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Comparative Structural Requirements of Brain Neuropeptide Y Binding Sites and Vas Deferens Neuropeptide Y Receptors

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SUMMARY

A series of fragments and analogues of neuropeptide Y (NPY), both human (hNPY) and porcine (pNPY), were synthesized and tested for their affinities at brain NPY receptor binding sites and their potencies in inhibiting the electrically stimulated twitch response of rat vas deferens. Results with N- and C-terminal fragments suggest that amino acid residues in the N-terminal portion of the molecule are mostly important for recognition of brain and vas deferens NPY receptors, in addition to being relevant for the maintenance of adequate receptor affinity. On the other hand, C-terminal amino acid residues appear to be responsible for triggering receptor activation in the rat vas deferens preparation, because full intrinsic activity is maintained with fragments up to NPY_{18-36}. C-terminal fragment NPY_{25-36} and N-terminal fragment NPY_{1-15} were devoid of affinity for [^{3}H]NPY brain receptor sites and showed no activity in the rat vas deferens preparation. Similarly, N-terminal fragment $hNPY_{1-24}CONH_2$ showed no affinity toward [^{3}H]NPY brain receptor sites and no inhibition of the twitch response in the rat vas deferens preparation at concentrations up to 1.0 μM. On the contrary, this fragment appears to selectively increase the amplitude of the twitch response to electrical stimulation at low micromolar concentrations, an effect opposite to that of NPY and all other NPY fragments and analogues studied here. The exact mechanism mediating this contractile action of $hNPY_{1-24}CONH_2$ remains to be established. Modifications of the tyrosine residue in position 20 led to the development of two analogues, [D-Tyr^{20}]hNPY and [D-Trp^{20}]hNPY, which show an apparent preference for the vas deferens NPY receptor. On the other hand, substitutions of the tyrosine residue in position 21 by a phenylalanine ([Phe^{21}]hNPY) or a methylated tyrosine residue ([Tyr-*O*-Me^{21}]hNPY) produced analogues demonstrating an apparent preference for the brain receptor site. This suggests that modifications of tyrosine residues at positions 20 and/or 21 may eventually lead to the development of NPY analogues distinguishing between the most abundant class of sites present in the brain and vas deferens, respectively.

NPY is a 36-amino acid peptide originally isolated by Tatemoto and co-workers (1, 2). This peptide shares important sequence homologies with a group of peptides known as the PPs (approximately 50% homology) (3) and with PYY (69% homology), a peptide originally isolated from porcine intestine (4). All these peptides have, therefore, been included in the PP superfamily (1). It has also been demonstrated that hNPY and pNPY are structurally identical except for residue 17 (methionine in hNPY, as compared with leucine in pNPY; see Table 1) (3).

Important quantities of NPY-like immunoreactivity are present throughout the CNS of all mammalian species studied thus far (3, 5, 6), including humans (7, 8). Important amounts of NPY-like immunoreactivity are found especially in hypothalamic nuclei, limbic structures such as cortex, hippocampus, and amygdala, striatum, and several brainstem nuclei (9–11).

Numerous pharmacological and behavioral effects of centrally administered NPY and homologues have been reported (3). For example, intracerebroventricular injections of NPY markedly stimulate water and food intake (12, 13), particularly when directly injected in the paraventricular nucleus (14). This effect of NPY can also be elicited by PYY (15) and other members of the PP family (16, 17). More recently, Drumheller and colleagues (18) have also demonstrated that central injections of NPY caused changes in body temperature, decreased motor activity, induced catalepsy, and increased muscular tone. As with other members of the PP family (19), NPY has been shown to shift circadian rhythms when injected into the suprachiasmatic nucleus (20). In the peripheral nervous system, NPY is often colocalized (21) and coreleased with noradrenaline and

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ABBREVIATIONS: NPY, neuropeptide Y; Cha, cyclohexylalanine; CNS, central nervous system; hNPY, human neuropeptide Y; HPLC, high pressure liquid chromatography; pNPY, porcine neuropeptide Y; PP, pancreatic polypeptide; PYY, peptide YY.

TABLE 1

Amino acids sequence of various PP homologues and fragments

Amino acids are represented by their one-letter symbols (68). pPYY, porcine peptide YY; a, avian; b, bovine; h, human; p, porcine; and r, rat PP. Bold amino acids, those shared with hNPY by other members of the PP family.

