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A Rational Approach for the Development of Reduced-size Analogues of Neuropeptide Y with High Affinity to the Y_1 Receptor

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Four sets of centrally truncated analogues of neuropeptide Y have been synthesized. In each series the N-terminal part was constant, while the C-terminal segment was systematically varied in length. The C- and N-terminal parts were linked by 6-aminohexanoic acid. The affinity to the Y_1 receptor was investigated on human neuroblastoma cells SK-N-MC. Significant differences were found between the series of peptides as well as within each set. Remarkably, the affinity did not solely depend on the length of the segment, and with increasing numbers of residues the IC_{50} values were not always decreased. With a given N-terminal segment, only one optimal length of the C-terminal segment was found, which suggests that it is not the amino acids themselves but their 3D arrangement and orientation that is important for high receptor affinity.

Keywords: Neuropeptide Y; centrally truncated analogues; Y_1 receptor binding

Abbreviations

Ahx, 6-aminohexanoic acid; Aoc, 8-aminooctanoic acid; NPY, neuropeptide Y.

INTRODUCTION

Neuropeptide Y (NPY) is a 36-amino acid peptide amide which belongs to the pancreatic polypeptide hormone family. It was isolated from pig brain and sequenced in 1982 [1]. Using the X-ray structure of the avian pancreatic polypeptide [2] which has been obtained by Blundell and coworkers, a model has been deduced for the 3D structure of NPY [3–5]. Molecular dynamic simulations and ^{1}H-NMR investigations [6] confirmed the hairpin-like structure. Accordingly, residues 1–8 form a type II proline helix, followed by a loop (residues 9–14), which is bound to an α-helix (amino acids 15–32). The four C-terminal amino acids are arranged flexibly (Figure 1).

NPY is present in the peripheral and central nervous system. In the periphery, NPY regulates its own release and inhibits the release of noradrenaline. Postsynaptically, NPY causes an increase in blood pressure by a direct vasoconstrictive effect as well as by potentiation of the activity of other vasoconstrictors, such as angiotensin II and noradrenaline [7–9]. Central applications of NPY into specific brain regions lead to a variety of effects. Blood pressure lowering, increase in food intake, sedation, enhancement of memory and modulation of sexual hormones have been attributed to NPY injections [7–9].

The activity of NPY has been shown to be mediated through at least two subtypes of NPY specific receptors – the so-called Y_1 and Y_2 receptors. Whereas the affinity of analogues to the Y_2 receptor has been investigated in great detail (for review see [10]), leading to the identification of the most

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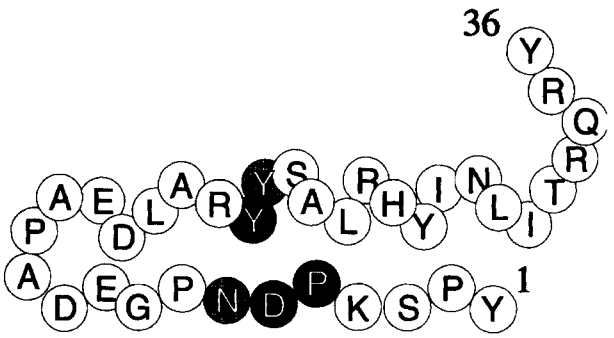

Figure 1 Sequence of neuropeptide Y (NPY) and a schematic illustration of the hypothetical arrangement of its C- and N-termini at the Y_1 receptor. The optimal linkage of the C- and N-terminal segments by Ahx is demonstrated by the light and dark marked amino acids. The systematical prolongation of the N-terminus led to the analogues [Ahx^{6-19}]NPY, [Ahx^{7-19}]NPY and [Ahx^{8-20}]NPY that exhibited the best Y_1 receptor affinity.

