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Synthesis and Characterization of Neurotensin Analogues for Structure/Activity Relationship Studies

Acetyl-neurotensin-(8—13) Is the Shortest Analogue with Full Binding and Pharmacological Activities

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Neurotensin and several sequence analogues have been synthesized using solid-phase technology. The purity of the following derivatives: neurotensin, neurotensin-(10–13), neurotensin-(9–13), neurotensin-(8–13), neurotensin-(6–13), neurotensin-(4–13), [Cit^8]neurotensin-(8–13), [Lys^8]neurotensin-(8–13), [Cit^9]neurotensin-(8–13), [Lys^9]neurotensin-(8–13), [Phe^{11}]neurotensin-(8–13), [Ala^{12}]neurotensin-(8–13) and [Ala^{13}]-neurotensin-(8–13) was verified by amino acid analyses after acid and enzymatic hydrolyses, reverse-phase high-performance liquid chromatography in two systems and Edman degradation. The above analogues, those obtained after *N*-acetylation of neurotensin-(6–13), neurotensin-(8–13), [Cit^8]neurotensin-(8–13), [Cit^9]-neurotensin-(8–13), [Lys^8]neurotensin-(8–13), [Lys^9]neurotensin-(8–13) and [Phe^{11}]neurotensin-(8–13), as well as native xenopsin, were all tested for binding competition with [^{3}H]neurotensin on the specific fixation sites of rat brain synaptosomal membranes and on those of HT 29 cells. In addition to these radioreceptor assays on neural and extraneural targets, a pharmacological test (contraction of guinea pig ileum in the presence of neostigmine) was used to compare the behavior of the synthetic analogues. The use of these three biological systems enabled us to obtain consistent results. A good parallel was observed between the degree of fixation and pharmacological effects for entire neurotensin and for C-terminal region analogues up to the size of neurotensin-(8–13). The two peptides neurotensin-(6–13) and neurotensin-(4–13) had an abnormally high affinity for rat brain synaptic membrane binding sites compared to a relatively low contracting activity. The C-terminal peptide -Arg-Arg-Pro-Tyr-Ile-Leu fulfills all the structural requirements for mimicking the entire sequence, provided its α-amino end is protected by acetylation. The guanidinium structure of residues 8 and 9 are not of vital importance, since they could be efficiently replaced by amino groups of lysyl side chains. Xenopsin, which can be considered as a natural analogue of neurotensin-(8–13), acts similarly to acetyl-neurotensin-(8–13). Removal of the phenolic function of residue 11 induces a decrease in neurotensin effects. The C-terminal isoleucyl and leucyl residues could not be replaced by alanine without complete loss of the three activities tested.

Neurotensin is a 13-residue peptide (< Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), originally isolated by Carraway and Leeman from calf hypothalamus [1] and subsequently purified from bovine [2] and human [3] small intestine. It has stimulated considerable research, since it displays a wide spectrum of pharmacological activities when injected peripherally (hypotension, hyperglycemia, gut contraction, increased vascular permeability, increased secretion of growth hormone) or centrally (hypothermic effect on cold-exposed rats, analgesic activity). There are both central and peripheral neurotensin fixation sites (for the above mentioned effects, see review of Fernstrom et al. [4]). The physiological role and the significance of its ubiquitous distribution, however, remain unknown.

In the last few years, several studies with synthetic analogues of neurotensin have been performed for investigating structure/activity correlations, resulting in some clear conclusions, but also often with divergent results. The first data obtained [5] indicated that the sequence could be considerably truncated at the N terminus without an important decrease of biological activity. It was subsequently shown that the last C-terminal residue of the peptide could not be removed [6], amidated [7] or *N*-methylamidated [8] without a complete loss of biological activity. Rivier et al. [8], after synthesizing a series of neurotensin analogues in which each residue was systematically replaced by its D-isomer, found that the important amino acids for binding to mast cells were located in the C-terminal part of the molecule [9], thus extending the earlier observations of Carraway and Leeman [5].

The above results indicated that the N-terminal region of the peptide was apparently not crucial for the expression of biological activities. Discordant results were obtained, however, concerning the two truncated sequences neurotensin-(8–13) and neurotensin-(9–13). Carraway and Leeman [5] measured the ability of neurotensin and partial sequences to

Abbreviations. Ac, acetyl; Boc, *tert*-butoxycarbonyl; Cit, citrulline; EC_{50}, half-maximal effective concentration; IC_{50}, concentration inhibiting 50% of the binding; mS, millisiemens; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; < Glu, pyroglutamic acid.

Enzymes. Aminopeptidase M, microsomal (EC 3.4.11.2); proline dipeptidase or imidodipeptidase (EC 3.4.13.9); papain (EC 3.4.22.2).