Peptide	Amino acid																															
	1				5					10					15					20					25					30					35				
hNPY	**Y**	**P**	**S**	**K**	**P**	**D**	**N**	**P**	**G**	**E**	**D**	**A**	**P**	**A**	**E**	**D**	**M**	**A**	**R**	**Y**	**Y**	**S**	**A**	**L**	**R**	**H**	**Y**	**I**	**N**	**L**	**I**	**T**	**R**	**Q**	**R**	**Y**[a]
pNPY	**Y**	**P**	**S**	**K**	**P**	**D**	**N**	**P**	**G**	**E**	**D**	**A**	**P**	**A**	**E**	**D**	**L**	**A**	**R**	**Y**	**Y**	**S**	**A**	**L**	**R**	**H**	**Y**	**I**	**N**	**L**	**I**	**T**	**R**	**Q**	**R**	**Y**[a]
pPYY	**Y**	**P**	**A**	**K**	**P**	**E**	**A**	**P**	**G**	**E**	**D**	**A**	**S**	**P**	**E**	**E**	**L**	**S**	**R**	**Y**	**Y**	**A**	**S**	**L**	**R**	**H**	**Y**	**L**	**N**	**L**	**V**	**T**	**R**	**Q**	**R**	**Y**[a]
aPP	**G**	**P**	**S**	**Q**	**P**	**T**	**Y**	**P**	**G**	**D**	**D**	**A**	**P**	**V**	**E**	**D**	**L**	**I**	**R**	**F**	**Y**	**D**	**N**	**L**	**Q**	**Q**	**Y**	**L**	**N**	**V**	**V**	**T**	**R**	**H**	**R**	**Y**[a]
bPP	**A**	**P**	**L**	**E**	**P**	**E**	**Y**	**P**	**G**	**D**	**N**	**A**	**T**	**P**	**E**	**Q**	**M**	**A**	**Q**	**Y**	**A**	**A**	**E**	**L**	**R**	**R**	**Y**	**I**	**N**	**M**	**L**	**T**	**R**	**P**	**R**	**Y**[a]
hPP	**A**	**P**	**L**	**E**	**P**	**V**	**Y**	**P**	**G**	**D**	**N**	**A**	**T**	**P**	**E**	**Q**	**M**	**A**	**Q**	**Y**	**A**	**A**	**D**	**L**	**R**	**R**	**Y**	**I**	**N**	**M**	**L**	**T**	**R**	**P**	**R**	**Y**[a]
pPP	**A**	**P**	**L**	**E**	**P**	**V**	**Y**	**P**	**G**	**D**	**N**	**A**	**T**	**P**	**E**	**Q**	**M**	**A**	**Q**	**Y**	**A**	**A**	**E**	**L**	**R**	**R**	**Y**	**I**	**N**	**M**	**L**	**T**	**R**	**P**	**R**	**Y**[a]
rPP	**A**	**P**	**L**	**E**	**P**	**M**	**Y**	**P**	**G**	**D**	**Y**	**A**	**T**	**H**	**E**	**Q**	**R**	**A**	**Q**	**Y**	**E**	**T**	**E**	**L**	**R**	**R**	**Y**	**I**	**N**	**T**	**L**	**T**	**R**	**P**	**R**	**Y**[a]
$pNPY_{2-36}$		**P**	**S**	**K**	**P**	**D**	**N**	**P**	**G**	**E**	**D**	**A**	**P**	**A**	**E**	**D**	**L**	**A**	**R**	**Y**	**Y**	**S**	**A**	**L**	**R**	**H**	**Y**	**I**	**N**	**L**	**I**	**T**	**R**	**Q**	**R**	**Y**[a]
$pNPY_{5-36}$					**P**	**D**	**N**	**P**	**G**	**E**	**D**	**A**	**P**	**A**	**E**	**D**	**L**	**A**	**R**	**Y**	**Y**	**S**	**A**	**L**	**R**	**H**	**Y**	**I**	**N**	**L**	**I**	**T**	**R**	**Q**	**R**	**Y**[a]
$pNPY_{11-36}$											**D**	**A**	**P**	**A**	**E**	**D**	**L**	**A**	**R**	**Y**	**Y**	**S**	**A**	**L**	**R**	**H**	**Y**	**I**	**N**	**L**	**I**	**T**	**R**	**Q**	**R**	**Y**[a]
$pNPY_{13-36}$													**P**	**A**	**E**	**D**	**L**	**A**	**R**	**Y**	**Y**	**S**	**A**	**L**	**R**	**H**	**Y**	**I**	**N**	**L**	**I**	**T**	**R**	**Q**	**R**	**Y**[a]
NPY_{16-36}																**D**	**L**	**A**	**R**	**Y**	**Y**	**S**	**A**	**L**	**R**	**H**	**Y**	**I**	**N**	**L**	**I**	**T**	**R**	**Q**	**R**	**Y**[a]
NPY_{18-36}																		**A**	**R**	**Y**	**Y**	**S**	**A**	**L**	**R**	**H**	**Y**	**I**	**N**	**L**	**I**	**T**	**R**	**Q**	**R**	**Y**[a]
NPY_{25-36}																									**R**	**H**	**Y**	**I**	**N**	**L**	**I**	**T**	**R**	**Q**	**R**	**Y**[a]
NPY_{1-15}	**Y**	**P**	**S**	**K**	**P**	**D**	**N**	**P**	**G**	**E**	**D**	**A**	**P**	**A**	**E**																					
$hNPY_{1-24}CONH_2$	**Y**	**P**	**S**	**K**	**P**	**D**	**N**	**P**	**G**	**E**	**D**	**A**	**P**	**A**	**E**	**D**	**M**	**A**	**R**	**Y**	**Y**	**S**	**A**	**L**[a]													

[a] Presence of an amidated c-terminus.

has been shown to modulate catecholaminergic response in various tissues (22–24).

Both NPY and PYY possess direct vasoconstrictive effects on blood vessels, in addition to potentiating the response of these tissues to various constrictors (24–28). Moreover, NPY and PYY also act prejunctionally to inhibit the release of noradrenaline from sympathetic nerve endings (23, 24, 26–28). Based on the specificity of the C-terminal fragment PYY_{13-36} for the prejunctional effects of NPY and PYY, Wahlestedt and colleagues (24, 29) have suggested the existence of NPY/PYY receptor subtypes. They classified the prejunctional PYY_{13-36}-sensitive site as the Y_2 receptor, whereas the other receptor type (Y_1) is only recognized by the entire molecule. However, full specificity of either NPY_{13-36} or PYY_{13-36} for prejunctional Y_2 receptors has not been observed in some preparations, suggesting further receptor heterogeneity (30–33).

Clarification of the existence of NPY subtypes will require the development of highly specific agonists and/or antagonists for each receptor class. For the eventual development of such tools, a better knowledge of the structural requirements of NPY receptors (and the putative subtypes) is needed. This study was, therefore, undertaken to evaluate the effects of deletions of N- and C-terminal portions of the peptide, as well as modifications of tyrosine residues in position 20 or 21, on NPY receptor binding in the rat brain and on biological activity in the electrically stimulated rat vas deferens. Our results provide further evidence for possible NPY receptor heterogeneity in these two preparations.

Experimental Procedures

Materials. Male Sprague-Dawley CD rats were obtained from Charles River Canada (St. Constant, Québec, Canada) and kept on a 12-hr light-dark cycle (light on at 7:00 a.m.). Animals were given standard laboratory chow and tap water *ad libitum*.

Analogues and fragments of hNPY or pNPY were synthesized in our laboratories, as summarized below. Bacitracin and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO) and ECO-lite scintillation cocktail was purchased from ICN Biomedical (Montréal, Québec, Canada). [^{3}H]Propionyl-pNPY (60–80 Ci/mmol) was obtained from Amersham Canada (Oakville, Ontario, Canada). All other chemicals were of analytical grade and were obtained from Fisher Scientific (Montréal, Québec, Canada).

Peptide synthesis. Analogues and fragments of hNPY and pNPY were synthesized by the solid phase method, using a home-made multireactor synthesizer. The syntheses were carried out using a benzhydrylamine resin (34) for the peptides having an amide C-terminal function and with a chloromethylated resin (35) for those having a C-terminal carboxylic function. All amino acids were coupled via the method described by Fournier and co-workers (36). The T-butyloxycarbonal-amino acids with appropriate side chain protection were obtained from Bachem (Torrance, CA). Completed peptides were cleaved from the resin support and deprotected by a 90-min treatment at 0° with liquid hydrofluoric acid containing *m*-cresol and dimethyl sulfide as scavengers (10:1:1, v/v).