important residues, little is yet known about the structure–activity relationships at the Y_1 receptor. However, it is clear that a different binding site and perhaps a different conformation of NPY is recognized by the two subtypes [10]. While short C-terminal segments and small centrally truncated analogues (such as NPY (13–36 [11], 18–36 [12], 25–36 [13, 14]) and [Ahx^{5-24}]NPY [15, 16]), bind with high affinity to the Y_2 receptor, the entire NPY molecule is needed for substantial Y_1 receptor binding. So far, no small peptidic analogues with high affinity to the Y_1 receptor have been reported. Although neither N- [17] nor C-terminal [11, 18] segments show any Y_1 receptor affinity, the results of the L-Ala scan [19] indicate that the entire sequence of the hormone is not required. Replacement of residues between positions 8 and 18 leads only to a small loss of affinity, whereas Pro^2 and Pro^5 are the most sensitive amino acids in the N-terminal segment. In the C-terminal region of the α-helix, the hydrophobic amino acids Tyr^{20}, Leu^{24}, Tyr^{27} and Ile^{31} are sensitive to replacement by L-Ala. It is suggested that the C-terminal pentapeptide plays an important role in Y_1 receptor binding. This is in good agreement with the fact that centrally truncated cyclic peptides of medium size have high Y_1 receptor binding [20]. The analogue [Aoc^{8-17}, D-Cys^7-D-Cys^{20}] in which the C- and N-terminal parts are additionally linked by a disulphide bridge shows 10% of the affinity of NPY to the Y_1 receptor in the mouse brain and in SK-N-MC cells. Kirby *et al.* [21] succeeded in optimizing the affinity of this analogue by exchanging Aoc with Pro-Gly and through the replacement of D-Cys^7 by L-Cys^7 at the same time as shifting the second bridge head from amino acid 20 up to 21.

The results with the large discontinuous peptides and also the results from the L-Ala scan [19] suggest that the conformation and especially the orientation of the C- and N-termini are important for high Y_1 receptor binding. In order to identify the minimal centrally truncated Y_1 receptor binding peptide, and to characterize the interaction, a rational approach of minimisation was established. 6-Aminohexanoic acid (Ahx, X) was used for spacing the N- and C-terminal segments. We started with an N-terminal segment of five amino acids in order to include Pro^5, which was found to be important. In the following, the N-terminal part was elongated to up to eight amino acids. For a given length of the N-terminal segment a systematic variation of the C-terminus was performed. Binding to the Y_1 receptor was investigated at human SK-N-MC neuroblastoma cells.

MATERIALS AND METHODS

Materials

L-Amino acids protected with fluoren-9-ylmethoxycarbonyl were obtained from Nova Biochem (Läufelfingen, Switzerland), aminomethylated polystyrene from Rapp Polymere (Tübingen, Germany) diisopropylcarbodiimide and thiocresol from Aldrich, 1-hydroxybenzotriazole, trifluoroacetic acid, thioanisole and piperidine from Fluka and dimethylformamide (p.a. grade), diethyl ether, acetonitrile and *tert*-butyl alcohol from Merck. Hepes, bovine serum albumin, bacitracin and pentamethylsulphonylfluoride were purchased from Sigma, Fmoc-aminomethyl-dimethoxyphenoxy valeric acid from Serva, sodium pyruvate, minimum essential medium with Earle's salts from Gibco, fetal calf serum, glutamine and non-essential amino acids from Boehringer Mannheim. EDTA and all salts for preparing the buffers were from either Fluka or Merck. ^{125}I-Bolton Hunter NPY was ordered from Anawa (Zürich, Switzerland) and specific activity was 81 TBq/mM.

Peptide Synthesis and Analysis

The peptides were synthesized by automated, multiple solid-phase peptide synthesis using a robot system (Syro, MultiSynTech, Bochum). In order to obtain peptide amides, 5-(4′-aminomethyl-3′5′-dimethoxyphenoxy)-pentanoyl-alanine was used for

anchoring to polystyrene–1%-divinylbenzene (30 mg, 15 μM/peptide). For the sidechain protection Tyr(*t*-butyl), Asp(*t*-butyl) Glu(*t*-butyl), Ser(*t*-butyl), Thr(*t*-butyl), Arg(2,2,3,5,5-pentamethylchromanesulphonyl), His(trityl), Gln(trityl) Asn(trityl) and Lys(*t*-butyloxycarbonyl) were used. The C-terminal 18 residues were incorporated using single couplings with diisopropyl-carbodiimide/1-hydroxybenzotriazole activation, 10-fold excess and a coupling time of 1 h. To extend the peptides, double coupling cycles of two times 40 min were chosen.