Nomenclature. The synthetic derivatives of neurotensin have been named following IUPAC-IUB Recommendations for amino-acid derivatives and peptides [*Eur. J. Biochem. 27*, 201–207 (1972)] and for naming synthetic modifications of natural peptides [*Eur. J. Biochem. 1*, 379–381 (1967) and *45*, 3 (1974)].

induce hyperglycemia and hypotention in rats and found that neurotensin-(8–13) retained 55% of the biological potency of neurotensin and that shortening the sequence from neurotensin-(8–13) to neurotensin-(9–13) led to a 60-fold reduction in activity. In contrast, Rivier et al. [8] reported that neurotensin-(8–13) and neurotensin-(9–13) were equipotent, with about 20–25% of the biological potency of neurotensin for the expression of the hypothermic effect in rats after intracisternal injection. Using this same bioassay *in vivo*, however, Loosen et al. [10] observed that neurotensin-(9–13) had only moderate activity. Assays *in vitro*, in which the contracting activity of neurotensin and its analogues was measured on gastrointestinal and cardiovascular preparations [11–13], showed that the potencies of neurotensin-(8–13) and neurotensin-(9–13) relative to neurotensin ranged between 20% and 100% for the hexapeptide and between 1% and 11% for the pentapeptide. Neurotensin-(8–13) was always 7–50-times more potent than neurotensin-(9–13), a result that agrees roughly with the report of Carraway and Leeman [5] but not with that of Rivier et al. [8]. Divergent results were also obtained when the activities of neurotensin-(8–13) and neurotensin-(9–13) were measured in various binding assays. Neurotensin-(8–13) was found to be more potent than the complete sequence itself in its ability to inhibit the fixation of [^{3}H]neurotensin to rat brain synaptic membranes [6], whereas a dramatic decrease (500-fold) in binding activity was observed when the structure was shortened from neurotensin-(8–13) to neurotensin-(9–13). Uhl et al. [14] used the same source of receptors and ^{125}I-neurotensin as the labeled ligand, but reported that neurotensin-(8–13) had only about 10% of the affinity of native neurotensin and that neurotensin-(9–13) had 0.5%. In a similar binding assay on rat brain membranes with ^{125}I-neurotensin, Lazarus et al. [15] found very low binding activities (relative to neurotensin): 0.03% and 0.01% for neurotensin-(8–13) and neurotensin-(9–13). They also reported [9] values of 67% for neurotensin-(8–13) and 76% for neurotensin-(9–13) when using mast cells as a source of extraneural receptors.

In order to clarify further this broad spectrum of results and determine the smallest chain length required for full activity, new well-defined synthetic truncated sequences of neurotensin were obtained and used in three biological tests believed to be specific and possibly physiologically relevant. Other analogues were synthesized to determine the chemical importance of the side chains in the C-terminal part of the peptide.

MATERIALS AND METHODS

All chemicals were of the best available grade (Merck or Fluka). In addition to our own synthetic derivatives, we also tested xenopsin (Peninsula Laboratories, Belmont, CA).

Peptide Synthesis

tert-Butoxycarbonyl (Boc) derivatives of amino acids were obtained from Bachem. Dichloromethane was redistilled daily over potassium carbonate. Trifluoroacetic acid was treated with Cr_2O_5 and redistilled before use. *N,N'*-Dimethylformamide was stored over a 0.4-nm molecular sieve in dark bottles. Trifluoroacetic acid and diisopropylethylamine solutions were prepared daily. Solid-phase syntheses were performed on chloromethylated 1% divinylbenzene polystyrene resins (U.C.B.). The esterification of the C-terminal residue was performed according to the procedure of Monahan and Gilon [16]. The synthetic protocol involved basically a double dicyclohexylcarbodiimide-mediated coupling (2.5-fold excess; 90-min mixing), followed by a ninhydrin test [17]. In the case of a positive result, either a third coupling or an acetic anhydride acetylation in the presence of an equivalent amount of diisopropylethylamine was performed. The deprotection of Boc-peptidyl resins was performed by a 30-min treatment with 30% trifluoroacetic acid in CH_2Cl_2 and subsequent neutralization with 5% diisopropylethylamine in CH_2Cl_2. A Beckman 990 C peptide synthesizer was used throughout.

Side-chain protections were tosyl for the guanidino group of arginine, 2,6-dichlorobenzyl for the phenolic function of tyrosine and benzyloxycarbonyl for the ε-NH_2 of lysine. The citrulline side chain was left unmasked and the incorporation of this residue was performed through its 4-nitrophenylester. L-Pyroglutamic acid (< Glu) was incorporated by a standard dicyclohexylcarbodiimide double coupling. Anhydrous HF (Matheson) treatment of peptidyl resins was in a Toho Kasei Corp. apparatus. The reaction took place at 0 °C, for 60 min in the presence of 10% (v/v) anisole. The crude peptide was extracted with glacial and then dilute acetic acid.

Purification and Chemical Characterization

Lyophilized crude peptides were first subjected to gel filtration on Bio-Gel P2 or P4 (150 × 2.5 cm column; eluted with 0.1 M acetic acid) or on Sephadex G-15 (200 × 4 cm column; eluted with 0.1 M ammonium acetate pH 8.6). The next purification step was an ion-exchange chromatography on carboxymethylcellulose (CM-52 Whatman). Ammonium acetate elution gradients were used with initial and final buffer concentrations chosen as a function of the calculated electrical charge of the peptide at neutral pH (see Table 1). Column dimensions were 25 × 2.5 cm and a three-vessel gradient-forming device (Kontess) was used. In two cases, an equilibrium chromatography was performed with a 125 × 1.5 cm column packed with CM-cellulose and run with an ammonium acetate buffer of constant composition. Elution profiles were monitored by measuring absorbance at 230 nm with a Beckman DB-GT spectrophotometer connected to a Gilson sampler.