After extraction from the resin and lyophilization, the peptides were purified by reverse phase HPLC on a Waters Deltapak column, using an eluant of H_2O (0.06% trifluoroacetic acid) and acetonitrile-H_2O (0.06% trifluoroacetic acid). Peptides were eluted with successive linear gradients of the second solvent. Analytical HPLC of the individual fractions was carried out and the fractions corresponding to the purified peptides were lyophilized. The purified material was characterized by analytical HPLC and amino acid analysis. A detailed description of the synthesis, purification, and chemical characterization of the peptides will be presented elsewhere.[1]

[^{3}H]NPY CNS binding assay. Membrane binding assays were performed as described before (37). Briefly, male Sprague-Dawley rats (250–300 g) were killed by decapitation and the brain (minus cerebellum) was rapidly removed, placed on ice, homogenized (25:1, w/v) in Krebs/Ringer buffer (38), pH 7.4 at 25°, using a Brinkman Polytron (setting 6 for 15–20 sec), and centrifuged at 35,000 × *g* for 15 min. The supernatant was discarded and the pellet was washed, resuspended, and centrifuged again. The final pellet was rinsed and resuspended to give a protein concentration (39) of 6–9 mg/ml of membrane preparation. For binding assays, 100 μl of membrane preparation were incubated at 25° for 120 min, in a final volume of 500 μl containing Krebs buffer, pH 7.4, 0.1% bovine serum albumin, 0.05% bacitracin, and 0.5 nM [^{3}H]propionyl-pNPY ([^{3}H]NPY). Incubations were terminated by

[1] A. Fournier, J-C. Martel, R. Quirtion A. Cadiroz,F.B. Jolndur, and S. St-Pierre. Synthesis and structure activity study analogues of neuropeptide Y in various tissues. Manuscript in preparation.

rapid filtration through Schleicher & Schuell no. 32 filters (previously soaked in 1.0% polyethyleneimine), using a cell harvester filtering apparatus (Brandel Instruments, Gaithersburg, MD). Filters were rinsed three times with 3 ml of ice-cold buffer and radioactivity remaining on the filters was quantified by counting filters in 5 ml of ECO-lite scintillation cocktail. Specific binding was calculated as the difference in radioactivity bound in the presence and absence of 1.0 μM hNPY or pNPY and represented 70–80% of total binding. The competition curves were constructed by using concentrations of NPY fragments or analogues ranging from 0.01 to 10,000 nM, and relative affinities were calculated on the basis of the affinity of hNPY or pNPY being taken as 100%.

Electrically stimulated rat vas deferens. Male Sprague-Dawley rats (175–225 g) were sacrificed by decapitation, the abdominal cavity was opened, and vasa deferentia were dissected out and placed in oxygenated (95%/5%, O_2/CO_2) Krebs-Ringer buffer (38) at 37°. Vasa deferentia were carefully freed from connective tissue, and the prostatic part was cut, mounted in a tissue bath containing oxygenated Krebs buffer, and maintained at 37°. The tissues were equilibrated under a resting tension of 0.5–1.0 g for 1 hr before the experiment began. Tissues were simulated by electric pulses using a Grass stimulator (model S44) (frequency, 0.15 Hz; duration, 0.5 msec; amplitude, 40–90 V), and the twitch response was recorded on a Grass polygraph (model 79D) using a force transducer (Grass model FT03). The effects of NPY, NPY fragments, and analogues were measured by constructing cumulative dose-response curves using concentrations ranging from 0.5 to 1000 nM, and the relative potencies of the various analogues were derived from comparison of their EC_{50} values with that of hNPY or pNPY. Inactive analogues and fragments (at 1000 nM) were tested for possible antagonistic properties by coincubation with increasing concentrations of pNPY.

Statistics. Binding and bioassay data were analyzed by computerized linear regression analysis (Bio-Soft Elsevier, Cambridge, UK). For both binding and bioassay data, values were expressed as means ± standard errors. For evaluation of possible antagonistic properties, comparisons were made using Student's *t* test for paired or unpaired values as required. The level of statistical significance was accepted at $p < 0.05$.

Results

The relative affinities and potencies of NPY, fragments, and analogues have been determined in the brain and vas deferens preparations. In the vas deferens, we also attempted to directly determine the relative affinities with a binding assay using a rat vas deferens membrane preparation. However, the percentage of specifically bound [^{3}H]NPY was too low (10–15%) to allow for accurate determination of receptor binding parameters.[2] Thus, relative potency values are derived from bioassay data. However, we are well aware that this parameter may be affected both by the affinity of the molecule for its receptor and by its efficacy for receptor activation (40). On the other hand, it is also clear that important information regarding amino acid residues relevant for receptor affinity and/or receptor triggering can be obtained from bioassay data. Thus, although we are still working to establish proper conditions in order to perform binding assays in the rat vas deferens, we believe that the present results reveal interesting and new information regarding NPY receptors in these two preparations.

The intrinsic activity (α) of the various NPY-related compounds studied was also determined in the rat vas deferens preparation, with this measure being related to receptor activation (40, 41). Apparent full intrinsic activity is given by a value of 1.0 (full agonist). In this study, we used maximal concentrations of 1–10 μM, because of limited peptide quantities. Consequently, apparent full intrinsic activities would most likely have been reached for analogues displaying an α of 0.8–0.9 at 1.0 μM concentrations (see Tables 2 and 3). This assumption is also supported by the parallelism of the dose-response curves observed with these analogues and fragments (see Fig. 3).

[^{3}H]NPY ligand selectivity pattern in rat brain. Estimations of relative affinities of fragments and analogues have been made in comparison with the IC_{50} obtained from the relevant native peptide (hNPY or pNPY), depending on from which of those two peptides the synthetic fragments or analogues were derived (Tables 2 and 3). The data were fit to a single-affinity state model, because the two-affinity state model did not give a statistically better fit for hNPY and pNPY. In addition, most Hill coefficients for the fragments and analogues are close to unity (Tables 2 and 3).