The peptide amides were cleaved with trifluoroacetic acid/thionanisole/thiocresol within 2 h, collected by centrifugation and lyophilized from water. The peptides were analysed and purified, if necessary to obtain homogeneity >95%, by reversed-phase HPLC (Merck-Hitachi, Darmstadt) on a Nucleosil 5 μm, C-18 column (analytical: 125 × 3 mm, preparative 250 × 20 mm). As eluent three different gradients of acetonitrile/0.1% trifluoroacetic acid in water were used. Furthermore the peptides were analysed by electrospray mass spectrometry (API III, Sciex, Toronto). The analytical data are shown in Table 1. Purity was >95% for each peptide according to MS and HPLC. Exact concentrations were determined by quantitative amino acid analysis after gas-phase hydrolysis (ABI 140/420, Applied Biosystems, Weiterstadt).

Table 1 Sequences and Analytical Data (Electrospray Mass Spectra, Retention Time of Reversed Phase HPLC)

Peptide	Sequence	Molecular mass (a.m.u.) Theoretical	Molecular mass (a.m.u.) Experimental	Retention times (min)
[Ahx$^{5-24}$]NPY	YPSKXRHYINLITRQRY	2220.6	2220 ± 0.5	21.8 (III)
[Ahx$^{6-22}$]NPY	YPSKPXALRHYINLITRQRY	2501.8	2501 ± 0.4	21.9 (II)
[Ahx$^{6-21}$]NPY	YPSKPXSALRHYINLITRQRY	2589.1	2588 ± 0.8	17.2 (I)
[Ahx$^{6-20}$]NPY	YPSKPXYSALRHYINLITRQRY	2752.2	2752 ± 0.7	23.0 (II)
[Ahx$^{6-19}$]NPY	YPSKPXYYSALRHYINLITRQRY	2915.4	2915 ± 0.7	19.8 (I)
[Ahx$^{6-18}$]NPY	YPSKPXRYYSALRHYINLITRQRY	3071.6	3070 ± 0.3	23.7 (II)
[Ahx$^{7-22}$]NPY	YPSKPDXALRHYINLITRQRY	2617.1	2617 ± 0.9	17.1 (I)
[Ahx$^{7-21}$]NPY	YPSKPDXSALRHYINLITRQRY	2704.2	2704 ± 0.8	16.9 (I)
[Ahx$^{7-20}$]NPY	YPSKPDXYSALRHYINLITRQRY	2867.3	2866 ± 0.3	22.9 (II)
[Ahx$^{7-19}$]NPY	YPSKPDXYYSALRHYINLITRQRY	3030.5	3029 ± 0.7	19.0 (I)
[Ahx$^{7-18}$]NPY	YPSKPDXRYYSALRHYINLITRQRY	3186.7	3186 ± 0.6	24.0 (II)
[Ahx$^{8-22}$]NPY	YPSKPDNXALRHYINLITRQRY	2731.2	2730 ± 0.1	17.0 (I)
[Ahx$^{8-21}$]NPY	YPSKPDNXSALRHYINLITRQRY	2818.3	2817 ± 0.8	17.5 (I)
[Ahx$^{8-20}$]NPY	YPSKPDNXYSALRHYINLITRQRY	2981.4	2980 ± 0.8	23.1 (II)
[Ahx$^{8-19}$]NPY	YPSKPDNXYYSALRHYINLITRQRY	3144.6	3144 ± 0.1	19.3 (I)
[Ahx$^{8-18}$]NPY	YPSKPDNXRYYSALRHYINLITRQRY	3300.8	3300 ± 0.8	23.5 (II)
[Ahx$^{9-22}$]NPY	YPSKPDNPXALRHYINLITRQRY	2828.3	2827 ± 0.8	21.9 (II)
[Ahx$^{9-21}$]NPY	YPSKPDNPXSALRHYINLITRQRY	2915.4	2914 ± 0.3	22.1 (II)
[Ahx$^{9-20}$]NPY	YPSKPDNPXYSALRHYINLITRQRY	3078.6	3078 ± 0.6	23.3 (II)
[Ahx$^{9-19}$]NPY	YPSKPDNPXYYSALRHYINLITRQRY	3241.7	3240 ± 0.0	24.1 (II)
[Ahx$^{9-18}$]NPY	YPSKPDNPXRYYSALRHYINLITRQRY	3397.9	3396 ± 1.0	23.9 (II)
[Ahx$^{9-17}$]NPY	YPSKPDNPXARYYSALRHYINLITRQRY	3469.0	3470 ± 0.2	18.5 (I)
[des-AA$^{9-22}$]NPY	YPSKPDNPALRHYINLITRQRY	2715.1	2714 ± 0.6	20.8 (II)
[des-AA$^{9-21}$]NPY	YPSKPDNPSALRHYINLITRQRY	2802.2	2801 ± 1.2	21.0 (II)
[des-AA$^{9-20}$]NPY	YPSKPDNPYSALRHYINLITRQRY	2965.4	2964 ± 0.0	24.3 (II)
[des-AA$^{9-19}$]NPY	YPSKPDNPYYSALRHYINLITRQRY	3128.5	3127 ± 0.5	24.5 (IV)
[des-AA$^{9-18}$]NPY	YPSKPDNPRYYSALRHYINLITRQRY	3284.7	3283 ± 0.6	22.5 (II)

