. Male Sprague–Dawley rats (200–250 g) were killed by decapitation, and the brain (without the cerebellum) was rapidly removed and placed on ice. The cerebral tissue was homogenized in Krebs–Ringer buffer (25:1, m/v), pH 7.4, at 25 °C using a Brinkman Polytron (set at 6, for 15–20 s) and the homogenate was centrifuged at 18000*g* for 15 min. The supernatant was discarded and the resulting pellet was washed, resuspended, and centrifuged again. The final pellet was rinsed and resuspended in Krebs–Ringer buffer to give a protein concentration[33] of 6–9 mg/mL of membrane preparations. For binding assays, 100 μL of membrane preparation was incubated at 25 °C for 120 min in a final volume of 500 μL of Krebs–Ringer buffer, pH 7.4, containing 0.1% bovine serum albumin (ICN Biomedicals, Montréal, Québec), 0.05% bacitracin (Sigma, St. Louis, MO), and 0.5 nM [^{3}H]pNPY (70 Ci/mmol), Amersham Canada, Oakville, Ontario, Canada). Incubations were terminated by rapid filtration through Schleicher & Schuel #32 filters (previously soaked in 1.0% polyethyleneimine) using a cell harvester (Brandel, Gaithersburg, MD). The filters were rinsed three times with 3 mL of ice-cold buffer. Binding of the ligand to the filters was quantitated by counting filters in 5 mL of Eco-lite scintillation cocktail (Fisher Scientific, Montréal, Québec, Canada). Specific binding was calculated as the differences in radioactivity bound in the presence and absence of 1.0 μM hNPY. The displacement curves were evaluated by using a concentration of NPY analogues ranging from 1 to 1000 nM.

3. Statistics. Binding data were analyzed by computerized linear-regression analysis (Bio-Soft Elsevier, Cambridge, U.K.). Values are expressed as means ± SEM.

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