Progressive shortening of pNPY from its N-terminal end rapidly decreased the affinity for [^{3}H]NPY brain sites; $pNPY_{2-36}$ demonstrated less than 10% of the affinity of the full peptide, whereas other C-terminal fragments ($pNPY_{5-36}$ to NPY_{18-36}) possessed less than 1% of pNPY affinity (Fig. 1A; Table 2). The parallelism of the curves suggests competitive interactions between C-terminal fragments and [^{3}H]NPY binding sites, without apparent evidence for the existence of cooperativity or multiple receptor states (Fig. 1A). NPY_{25-36} was virtually inactive at concentrations up to 1.0 μM (Fig. 1A; Table 2). The two N-terminal fragments tested here (NPY_{1-15} and $hNPY_{1-24}CONH_2$) were completely inactive in competing for [^{3}H]NPY binding to brain membranes at concentrations up to 10 μM (Fig. 1A; Table 2).

In regard to NPY analogues modified at position 20, it appears that the oxygen moiety of the benzyl ring of the tyrosine residue is important for adequate receptor affinity, because the analogue [Phe^{20}]hNPY demonstrated only 38% of the relative affinity of the native peptide, whereas the [Tyr-*O*-Me^{20}]hNPY analogue retained most of the affinity of the parent peptide (Fig. 1B; Table 3). An unsaturated ring of adequate size also appears to be important, on the basis of the relative affinities demonstrated by analogues [Cha^{20}]hNPY and [Trp^{20}]hNPY (Fig. 1B; Table 3). Appropriate stereochemistry at this position is also critical, as shown by the marked loss of affinities with analogues [D-Tyr^{20}]hNPY and [D-Trp^{20}]hNPY (Fig. 1B; Table 3).

Most modifications of the tyrosine residue at position 21 also had detrimental effects on affinity for [^{3}H]NPY binding sites, although not as dramatic as those seen at position 20 (Fig. 1C; Table 3). As shown in Table 3, the hydroxyl group of the tyrosine residue at position 21 does not appear to be greatly involved in ensuring adequate brain receptor recognition, because modifications with either a phenylalanine, methylated tyrosine, or cyclohexylalanine residue had minor or no effects on receptor affinity. On the other hand, modification with a bulkier residue (tryptophan) markedly reduced affinity (Fig. 1C; Table 3). Appropriate stereochemistry is also important in this position, as can be seen by the great loss of affinities with analogues [D-Tyr^{21}]hNPY and [D-Trp^{21}]hNPY (Fig. 1C; Table 3). As for the C-terminal fragments, competition curves for the various analogues with modifications at positions 20 and 21 are

[2] J-C. Martel and R. Quirion. Unpublished observations.

TABLE 2

Relative potencies of NPY fragments in [^{3}H]NPY CNS binding assay and in the electrically stimulated rat vas deferens bioassay

IC_{50}, concentration of competitor needed to inhibit 50% of specific [^{3}H]NPY binding in rat CNS membranes, expressed as mean ± standard error of three to six determinations. IC_{50} values should approximate K_i, because the concentration of ligand used in assays was much lower than the IC_{50} of hNPY. EC_{50}, concentration of NPY or NPY fragment necessary to inhibit 50% of the twitch response in rat vas deferens, expressed as mean ± standard error of three to seven determinations. RA, relative affinity as compared with pNPY; RP, relative potency as compared with pNPY; α, intrinsic activity, which refers to the capacity to trigger receptor activation (apparent full intrinsic activity being 1.0); N_H, Hill coefficient.

Peptide	[^{3}H]NPY CNS binding assay			Rat vas deferens bioassay		
	IC_{50}	RA	N_H	EC_{50}	RP	α
	nM	%		nM	%	
pNPY	4.4 ± 1.2	100	0.85 ± 0.07	22.2 ± 3.4	100	1.0
$pNPY_{2-36}$	66.7 ± 5.5[a]	7	0.77 ± 0.05	112 ± 3[b]	20	1.0
$pNPY_{5-36}$	1,824 ± 84[a]	0.2		238 ± 15[b]	9	0.9–1.0
$pNPY_{11-36}$	1,260 ± 170[a]	0.4	0.72 ± 0.09	308 ± 49[b]	7	0.8–1.0
$pNPY_{13-36}$	708 ± 112[a]	0.6	0.75 ± 0.12	337 ± 57[b]	6	0.8–1.0
$pNPY_{16-36}$	942 ± 164[a]	0.5	0.91 ± 0.08	210 ± 29[b]	10	0.9–1.0
NPY_{18-36}	642 ± 159[a]	0.7	0.83 ± 0.12	322 ± 13[b]	7	0.8–1.0
NPY_{25-36}	>10,000	<0.04		>1,000	<2.0	<0.1
NPY_{1-15}	>10,000	<0.04		>1,000	<2.0	<0.1
$hNPY_{1-24}CONH_2$	>10,000	<0.04[c]		>1,000	<4.0[c]	<0.1

[a] IC_{50} significantly higher than that of pNPY ($p < 0.05$).
[b] EC_{50} statistically different from pNPY ($p < 0.05$).
[c] RA and RP values for $hNPY_{1-24}CONH_2$ are derived from comparison with hNPY.

TABLE 3

Relative potencies of NPY analogues in [^{3}H]NPY CNS binding assay and in the electrically stimulated rat vas deferens bioassay

IC_{50}, concentration of competitor needed to inhibit 50% of specific [^{3}H]NPY binding in rat CNS membranes, expressed as mean ± standard error of three to six determinations. IC_{50} values should approximate K_i, because the concentration of ligand used in assays was much lower than the IC_{50} of hNPY. EC_{50}, concentration of hNPY or analogue necessary to inhibit 50% of the twitch response in rat vas deferens, expressed as mean ± standard error of three to seven determinations. RA, relative affinity as compared with pNPY; RP, relative potency as compared to hNPY; α, intrinsic activity, which refers to the capacity to trigger receptor activation (apparent full intrinsic activity being 1.0); N_H, Hill coefficient.