Quantities of peptides were calculated from the mean residue value of stable amino acids after acid hydrolysis (6 M, HCl, 20 h, 110 °C) of an aliquot and amino acid analysis by a Beckman 120 C. Enzymatic digestions involved incubation with papain and then with aminopeptidase M (both from Sigma) and imidodipeptidase (Miles Laboratories). Reverse-phase high-performance liquid chromatography was performed on a Waters Associates instrument model 204. Columns were either C_{18} μBondapak (Waters) or Licrosorb RP_{18} (Merck). Two homologous columns were fitted in series and run with 10 mM triethylammonium phosphate (pH 3)/methanol (50/50, v/v) at a flow rate of 2 ml/min or with 1% phosphoric acid/acetonitrile (74/26, v/v) at 1.5 ml/min. Peptides were dissolved in the eluting buffer and about 20 nmol were injected. Column effluents were continuously monitored by absorbance at 230 nm with a Waters Model 450 variable-wavelength detector. Automatic integration of peaks areas was performed with a Waters Data Module.

Sequencing data of the [Lys^8]neurotensin-(8–13) and [Lys^9]neurotensin-(8–13) analogues were obtained after automatic Edman degradation in a Socosi PS 100 sequenator. About 500 nmol of peptide were placed in the spinning cup in the presence of hake parvalbumin as a protecting protein and were subjected to four degradation cycles

using the peptide program. Phenylthiohydantoins were identified by high-performance liquid chromatography with a Waters model 204, a C_{18} μBondapak column and eluting conditions described by Julien et al. [18].

Chemical Modifications of Peptides

Acetylation was performed with acetic anhydride added in 20-μl aliquots every 20 min to a stirred and cold (4 °C) solution of the peptide (about 1 mg/ml) in 0.1 M Tris/HCl (pH 9.0). After a 100-min reaction, the mixture was filtered through a Bio-Gel P4 column (150 × 2.5 cm; elution with 0.1 M acetic acid). CM-cellulose chromatography was then performed in order to separate any residual unreacted initial peptide from the desired product. Reversal of the *O*-acetylation of tyrosine side chain was done by 0.1 M hydroxylamine at pH 7.4. After 1 h, the peptide was separated from excess reagent by Bio-Gel P2 gel filtration (100 × 1.5 cm column; elution with 0.1 M acetic acid). The ultraviolet spectrum of the recovered material was recorded on a Jobin-Yvon 201 spectrophotometer.

BIOLOGICAL MEASUREMENTS

Tritiated neurotensin was prepared by J. L. Morgat at the Centre d'Etudes Nucléaires de Saclay (Gif sur Yvette, France) as previously described [6]. Two different batches were used with specific radioactivities of 77 and 65 Ci/mmol.

Biological activities of the neurotensin analogues were tested in the following systems, which have been characterized in details elsewhere (see [6, 19, 20]).

Inhibition of [^{3}H]Neurotensin Binding on Synaptic Membranes of Rat Brain with Non-radioactive Neurotensin or with Analogues

Specific fixation of tritiated neurotensin to rat brain synaptic membranes has been demonstrated [6]. In the present study [^{3}H]neurotensin (1.5 – 2 nM) and increasing concentrations of an analogue (or non-radioactive neurotensin) were incubated in 250 μl of 50 mM Tris/HCl (pH 7.5) containing 1% bovine serum albumin and 0.4 mg/ml of membrane proteins. At equilibrium (30 min at 24 °C), free peptide was filtered from membrane-bound ligand through Millipore EGWP 0.2-μm filters. After several rinses with chilled incubation buffer, the filters were placed in vials containing 6 ml of scintillation liquid (Unisolve 1) and radioactivity was determined (Packard spectrometer).

Inhibition of [^{3}H]Neurotensin Binding to HT 29 Cells

The specificity of fixation of tritiated neurotensin to HT 29 cells (line derived from a human colon carcinoma) has been established by Kitabgi et al. [19]. In the present study, cells (3×10^6/ml, determined in a Nageotte counting chamber) were incubated with non-radioactive neurotensin or an analogue at known concentrations in a final volume of 250 μl of Krebs-Ringer/Hepes solution (114 mM NaCl, 4 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 20 mM Hepes, pH 7.4) containing 1% bovine serum albumin. After 30 min at 24 °C, cells were centrifuged at 1000 × *g* for 90 s in 1.5-ml conical tubes filled with 1 ml of the chilled incubation buffer. Pellets were resuspended in 100 μl of distilled water, were dissolved in 1.2 ml of Unisolve 1 and radioactivity was determined.