[a] The following gradients were used (I) 20–70% acetonitrile/trifluoroacetic acid within 30 min; (II) 15–60% acetonitrile/trifluoroacetic acid within 30 min; (III) 20–60% acetonitrile/trifluoroacetic acid within 30 min; (IV) 10–50% acetonitrile/trifluoroacetic acid within 30 min.

Membrane Preparation

SK-N-MC cells were grown in minimum essential medium containing Earle's salt, 10% fetal calf serum, 1 mM sodium pyruvate, 1% non-essential amino acids and 4 mM glutamine. Confluent cells are removed with 0.02% EDTA in phosphate-buffered saline (1 min incubation) and resuspended in 10 ml incubation buffer (minimum essential medium, 25 mM Hepes, 0.5% bovine serum albumin, 50 μM phenylmethylsulphonyl fluoride, 0.1% bacitracin, 3.75 mM $CaCl_2$). After a 5 min centrifugation (150 × *g*), the pellet was resuspended in an equal volume and, after further centrifugation in incubation buffer, the cells are counted and diluted to a final concentration of 1.25×10^6 cells/ml.

Receptor Binding

Y_1 receptor affinity was tested by incubation of SK-N-MC cell suspension (0.2 ml, 1.25×10^6 cells/ml) with 30 pM ^{125}I-Bolton Hunter NPY and with the peptides at 11 different dilutions (covering six orders of magnitude) in minimum essential medium, 25 mM Hepes, 0.5% bovine serum albumin, 50 μM phenylmethylsulphonylfluoride, 0.1% bacitracin, 3.75 mM $CaCl_2$ at room temperature for 3 h in a total volume of 0.25 ml. Non-specific binding was defined in the presence of 100 nM NPY in the incubation mixture. At the end of the incubation period, cells were centrifuged (5 min, 150 × *g*), the pellets were washed with phosphate-buffered saline and recentrifuged. Bound radioactivity was determined by gamma-counting. Half maximal inhibition of the specific binding of the ^{125}I-BH-NPY of two to three separate experiments is given as the IC_{50} value.

RESULTS

The centrally truncated analogues of NPY were obtained by multiple automatic solid-phase peptide synthesis. The peptides shown in Table I and the analogues [des-AA$^{5-23}$]NPY, [des-AA$^{6-22}$]NPY were synthesized by the Fmoc strategy. The Y_1 receptor affinity of all the analogues was investigated on human neuroblastoma cells SK-N-MC. The two analogues [des-AA$^{5-23}$]NPY and [des-AA$^{6-22}$]NPY exhibited no binding to the Y_1 receptor ($IC_{50} > 4000$ nM), like the well-known analogue [Ahx$^{5-24}$]NPY [22].