Peptide	[^{3}H]NPY CNS binding assay			Rat vas deferens bioassay		
	IC_{50}	RA	N_H	EC_{50}	RP	α
	nM	%		nM	%	
hNPY	3.8 ± 0.4	100	0.89 ± 0.07	44.3 ± 2.4	100	1.0
[D-Tyr^{20}]hNPY	656 ± 86[a]	1	0.91 ± 0.08	131 ± 9[b]	34	1.0
[Trp^{20}]hNPY	22.4 ± 1.2[a]	17	0.91 ± 0.09	139 ± 29[b]	32	0.9–1.0
[D-Trp^{20}]hNPY	492 ± 92[a]	1	0.69 ± 0.09	179 ± 24[b]	25	0.8–1.0
[Phe^{20}]hNPY	10.1 ± 4.3[a]	38	1.02 ± 0.09	234 ± 11.3[b]	19	0.9–1.0
[Tyr-*O*-Me^{20}]hNPY	4.1 ± 1.0	93	0.77 ± 0.09	74.3 ± 5.4[b]	60	1.0
[Cha^{20}]hNPY	503 ± 112[a]	1	1.01 ± 0.10	532 ± 82[b]	8	0.8–1.0
[D-Tyr^{21}]hNPY	107 ± 31[a]	4	0.80 ± 0.10	178 ± 3[b]	25	1.0
[Trp^{21}]hNPY	35.6 ± 7.7[a]	11	1.03 ± 0.09	271 ± 10[b]	16	0.9–1.0
[D-Trp^{21}]hNPY	184 ± 63[a]	2	1.28 ± 0.09	723 ± 183[b]	6	0.8–1.0
[Phe^{21}]hNPY	4.7 ± 0.7	81	0.80 ± 0.10	310 ± 3[b]	14	1.0
[Tyr-*O*-Me^{21}]hNPY	1.9 ± 1.0[c]	200	0.90 ± 0.09	572 ± 15[b]	8	0.9–1.0
[Cha^{21}]hNPY	4.6 ± 2.9	83	0.93 ± 0.10	81.7 ± 2.9[b]	54	1.0

[a] IC_{50} significantly higher than that of hNPY ($p < 0.05$).
[b] EC_{50} significantly higher than that of hNPY ($p < 0.05$).
[c] IC_{50} significantly lower than that of hNPY ($p < 0.05$).

parallel (Fig. 1, B and C), suggesting the absence of cooperativity under our incubation conditions.

Structure-activity relationships in vas deferens. As reported earlier (for example, see Refs. 22, 23, and 42), pNPY and hNPY inhibit the twitch response of the electrically stimulated vas deferens in a dose-dependent manner (Figs. 2 and 3). pNPY was slightly more potent than hNPY in this bioassay (Tables 2 and 3). As described for the brain binding assay, relative potencies were estimated on the basis of the EC_{50} obtained for the native peptide used to design fragments (pNPY) or analogues (hNPY).

Deletion of the tyrosine residue in position 1 ($pNPY_{2-36}$) markedly decreased the relative potency of this peptide (Fig. 3A; Table 2). However, full intrinsic activity was maintained with this NPY fragment (Fig. 3A; Table 2). Additional shortening of the peptide sequence from its N-terminal side further decreased the relative potency without significantly affecting the capacity to trigger receptor activation (α close to unity), at least up to fragment NPY_{18-36} (Fig. 3A; Table 2). On the other hand, fragment NPY_{25-36} did not inhibit the twitch response of the rat vas deferens at concentrations up to 1.0 μM (Fig. 3A; Table 2). Moreover, this fragment did not display any antagonistic activity at 10.0 μM (not shown).

N-terminal fragment NPY_{1-15} were unable to reduce the twitch response of the rat vas deferens preparation at concentrations up to 1.0 μM (Table 2). NPY_{1-15} was also devoid of any antagonistic properties at 1.0 μM concentration (not shown). Similarly, no direct biological effect of the N-terminal fragment $hNPY_{1-24}CONH_2$ was observed at concentrations up to 1.0 μM in the tissue bath (Table 2). Interestingly, it appears that this fragment, at a concentration of 1.0 μM, may be able to weakly antagonize the effects of pNPY in this preparation

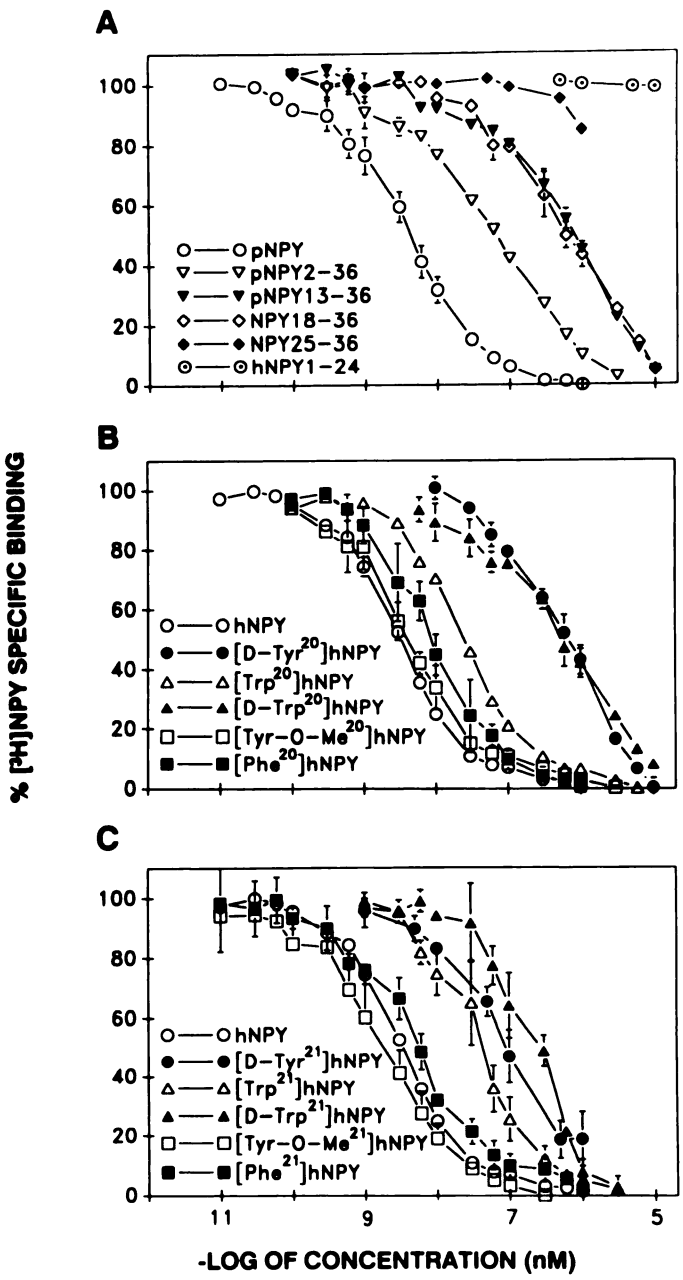

Fig. 1. Competition curves of [³H]NPY specific binding to rat brain membrane preparation by fragments (A) and analogues modified at position 20 (B) or 21 (C). Each *point* represents the mean ± standard error of results obtained from three to seven separate experiments, each performed in triplicate and expressed as percentage of specific binding at equilibrium.