Expression of Results

It has been demonstrated previously that neurotensin interacts with its receptors on rat brain synaptosomal membranes and HT 29 cells according to the law of a simple, reversible, bimolecular reaction [6, 19]. For non-radioactive neurotensin and each analogue the concentration of peptide inhibiting 50% of the binding of [^{3}H]neurotensin (IC_{50}) was calculated from inhibition curves and K_i was then deduced with the relation [19]:

$$K_i = \frac{K_d^*}{K_d^* + [L^*]} \times IC_{50}$$

where K_d^* represents the dissociation constant of [^{3}H]neurotensin. Although [L*] normally refers to the concentration of free [^{3}H]neurotensin, it can be approximated by total ligand concentration in the conditions of our binding experiments [6, 19]. Indeed, it has been shown that bound ligand represented less than 2% and 5% of total ligand with rat brain synaptic membranes [6] and HT 29 cells [19], respectively. In addition, degradation of free ligand was shown to be negligible in both binding systems [6, 19]. All of those analogues with a measurable level of activity, except neurotensin-(4 – 13) and neurotensin-(6 – 13), were found to produce inhibition curves parallel to that of neurotensin, as already mentioned for several analogues [19]. The extent of this parallelism was verified by computing the Hill coefficient [20], for each analogue in the two binding systems. Hill coefficient values between 0.8 and 1.1 were found, which confirms the high degree of parallelism of inhibition curves and indicates that no cooperativity is involved in the interactions of the peptides with the neurotensin receptors [6, 19], with the exception of neurotensin-(4 – 13) and neurotensin-(6 – 13).

Pharmacological Assay of Neurotensin and Analogues

The contracting effect of neurotensin on longitudinal smooth muscle of guinea pig ileum in the presence of neostigmine has been demonstrated by Kitabgi and Freychet [21]. After dissection of the guinea pig ileum, longitudinal strips (4 – 5 cm) of the smooth muscle were placed in a 10-ml organ bath for isometric recording and were equilibrated with Tyrode solution (136.8 mM NaCl, 2.7 mM KCl, 1 mM $MgSO_4$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 3.6 mM $CaCl_2$, 5.5 mM glucose, pH 7.4). Neostigmine methylsulfate was added to the incubated muscle at a concentration of 0.1 μM and response curves were established as a function of increasing concentrations of peptides. Each experiment consisted of complete concentration/response curves for neurotensin and a maximum of three analogues. EC_{50} values were determined graphically.

Activities of each analogue in each test are expressed both in absolute values and relative to neurotensin, taken as 100%.

RESULTS AND DISCUSSION

Preparation and Characterization of Entire and Truncated Sequences of Neurotensin

Solid-phase synthesis [22] of neurotensin-(10 – 13), neurotensin-(9 – 13), neurotensin-(8 – 13), neurotensin-(6 – 13), neurotensin-(4 – 13), neurotensin-(1 – 13), [Lys^8]neurotensin-(8 – 13), [Cit^8]neurotensin-(8 – 13), [Lys^9]neurotensin-(8 – 13), [Cit^9]neurotensin-(8 – 13), [Phe^{11}]neurotensin-(8 – 13), [Ala^{12}]-neurotensin-(8 – 13 and [Ala^{13}]neurotensin-(8 – 13) proceeded

Table 1. *Purification of neurotensin analogues*
e is the calculated electric charge at neutral pH. For molecular sieving, three types of columns have been used: A is Bio-Gel P4, B is Sephadex G-15 and C is Bio-Gel P2. For ion-exchange chromatography the initial buffer was at pH 5.1, final buffer pH 6.0. The last column gives the uncorrected yield of purified peptide taking crude peptide as 100%

Peptides		Purification conditions			Purification yield
		molecular sieving	ion-exchange chromatography on CM-cellulose		
			conductivity gradient	equilibrium conditions	
	e		mS		%
Neurotensin-(10–13)	0	A			79
Neurotensin-(9–13)	+1	A		1 (pH 6.0)	51
Neurotensin-(8–13)	+2	B	1 –10		56
Neurotensin-(6–13)	+3	C	15 –25		58
Neurotensin-(4–13)	+2	B	1 –10		55
Neurotensin	+1	B	1 –10	2.5 (pH 6.0)	50
[Cit^8]Neurotensin-(8–13)	+1	A	0.25– 8		76
[Lys^8]Neurotensin-(8–13)	+2	A	1 –20		71
[Cit^9]Neurotensin-(8–13)	+1	A	0.25– 8		58
[Lys^9]Neurotensin-(8–13)	+2	A	1 –10		57
[Phe^{11}]Neurotensin-(8–13)	+2	A	0.25– 8		65
[Ala^{12}]Neurotensin-(8–13)	+2	A	1 –10		66
[Ala^{13}]Neurotensin-(8–13)	+2	A	1 –10		52

smoothly with an efficient double-coupling procedure [23]. After HF treatment, crude peptides were fractionated by molecular sieving (see Table 1) leading to single peptide peaks in all cases, except for neurotensin-(6–13), neurotensin-(4–13) and neurotensin-(1–13)[1]. After lyophilizing, these main fractions were subjected to carboxymethylcellulose ion-exchange chromatography. The various gradient or equilibrium conditions used are reported in Table 1, together with the final yields of the purification procedure.