The peptides of the set containing five N-terminal amino acids and the spacer Ahx showed only low affinity to the Y_1 receptor although [Ahx$^{6-19}$]NPY was 30-fold more potent than [Ahx$^{5-24}$]NPY (IC_{50} 140 nM compared with 4000 nM). Interestingly, in this set a C-terminal segment of 17 amino acids seemed to be the optimum ([Ahx$^{6-19}$]NPY, IC_{50} 140 nM). Longer segments ([Ahx$^{6-18}$]NPY, IC_{50} 360 nM) as well as shorter ones ([Ahx$^{6-20}$]NPY, IC_{50} 272 nM; [Ahx$^{6-21}$]NPY, IC_{50} 350 nM) showed considerably lower affinity (Figure 2).

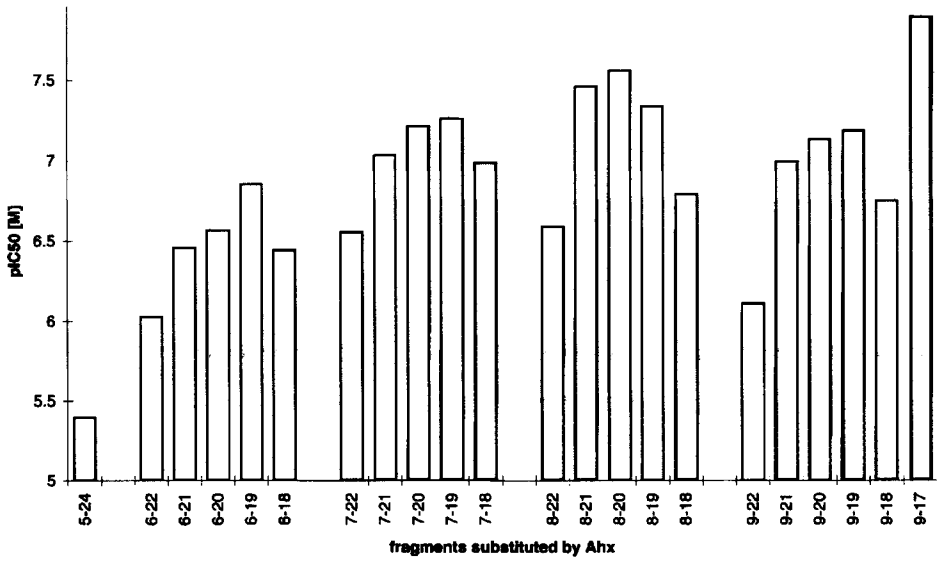

Figure 2 Y_1 receptor affinity of centrally truncated neuropeptide Y analogues, pIC_{50} were determined at human neuroblastoma cell line SK-N-MC.

The extension of the N-terminus by one amino acid to obtain [Ahx$^{7-19}$]NPY increased the affinity by a factor 2.5 (IC_{50} 55 nM). Again, the C-terminally longer [Ahx$^{7-18}$]NPY (IC_{50} 103 nM) and shorter [Ahx$^{7-20}$]NPY (IC_{50} 61 nM), [Ahx$^{7-21}$]NPY (IC_{50} 93 nM) sequences had reduced binding capacities. However, the difference is not as clear as in the case of [Ahx$^{6-19}$]NPY.