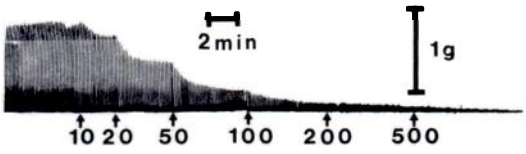

Fig. 2. Typical trace of the twitch response inhibition produced by pNPY on the isolated rat vas deferens. *Numbers*, final concentration of the peptide (nM) reached in the tissue bath after each addition of pNPY.

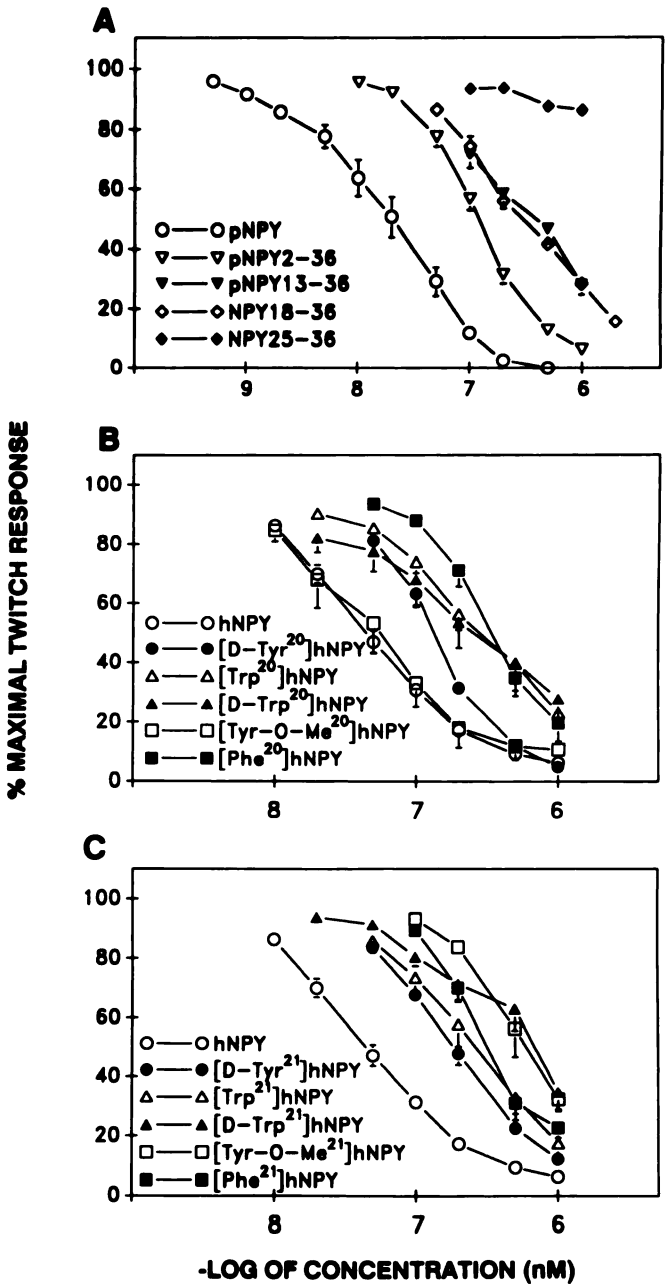

Fig. 3. Dose-response curves of the inhibition of the rat vas deferens twitch response by fragments (A) and analogues modified at position 20 (B) or 21 (C). Each *point* represents the mean ± standard error of data obtained from four to eight individual determinations expressed as a percentage of maximal twitch response.

(Fig. 4B; Table 4). Moreover, at a higher concentration (10 μM), $hNPY_{1-24}CONH_2$ was able to actually increase the amplitude of the twitch response of this tissue to electrical stimulation (Fig. 4A), an action opposite to that of NPY, C-terminal fragments, and analogues (Fig. 2).

Modifications of tyrosine residues at either position 20 or 21 reduced the relative potencies of NPY analogues without significantly altering intrinsic activities (Fig. 3, B and C; Table 3). As observed in the [³H]NPY binding assay, the presence of an oxygen moiety as well as the aromaticity and size of the residue at position 20 seem to be relatively important for the maintenance of NPY receptor recognition in the vas deferens, as shown by the decreased affinities of analogues [Phe²⁰]hNPY,

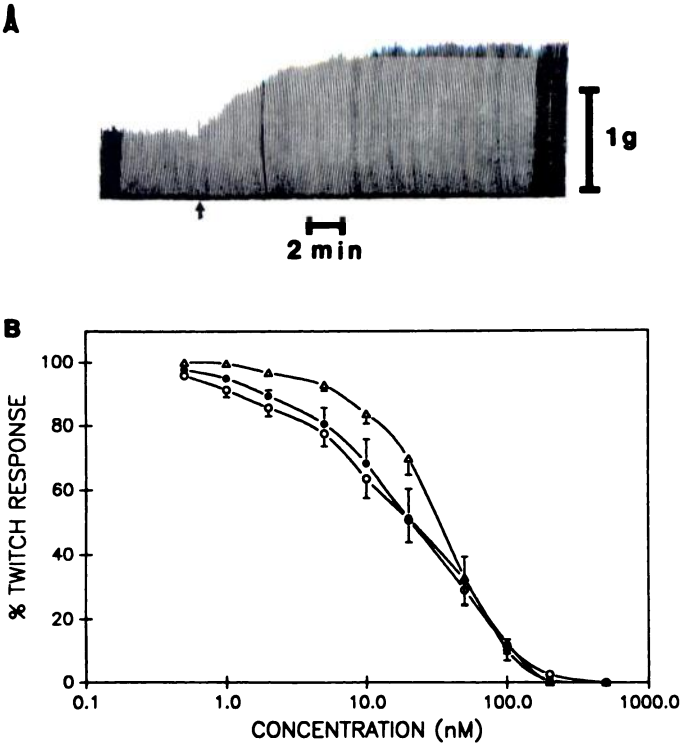

Fig. 4. Effect of $hNPY_{1-24}CONH_2$ on rat vas deferens twitch response (A). *Arrow*, addition of 10 μM (final concentration) levels of this fragment in the tissue bath. B, Dose-response curve of the inhibitory effect of pNPY on rat vas deferens twitch response in the absence (○) and presence of 0.1 μM (●) or 1.0 μM (△) $hNPY_{1-24}CONH_2$. Each *point* represents the mean ± standard error of data obtained from 8 to 10 individual determinations, expressed as percentage of maximal twitch response. $hNPY_{1-24}CONH_2$ was added at least 10 min before testing pNPY. Statistically different response from when pNPY was incubated alone.