The homogeneity of the synthetic derivatives was rigorously verified in order to avoid misinterpretation of biological assays resulting from major contamination by related structures. Tables 2A and B give the amino acid composition of every analogue as determined after acid and enzymatic hydrolysis. The tyrosine content is consistently low (0.94 average for 1 residue) after enzymatic digestion. This may be due to the presence of some of the 0 → C transposition product of 2,6-dichlorobenzyltyrosine [24]. Otherwise, experimental values are in full agreement with predicted values thus demonstrating both complete side-chain removal and lack of measurable racemization. Table 3 summarizes the high-performance liquid chromatography data. Synthetic derivatives were analyzed with two different commercially available reverse-phase columns, each run with its own elution conditions. A satisfactory correlation ($r = 0.903$) was observed between the retention times in the two systems. In our laboratory, system B (see legend of Table 3) exhibited better resolution than the other, since more ultraviolet-absorbing impurities were separated. All ultraviolet-absorbing peaks were automatically integrated (Table 3). Results show that in the samples of [Ala^{13}]neurotensin-(8–13) and [Cit^9]-neurotensin-(8–13), total 'contaminants' reached the level of 10%, but individually each of the 'contaminating' peaks accounted for less than 2%. The chemical nature of the ultraviolet(230 nm)-absorbing materials was not investigated and undoubtedly not all contain peptides. For all other analogues the total surface of 'impurities' was in the 0–5% range. Finally, [Lys^8]neurotensin-(8–13) and [Lys^9]neurotensin-(8–13) were subjected to automatic Edman degradation with parvalbumin protection [25]. The combination of high-performance liquid chromatography and hydrolysis of phenylthiohydantoins was used to evaluate the sequencing data quantitatively. The presence of phenylthiohydantoin $n + 1$ together with the expected one at step n of the degradation reveals a contamination of the target peptide by an intrusive sequence lacking residue n [26]. Phenylthiohydantoin $n + 1$ detected at step n is called the 'preview' phenylthiohydantoin. The results are shown in Tables 4A and B. The high-performance liquid chromatography analysis of phenylthiohydantoins obtained at step 3 of the degradation of [Lys^8]neurotensin-(8–13) showed a peak with a retention time approximately equal to that of tyrosine. The presence of a deletion peptide lacking a prolyl residue was eliminated, however, by back hydrolysis and amino acid analysis of the product obtained at step 3, and by the absence of a 'preview' phenylthiohydantoin at the following step (Table 4B). The chemical nature of the contaminating peak of step 3 was not determined. Finally, no 'preview' phenylthiohydantoin could be detected in amounts higher than background contamination for the two peptides analyzed even though the starting quantities of peptides subjected to degradation were high (about 500 nmol).

Acetylation of neurotensin-(6–13), neurotensin-(7–13), neurotensin-(8–13), [Cit^8]neurotensin-(8–13), [Lys^8]neurotensin-(8–13), [Cit^9]neurotensin-(8–13), [Lys^9]neurotensin-(8–13) and [Phe^{11}]neurotensin-(8–13) with acetic anhydride was uncomplicated and quantitative. It resulted in the cor-

[1] These derivatives were obtained in the course of a single synthesis of the entire sequence of neurotensin: all three crude peptides were contaminated by the same by-product Ac-neurotensin-(7–13) formed during the synthesis when, after a double coupling of Lys^6 onto Pro^5, we performed an automatic acetylation of possible remaining imino groups. Unexpected poor incorporation of Lys^6 yielded a 20% formation of Ac-neurotensin-(7–13).

Table 2A. *Amino acid analysis of neurotensin and its N-terminally truncated sequences, after acid and enzymatic hydrolysis*
Values are given as molar ratios and are the means of three separate analyses.

Amino acid	Neurotensin peptide										
	(4–13)	(6–13)	(7–13)[a]		(8–13)		(9–13)		(10–13)		(1–13)[b]
	acid hydrolysis	acid hydrolysis	acid hydrolysis	enzymatic hydrolysis	acid hydrolysis	enzymatic hydrolysis	acid hydrolysis	enzymatic hydrolysis	acid hydrolysis	enzymatic hydrolysis	acid hydrolysis
Aspartic acid	1.03										1.00
Glutamic acid	0.98										1.99
Proline	2.04	2.04	2.01	2.04	0.99	1.02	1.00	1.03	0.99	1.00	2.04
Isoleucine	0.96	0.99	0.99	1.03	1.00	1.02	0.97	1.03	1.00	1.02	0.94
Leucine	1.04	1.03	1.05	1.06	1.02	1.04	1.01	1.03	1.01	1.03	1.99
Tyrosine	0.88	0.98	0.90	0.99	0.81	0.94	0.80	0.94	0.71	0.93	2.05
Lysine	1.02	1.01	1.94	1.88							1.09
Arginine	1.99	2.01			1.99	1.96	1.01	0.99			2.04

[a] This peptide was synthesized by S. Geoffre (Laboratoire de Cristallographie, Université Bordeaux I, Talence, France) following our synthetic protocol and was characterized in our laboratory.
[b] Neurotensin.