[Ahx$^{8-19}$]NPY (IC_{50} 46 nM) bound slightly better than [Ahx$^{7-19}$]NPY (IC_{50} 55 nM). Nevertheless, by shortening the C-terminal segment to obtain [Ahx$^{8-20}$]NPY (IC_{50} 28 nM) and [Ahx$^{8-21}$]NPY (IC_{50} 35 nM) the affinity increased again. In contrast by lengthening the segment the binding was reduced: [Ahx$^{8-18}$]NPY (IC_{50} 163 nM)

In the set of [Ahx^{9-xx}]NPY peptides it was found that neither [Ahx$^{9-19}$]NPY (IC_{50} 65 nM) nor [Ahx$^{9-20}$]NPY (IC_{50} 74 nM) showed the best affinity. By shortening the peptide to obtain [Ahx$^{9-21}$]NPY (IC_{50} 102 nM) or [Ahx$^{9-22}$]NPY (IC_{50} 780 nM), a decrease in activity was observed. Extension of the C-terminus as in [Ahx$^{9-18}$]NPY (IC_{50} 178 nM) further reduced binding. However, the elongation of the C-terminus to 19 amino acids resulted in the analogue [Ahx$^{9-17}$]NPY (IC_{50} 13 nM) being the one with highest affinity.

The analogues corresponding to the [Ahx^{9-xx}]NPY peptide series, which were missing the Ahx spacer, mainly showed less Y_1 receptor affinity. The IC_{50} values of the peptides [des-AA$^{9-20}$]NPY (210 nM) and [des-AA$^{9-19}$]NPY (220 nM) were about three times higher and the affinity of [des-AA$^{9-22}$]NPY (IC_{50} 1000 nM) was lower compared with the analogues containing the spacer Ahx. The only exceptions were the analogues [des-AA$^{9-21}$]NPY, which bound with a concentration of 74 nM slightly better than [Ahx$^{9-21}$]NPY (102 nM), and the centrally truncated [des-AA$^{9-18}$]NPY (169 nM) exhibiting nearly the same affinity as [Ahx$^{9-18}$]NPY (178 nM).

It became evident that for a given N-terminus, there exists only one optimal length for the C-terminal segment. For an N-terminus consisting of five or six amino acids, a C-terminus should consist of the residues 20–36, for one with seven amino acids the latter should contain 21–36 residues. If there are eight N-terminal amino acids, the most favourable C-terminal segment is NPY (18–36) (Figure 1).

DISCUSSION

Discontinuous analogues of NPY are especially helpful tools in characterizing receptor binding interactions and investigating the binding site of the native hormone. Here we report on the synthesis and the Y_1 receptor binding studies of four sets of centrally truncated analogues. The sequence of NPY was preserved in the N- as well as in the C-terminal part. The segments were linked by Ahx. Investigations were performed to elucidate the interaction and the orientation of the C- and N-terminal parts of NPY adopted at the Y_1 receptor. All the peptides showed good Y_2 receptor affinity (data not shown). This is, however, not surprising, since much smaller analogues such as [Ahx$^{5-24}$]NPY have been identified as full Y_2 receptor agonists [5, 10].

With respect to the series of analogues [Ahx^{6-xx}]NPY, [Ahx^{7-xx}]NPY, [Ahx^{8-xx}]NPY and [Ahx^{9-xx}]NPY, the four peptides consisting of the C-terminal part NPY(23–36) showed the most reduced receptor affinity ([Ahx$^{6-22}$]NPY, IC_{50} 940 nM; [Ahx$^{7-22}$]NPY, IC_{50} 280 nM; [Ahx$^{8-22}$]NPY, IC_{50} 260 nM; [Ahx$^{9-22}$]NPY, IC_{50} 780 nM). In each case the extension of the C-terminal segment by one amino acid leads to considerably lower IC_{50} values. It seems that Ser in position 22 is required for high receptor ligand interaction. In the suggested 3D structure of NPY [3–5], Ser is localized at the hydrophilic side of the amphiphilic α-helix. The L-Ala scan of NPY [19] indicates that the sidechain of Ser^{22} is not directly involved in receptor interactions. Therefore the increase of binding affinity is either caused by direct interaction of the backbone, or just by stabilization of the α-helical conformation.