TABLE 4

Comparative potencies of NPY in the rat vas deferens preparation in the presence of $hNPY_{1-24}CONH_2$

EC_{50} values are mean ± standard error of six determinations and represent the concentration of pNPY needed to reduce by 50% the twitch response of the tissue; $hNPY_{1-24}CONH_2$ was added at least 10 min before NPY was tested.

$hNPY_{1-24}CONH_2$	pNPY EC_{50}
nM	*nM*
0	22.2 ± 3.4
100	22.5 ± 5.7
1000	39.4 ± 9.0[a]

[a] Significantly greater ($p < 0.05$) than when only pNPY was present.

[Tyr-*O*-Me^{20}]hNPY, [Cha^{20}]hNPY, and [Trp^{20}]hNPY (Fig. 3B; Table 3). However, analogues bearing an aromatic D-amino acid residue in this position ([D-Tyr^{20}]hNPY and [D-Trp^{20}]hNPY) displayed approximately 30-fold greater potencies in the rat vas deferens preparation than in the [^{3}H]NPY brain binding assay (Table 3), suggesting possible differential structural requirements of NPY receptors in these two preparations. The reverse situation was observed with some modifications of the tyrosine residue in position 21. As can be seen in Table 3, the analogues [Phe^{21}]hNPY and [Tyr-*O*-Me^{21}]hNPY were, respectively, 5 and 20 times more potent in the binding assay than in the rat vas deferens preparation. Other analogues such as [D-Tyr^{21}]hNPY, [Trp^{21}]hNPY, [D-Trp^{21}]hNPY, and [Cha^{21}]hNPY displayed similar potencies in both preparations (Figs. 1C and 3C; Table 3).

Discussion

The sequences of NPY, NPY homologues, and the fragments used in the present study are given in Table 1. Of particular interest is the conservation of structural amino acids such as proline in positions 2, 5, 8, and 13, leucine in positions 17, 24, 30, and 31, and tyrosine in positions 20 and 27. The importance of these residues in stabilizing the characteristic "hairpin-like" compact structure of the various members of the PP family has been previously discussed (43–46). In addition, the C-terminal third of the various PP members shows a particularly high degree of sequence homology (Table 1). Such sequence homology may imply a common evolutionary origin of the NPY/PYY/PP genes, an assumption that is supported by the similar intron organization of NPY and PP genes (47). Furthermore, the common evolutionary origin of the various PP members may suggest that these polypeptides act on receptors that were also derived from a single ancestral molecule, and a similar triggering mechanism may be postulated for these various receptors. This hypothesis is supported by the observation that various PPs can elicit several central and peripheral effects of NPY and PYY (16, 19, 25, 48) and may suggest that the amino acid sequence responsible for triggering NPY and PYY receptor activation lies in the highly conserved C-terminal portion of these molecules. Our previous results with NPY fragments (37, 42, 49, 50), as well as those of others (30, 32, 51, 52), support this hypothesis, because deletions of various N-terminal segments of up to half of the NPY molecule generally reduced the potency of the fragments in central and peripheral assays, but without eliminating the biological response. The data reported here, in addition to the recent finding that the central core of the NPY molecule is not necessary for maintaining the activities of this peptide (53, 54); further suggest that the amino acid sequence responsible for triggering NPY receptors most likely lies in the C-terminal third of the NPY molecule.

As previously reported (32, 33, 37, 50), the loss of affinity and potency observed with fragment $pNPY_{2-36}$ reveals that the tyrosine residue in position 1 is critical for the maintenance of appropriate NPY receptor recognition. However, this fragment maintained full intrinsic activity in the vas deferens preparation. Similar results have been obtained by replacement of tyrosine residue 1 by a neutral amino acid (55). This emphasized the importance of the tyrosine residue in this position to ensure proper NPY receptor recognition, while also demonstrating that this amino acid is most likely not directly involved in receptor activation. Interestingly, it has recently been suggested that this amino acid residue may also be important in stabilizing the hairpin-like tertiary structure of NPY (32). Thus, it is possible that a destabilization of the tertiary structure of the molecule by the removal of this residue may account for the major loss of affinity and potency generally observed with this NPY fragment.

Surprisingly, Magdalin and colleagues (56) and Jolicoeur and collaborators[3] have recently shown that $pNPY_{2-36}$ may be more potent than NPY and PYY themselves in stimulating food intake, while being much less active in other behavioral paradigms. This suggests that the receptors mediating the effects of NPY on food intake may have unique structural require-

[3] F. B. Jolicoeur, J. N. Michaud, R. Rivest, D. Menard, A. Fournier, and S. St-Pierre. In vivo structure activity study suggests the existence of heterogeneous neuropeptide receptors. Submitted for publication.

ments, in which the tyrosine residue in position 1 would play a minor role. We are currently investigating this interesting possibility.

The residues located between positions 2 and 18 also appear to be mostly associated with receptor recognition. Similar results have been reported by others for some postjunctional effects of NPY, suggesting that pre- and postjunctional NPY receptors may have relatively similar structural requirements (30, 32, 50). This is somewhat surprising, because fragment PYY_{13-36} has been used to classify NPY receptor subtypes on the basis of its selectivity for prejunctionally mediated effects of NPY/PYY (24). It is presently difficult to fully explain these differences, although it may suggest that PYY_{13-36} is more selective at the Y_2 receptor subtype than similar fragments of the NPY molecule. In that regard, Chang and colleagues (51) have demonstrated that PYY is approximately 30-fold more potent than NPY at inhibiting the rat vas deferens twitch response. Thus, it is possible that deletion of the first 12 amino acid residues of PYY or NPY molecules confers a much greater selectivity for the prejunctional NPY/PYY receptor to the PYY fragment than to the NPY fragment, with the latter showing only a relative selectivity toward prejunctional NPY/PYY receptors. Recent data from Potter and collaborators (33) tend to support this hypothsis, at least with regard to NPY inhibition of cardiac vagal action.