Table 2B. *Amino acid analyses of structural analogues of the 8–13 sequence of neurotensin*

Amino acid	[Cit[8]]Neurotensin-(8–13)		[Lys[8]]Neurotensin-(8–13)		[Cit[9]]Neurotensin-(8–13)		[Lys[9]]Neurotensin-(8–13)		[Phe[11]]Neurotensin-(8–13)		[Ala[12]]Neurotensin-(8–13)		[Ala[13]]Neurotensin-(8–13)	
	acid hydrolysis	enzymatic hydrolysis	acid hydrolysis	enzymatic hydrolysis	acid hydrolysis	enzymatic hydrolysis	acid hydrolysis	enzymatic hydrolysis	acid hydrolysis	enzymatic hydrolysis	acid hydrolysis	enzymatic hydrolysis	acid hydrolysis	enzymatic hydrolysis
Proline	1.00	1.05	1.00	1.01	1.02	0.97	1.03	0.96	1.19	1.09	1.02	1.07	1.00	1.04
Citrulline	n.d.[a]	1.01			n.d.[a]	1.03								
Alanine											1.07	1.09	1.10	1.07
Isoleucine	0.97	0.99	0.94	0.99	0.97	0.97	0.97	0.99	0.95	0.98			0.97	1.00
Leucine	1.01	1.01	0.98	0.99	1.02	0.99	0.99	1.00	0.99	0.99	0.98	1.00		
Phenylalanine									1.02	1.06				
Tyrosine	0.61	0.94	0.83	0.94	0.80	0.93	0.88	0.96			0.66	0.94	0.78	0.94
Lysine			0.96	1.04			0.99	1.02						
Arginine	1.01	0.99	1.04	0.99	1.04	1.04	1.01	1.02	2.03	1.99	1.92	1.89	1.92	1.92

[a] Not determined (n.d.) since citrulline is partially destroyed during acid hydrolysis.

responding N^{α}-blocked derivatives after reversal of *O*-acetylation by hydroxylamine, followed by desalting on Bio-Gel P4. The ultraviolet spectrum showed a typical tyrosine peak in all cases.

Biological Measurements and Structure/Activity Relationships

Table 5 shows the biological activities of all the synthetic analogues determined with two different binding assays and one pharmacological assay. As mentioned, neurotensin exhibits numerous effects, so that the specificity of the tests chosen to elucidate structure/activity relationships are of the utmost importance. The binding assays with neural receptors (the synaptic membranes) [6] and with extraneural receptors (the HT 29 cell line derived from a human colon carcinoma) [19] are highly specific. The constants (K_i) for inhibition of [^{3}H]neurotensin binding by the unlabeled peptide are low: 1.9 nM and 1.2 nM, respectively [19]. The half-maximal effective concentration (EC_{50}) of neurotensin determined for the smooth muscle contraction assay is also low and of the same order of magnitude (5 nM) [19], rendering the comparisons of analogue potencies among tests meaningful.

Table 5 shows that the shortest sequence which can measurably bind to both types of receptors and still be able to promote a consistent smooth muscle contraction is the C-terminal five-residue peptide neurotensin-(9–13). It should be noted that neurotensin-(9–13) is able to inhibit [^{3}H]neurotensin binding to its receptors completely, although with a low potency, and to produce the same maximal contraction as native neurotensin on the guinea-pig ileum. Therefore, neurotensin-(9–13), behaves as a full agonist. These observations agree with the conclusion drawn by Quirion et al. [11–13], who tested peptide activity with pharmacological assays *in vitro* on guinea-pig atria and on rat stomach, heart and portal vein. The addition of one residue to this 'shortest sequence' produced a 15-fold rise in binding to rat brain membranes (Table 5), a result similar to that reported by Uhl et al. [14] for neurotensin-(8–13) and neurotensin-(9–13) in a neural binding assay using ^{125}I-neurotensin as the labeled

Table 3. *High-performance liquid chromatography of purified synthetic derivatives of neurotensin*
System A consisted of two columns of C_{18} μBondapak in series; elution was at 2 ml/min with 10 mM triethylammonium phosphate pH 3/methanol (50/50, v/v). System B consisted of two columns of RP_{18} Lichrosorb in series; elution was at 1.5 ml/min with 1% phosphoric acid/acetonitrile (74/26, v/v). About 20 nmol of the peptide were chromatographed on system A and exactly 20 nmol on system B. Absorbance was measured at 230 nm. Values for the percentage of contaminants were obtained directly from automatic integration, so that even non-peptide ultraviolet-absorbing material was taken into account. n.d. = not determined

Peptide	Chromatographic data on system A		Chromatographic data on system B	
	retention time	maximum amount of ultraviolet-absorbing contaminants	retention time	maximum amount of ultraviolet-absorbing contaminants
	min	%	min	%
Neurotensin	5.9	5	10.5	1
Neurotensin-(4–13)	3.9	n.d.	4.6	4
Neurotensin-(6–13)	3.7	n.d.	4.5	3
Neurotensin-(8–13)	n.d.	n.d.	5.7	5
[Ala^{13}]Neurotensin-(8–13)	3.2	10	6.1[a]	8
[Ala^{12}]Neurotensin-(8–13)	3.5	5	11.4[a]	1
[Lys^{8}]Neurotensin-(8–13)	4.0	1	5.3	3
[Lys^{9}]Neurotensin-(8–13)	4.2	1	5	5
[Cit^{8}]Neurotensin-(8–13)	5.2	1	6.2	2
[Cit^{9}]Neurotensin-(8–13)	5.2	10	7.1	11
[Phe^{11}]Neurotensin-(8–13)	5.6	1	7.4	5
Ac-Neurotensin-(8–13)	n.d.	n.d.	7.4	n.d.

[a] In this case, the mobile phase contained 15% acetonitrile.