The importance of the C-terminal portion of the NPY molecule for both receptor recognition and activation is illustrated by the very low affinities and potencies of NPY_{1-15} and $hNPY_{1-24}CONH_2$ in our two preparations. In addition, we observed that fragment $hNPY_{1-24}CONH_2$ demonstrated very little affinity for brain [^{3}H]NPY receptor sites and no inhibitory effect on the twitch response of the vas deferens at concentrations up to 1.0 μM. In fact, this fragment apparently had slight antagonistic effects on NPY-induced inhibition of the twitch response in this tissue. This could be a useful starting point for the design of NPY receptor antagonists, although this blocking effect was observed at a single concentration of $hNPY_{1-24}CONH_2$. Rather surprisingly, we also observed that a higher concentration (10 μM) of $hNPY_{1-24}CONH_2$ is able to potentiate the effect of the electrical stimulation in the rat vas deferens preparation. The possible mechanism(s) by which this analogue produces its effects is currently being investigated.

The maintenance of full biological activity, albeit with much reduced affinity, with fragment $pNPY_{18-36}$ but not with NPY_{25-36} suggests that amino acid residues between positions 18 and 24 may be essential to ensure adequate receptor binding and/or activation. Of these various amino acid residues (-R-Y-Y-S-A-L-; see Table 1), the tyrosine doublet in position 20 and 21 has attracted our attention, because it is well known that aromatic amino acid residues are often critical for receptor recognition and activation (38, 41, 57, 58).

Crystallographic studies with avian PP (44, 45) have suggested the involvement of the residue in position 20 for stabilizing, through a hydrophobic interaction with the proline residue in position 8, the compact folding of the polyproline and α-helices responsible for the characteristic hairpin-like tertiary structure of this PP. Computer-aided simulations of the tertiary structures of other members of the PP family also suggest that this residue plays an important role in stabilizing the hairpin-like tertiary structure proposed by these models for all members of the PP family (43, 46, 59). This hypothesis has been recently investigated for NPY by Minakata and colleagues (60), who found that phenylalanine substitution at position 20, which removes the polar characteristics of the tyrosine residue normally present at that position, may actually stabilize the α-helix. Our results with analogues substituted at this position would also tend to support this hypothesis for the NPY molecule, particularly with regard to brain NPY receptor sites. For example, D-substitutions ([D-Tyr^{20}]hNPY and [D-Trp^{20}]hNPY) or replacement of the aromatic ring by a saturated ring ([Cha^{20}]hNPY) greatly reduced the affinity of these analogues for brain [^{3}H]NPY receptor sites. On the other hand, modification of the size of the aromatic ring ([Trp^{20}]hNPY) or alterations at the oxygen moiety on the phenyl ring ([Phe^{20}]hNPY or [Tyr-*O*-Me^{20}]hNPY) did not have as large deleterious effects on the affinity of these analogues for brain NPY receptor sites. Thus, an aromatic ring at this position, having the appropriate orientation, appears to be critical to ensure appropriate recognition of CNS NPY receptors, suggesting that these receptors may be particularly sensitive to modification of the compact tertiary structure of the NPY molecule.

In general, the results obtained in the rat vas deferens preparation with analogues substituted at position 20 parallel those obtained in the brain binding assay, except for the two D-substitutions. These two analogues (D-Tyr^{20}]hNPY and [D-Trp^{20}]hNPY) displayed an apparently greater potency in the rat vas deferens preparation than in the brain binding assay, suggesting differential requirements for these two NPY receptor populations.

In contrast to the tyrosine residue in position 20, crystallographic and computer simulation data suggest that the tyrosine residue in position 21 is located at the hydrophilic surface of the NPY molecule and may, therefore, be directly involved with NPY receptor recognition and/or activation (43, 46, 59). Our results indicate that proper orientation and size of the side chain of this amino acid residue are critical to ensure appropriate receptor recognition. For example, D-substitution in this position ([D-Tyr^{21}]hNPY and [D-Trp^{21}]hNPY) resulted in major loss of affinities and potencies in the two preparations. Similarly, substitution by a larger residue ([Trp^{21}]hNPY) greatly reduced affinities and potencies in the two preparations. Interestingly, two other modifications at this position ([Phe^{21}]hNPY and [Tyr-*O*-Me^{21}]hNPY) had little effect on brain binding affinities, while markedly reducing potencies in the rat vas deferens preparation.

We observed that analogues [Phe^{21}]hNPY and [Tyr-*O*-Me^{21}]hNPY demonstrated approximately 10-fold greater potencies in the rat brain binding assay, as compared with the rat vas deferens bioassay, whereas the reverse situation was observed for two analogues with modifications at position 20, namely [D-Tyr^{20}]hNPY and [D-Trp^{20}]hNPY, which were approximately 30 times more potent in the bioassay preparation. To our knowledge, this is the first evidence suggesting differential importance of tyrosine residues in positions 20 and 21 in the recognition of NPY receptors in brain versus vas deferens preparations. The possible existence of CNS versus peripheral tissue receptor heterogeneity has also been suggested by Chang and collaborators (51, 61) on the basis of the differential affinities of NPY, PYY, rat PP, and human PP in preparations similar to those used in the present study. In addition, Minakata and colleagues (60) have also observed that a series of NPY analogues demonstrated differential potencies in binding

and bioassay preparations comparable to those used here. Moreover, other groups have recently suggested the existence of NPY receptor subtypes in the CNS, using receptor binding techniques (62–66). The development of selective NPY receptor subtype agonists and/or antagonists will be necessary to confirm this hypothesis. In that regard, the recent characterization of [Leu^{31},Pro^{35}]NPY as a selective Y_1 receptor agonist (67) is of great interest. However, it is not clear whether brain [^{3}H] NPY binding sites characterized in our study are of this type, whereas those present in the vas deferens are likely of the Y_2 class (24).

In summary, our results further demonstrate the importance of the C-terminal portion of the NPY molecule for receptor activation. Moreover, it appears that certain specific amino acids, particularly those in positions 1, 20, and 21, are critical in ensuring the maintenance of appropriate receptor recognition. Additionally, certain analogues with modifications at positions 20 and 21 may allow further characterization of NPY receptor subtypes. Finally, certain N-terminal fragments such as $hNPY_{1-24}CONH_2$ may be useful for the characterization of NPY receptor sites demonstrating preferential affinity for the N-terminal portion of the molecule and/or for the eventual design of selective NPY receptor antagonists.

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