Table 4A. *Quantitative data of a four-step sequential Edman degradation of [Lys^{9}]neurotensin-(8–13)*
Phenylthiohydantoins were identified and quantified first using high-performance liquid chromatography, then by amino acid analysis after hydrolysis. The 'preview' amino acid is defined in the text; 'other' amino acids are due to peptide extracted from the sequencer cup together with the phenylthiocarbamoyl derivative. n.d. = not determined

Method	Amount of phenylthiohydantoin found in											
	step 1			step 2			step 3			step 4		
	expected (Arg)	'preview' (Lys)	other	expected (Lys)	'preview' (Pro)	other	expected (Pro)	'preview' (Tyr)	other	expected (Tyr)	'preview' (Ile)	other
	nmol											
High-performance liquid chromatography	n.d.	7.5	–	176	8.9	–	117	6.1	–	106	8.5	–
Amino acid analysis	–	10.2	10.5	–	13.7	12.6	–	6.3	8.7	–	1.5	1.7

Table 4B. *Quantitative data of a four-step sequential Edman degradation of [Lys^{8}]neurotensin-(8–13)*

Method	Amount of phenylthiohydantoin found in											
	step 1			step 2			step 3			step 4		
	expected (Lys)	'preview' (Arg)	other	expected (Arg)	'preview' (Pro)	other	expected (Pro)	'preview' (Tyr)	other	expected (Tyr)	'preview' (Ile)	other
	nmol											
High-performance liquid chromatography	94	–	–	n.d.	5	–	43	25[a]	–	37	8.4	–
Amino acid analysis	–	1.5	7.1	–	6.5	8.1	–	8.7	8.6	–	6.5	7.5

[a] See text.

Table 5. *Comparison of binding and pharmacological potencies of neurotensin analogues*
Calculations of K_i values were done according to the procedure described in Materials and Methods and using K_d values of 2 nM for [^{3}H]neurotensin fixation to synaptic membranes [6] and 1.5 nM for [^{3}H]neurotensin binding to HT 29 cells [19]. Relative activities are $100 \times K_i$ (or EC_{50}) of neurotensin/K_i (or EC_{50}) of the analogue. n.d. = not determined

Peptide	Binding assay on rat brain synaptic membranes		Binding assay on HT 29 cells		Pharmacological assay with guinea pig ileum	
	K_i	relative activity	K_i	relative activity	EC_{50}	relative activity
	nM		nM		nM	
Neurotensin	1.9	100	1.2	100	5	100
Neurotensin-(1–12)[a]	>4000	<0.05	>4000	<0.05	>5000	<0.1
Neurotensin-(10–13)	>4000	<0.05	>4000	<0.05	>5000	<0.1
Neurotensin-(9–13)	200	1	90	1.3	170	3
Neurotensin-(8–13)	13	15	1	120	25	20
Ac-Neurotensin-(8–13)	1.1	170	1	120	5.6	90
Neurotensin-(7–13)	2.7	70	1.3	92	15	35
Ac-Neurotensin-(7–13)	2.1	90	n.d.	n.d.	11	46
Neurotensin-(6–13)	0.0014[b]	10^5	n.d.	n.d.	17	29
Ac_2-Neurotensin-(6–13)	13.5	15	n.d.	n.d.	26	19
Neurotensin-(4–13)	0.02[b]	10^4	n.d.	n.d.	16	30
Ac-[Cit^8]Neurotensin-(8–13)	90	2.1	27	4.4	30	17
Ac-[Cit^9]Neurotensin-(8–13)	85	2.3	20	6	55	9
[Lys^8]Neurotensin-(8–13)	13	15	0.6	200	n.d.	n.d.
Ac_2-[Lys^8]Neurotensin-(8–13)	110	1.7	n.d.	n.d.	n.d.	n.d.
[Lys^9]Neurotensin-(8–13)	7.5	25	1	120	n.d.	n.d.
Ac_2-[Lys^9]Neurotensin-(8–13)	80	2.4	n.d.	n.d.	n.d.	n.d.
[Ala^{13}]Neurotensin-(8–13)	>2000	<0.1	400	0.3	>5000	<0.1
[Ala^{12}]Neurotensin-(8–13)	>2000	<0.1	300	0.4	>5000	<0.1
[Phe^{11}]Neurotensin-(8–13)	120	1.6	n.d.	n.d.	160	3
Ac-[Phe^{11}]Neurotensin-(8–13)	26	7	n.d.	n.d.	75	7
Xenopsin	1.5	127	n.d.	n.d.	9	55

[a] Synthesis and characterization of this peptide have been already described elsewhere [6].
[b] Inhibition curve for this analogue is not parallel to that of neurotensin and therefore its K_i value does not represent a true inhibition constant.

ligand. These observations are markedly different, however, from those of Lazarus et al. [15], who found that both neurotensin-(8–13) and neurotensin-(9–13) were 10000–30000 times less potent than unlabeled neurotensin for inhibiting ^{125}I-neurotensin binding to rat brain membranes. The explanation for these discrepancies is apparently independent of the different origins of the analogues, since the partial sequences neurotensin-(8–13) and neurotensin-(9–13) of River [8] which were used by Lazarus et al. [15] were tested in our neural radioreceptor assay [19] and showed the same activity as the analogues used in the present study. The differences between our results and those of Lazarus et al. [15] may rather be related to the fact that they used a highly unstable ^{125}I-neurotensin [27] as their labeled ligand, instead of [^{3}H]neurotensin as in the present study. Marked differences also appear when the binding potency values for neurotensin-(8–13) and neurotensin-(9–13) in our extraneural radioreceptor assay using HT 29 cells are compared to the corresponding values obtained by Lazarus et al. [9] in their extraneural radioreceptor assay with rat mast cells. It should be noted that the low K_d value (154 nM) determined for the fixation of neurotensin to rat mast cells [27] is far above the concentrations of neurotensin that are active *in vitro*.

The *N*-acetylation of the C-terminal six-residue peptide of neurotensin generated a derivative, Ac-neurotensin-(8–13), whose effects mimic that of the complete structure of neurotensin (Table 5). Since neurotensin-(1–12) lacked any of the three activities tested, we can reasonably consider that the structure -Arg-Arg-Pro-Tyr-Ile-Leu is the minimal sequence required for expression of full biological potency and effectiveness, provided that its N-terminal extremity is protected. The results obtained with xenopsin, a natural analogue of the C-terminal portion of neurotensin (< Glu-Gly-Lys-Arg-Pro-Trp-Ile-Leu) are in good agreement with this conclusion. Our results appear to indicate an influence of an enzymatic action on the biological measurements. The data are consistent with the existence of an aminopeptidase activity in rat brain membranes and in the smooth muscle preparation, shortening the fully efficient sequence 8–13 into derivatives with low activities. This mechanism, which remains to be formally demonstrated, is apparently not a factor in the HT 29 cell preparations, since all three peptides neurotensin-(8–13), [Lys^8]neurotensin-(8–13) and [Lys^9]neurotensin-(8–13) had about the same activities as Ac-neurotensin-(8–13) in the corresponding fixation test (Table 5).

Truncated sequences including lysine in position 6 [neurotensin-(6–13), neurotensin-(4–13)] led to surprising results in the fixation assay on synaptosomal membranes. We observed a marked increase of the affinity of the peptide for the membranes and although the binding curve was not parallel to those obtained with neurotensin and all other analogues, a 50% inhibition of [^{3}H]neurotensin binding was obtained with concentrations at least 100-times lower than that of native neurotensin. This increased affinity is an artifact and may be due to a salt bridging near the receptor involving lysine at position 6, since acetylation of this residue brings back the binding affinity to the level of neurotensin-(8–13). The contracting activities of neurotensin-(7–13), neurotensin-(6–13), Ac_2-neurotensin-(6–13) and neurotensin-(4–13) were all in the range of 30% of that of entire neuro-

tensin and no inhibition was obtained when neurotensin-(6–13) and neurotensin-(4–13) were added to neurotensin in concentrations at which the partial sequences have no effect on the guinea-pig ileum and yet are able to inhibit more than 50% of the binding of [^{3}H]neurotensin to brain membranes. The difference in binding and contracting activities suggest that although the receptor sites themselves may be of the same kind in brain membranes and in guinea-pig ileum, their environment may be different.

Thus, the effects of neurotensin chain length on binding or contracting activities may be interpreted as follows. The 8–13 portion of neurotensin contains the necessary structure for expressing complete activity and the N-terminal part would play a role in conformation, minimizing the effect of the α-amino group of Lys^6 and/or a protective role against exopeptidases.

The results obtained with Ac-[Cit^8]neurotensin-(8–13) and Ac-[Cit^9]neurotensin-(8–13) show the importance of two positively charged residues in positions 8 and 9 for binding and for the expression of contracting activity, since the replacement of the guanidino function of arginine by the isosteric but uncharged urea group of citrulline led to decreased activity. The results obtained with Ac-[Lys^8]neurotensin-(8–13) or Ac-[Lys^9]neurotensin-(8–13) are in perfect agreement. Since [Lys^8]neurotensin-(8–13) and [Lys^9]neurotensin-(8–13) exhibit binding properties very similar to the unsubstituted parent peptide [neurotensin-(8–13)] we may conclude that there is no prerequisite for the presence of a guanidinium structure at positions 8 and 9.

Peptides [Ala^{12}]neurotensin-(8–13) and [Ala^{13}]neurotensin-(8–13) were prepared and tested in their N^{α}-free form and the data are unambiguous. The side chains of the C-terminal and penultimate residues (Leu and Ile) cannot be replaced by a simple methyl group without a dramatic loss of biological properties. Clearly, Ile^{12} and Leu^{13} are key amino acids for the expression of neurotensin activities, either because they are part of an hydrophobic C-terminal area or because of specific requirements for aliphatic branched side chains. The role of Tyr^{11} is not obvious. A report by Rivier et al. [8] showed that [Phe^{11}]neurotensin and neurotensin are equipotent for binding to mast cells and for inducing hypothermia, and that [DPhe^{11}]neurotensin and [DTyr^{11}]neurotensin have reduced fixation activity, but a 10-fold increase of bio-activity. In the present study, we prepared [Phe^{11}]neurotensin-(8–13) and Ac-[Phe^{11}]neurotensin-(8–13) and found them to express only partial neurotensin properties and no discrepancy between binding and pharmacological activities. The phenol function at position 11 is probably also involved in the interaction with receptors, supporting the recent proposal of Quirion et al. [28] of hydrogen bonding between the receptor and the hydroxyl function of Tyr^{11}.

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