In order to evaluate the role of the N-terminus, the absolute IC_{50} values of the four analogues [Ahx^{x-22}]NPY should be considered. A short N-terminus of five amino acids is very sensitive to changes of the optimal C-terminal length. In the first series, the differences between IC_{50} values were most pronounced when comparing the optimal and the shorter C-terminal segments. In the case of [Ahx$^{6-22}$]NPY the N-terminus was too short to compensate for the lack of the amino acids in the C-terminal part. The analogues [Ahx$^{7-22}$]NPY and [Ahx$^{8-22}$]NPY show nearly the same Y_1 affinity, which is about 3.5-fold better than [Ahx$^{6-22}$]NPY. In [Ahx$^{9-22}$]NPY, the N-terminal part is too long to fit with proper receptor interaction leading to a decrease of affinity. These results indicate that a relatively long N-terminus linked to a short C-terminus is necessary for the formation of the receptor binding conformation. Whether this is achieved by preserving a more flexible N-terminus which is able to bind to the receptor or by the improvement of the intramolecular interactions to stabilize a certain conformation of the C-terminal segment, is unclear.

Main results were obtained by the series [Ahx^{7-xx}]NPY and [Ahx^{8-xx}]NPY. In the former the optimum of the C- and N-termini were found with [Ahx$^{7-19}$]NPY, in the latter, [Ahx$^{8-20}$]NPY showed the highest affinity. Interestingly extension of the N-terminus forces a shortening of the C-terminus, and vice versa. This leads to the conclusion that no definite amino acid in the region of the linker is required, although the orientation of the N- and C-termini to each other is of great significance.

The irrelevance of the sidechains of amino acids 6, 7 and 21 for direct receptor interactions is in agreement with the results of the L-Ala scan [19]. The substitution of Asn^6 or Tyr^{21} led only to a slight decrease of affinity. [Ala^7]NPY binds to the Y_1 receptor nearly as well as NPY itself. In contrast, Tyr^{20} is known as a sensitive position like Leu^{24}, Tyr^{27} and Ile^{31}. In this case the sensitivity could be correlated with the hydrophobic side of the amphiphilic helix. According to former speculations that the hydrophobicity itself or its influence on the orientation with the N-terminus is decisive for Y_1 receptor affinity, the substitution of Tyr^{20} by the hydrophobic linker Ahx is well tolerated. The truncated analogue [Ahx$^{8-20}$]NPY is one of the peptides with highest Y_1 receptor affinity.

In the series [Ahx^{9-xx}], the N-terminus consists of the eight amino acids that are known to build up the whole typical type II proline helix. Although the analogue [Ahx$^{9-19}$]NPY binds relatively well to the Y_1 receptor, the affinity is strongly increased by two additional amino acids, at the C-terminal part, for [Ahx$^{9-17}$]NPY. Because of this lengthening an additional α-helical turn could be formed.

It has been proven that extension of the C-terminal part itself serves to stabilize the α-helical conformation. Therefore it seems to be difficult to explain the 2.5-fold reduced affinity of [Ahx$^{9-18}$] (IC_{50} 178 nM) compared with [Ahx$^{9-19}$] (IC_{50} 65 nM). In the former even the sensitive amino acid Arg^{19} would be available for binding. These results indicate that a good Y_1 receptor affinity is less a problem of helicity or essential amino acids than a problem of orientation and stabilization by intramolecular interaction.

Hu *et al.* [23] investigated the Y_1 binding sites in rat brain cortex using a series of N-terminal deletion fragments of NPY. Tyr^1 and the residues around Pro^5 were identified as important in maintaining the overall tertiary structure. The significance of Pro^5 is confirmed by our studies. This is in contrast to the results of Fournier *et al.* [24], who found that [Ahx$^{5-19}$]NPY shows some binding to Y_1 receptor-enriched tissue. The extension of the type II proline helix up to six amino acids, as in the [Ahx^{7-xx}]NPY analogues, led to a general increase of Y_1 receptor affinity. Whether this is due to a stabilization of the type II proline helix itself or to the extension serving to present another functional group in a position which is necessary for receptor interaction is not clear.

Investigations in which the N-terminal type II proline helix is directly linked to the C-terminal α-helix have been made by other groups [21, 25]. Kirby *et al.* synthesized [cyclo(7/21)-des-AA$^{10-17}$(Cys^7, Cys^{21})]NPY, which exhibits slightly reduced binding properties compared with native NPY. The tertiary structure of this analogue is probably very similar to our peptide [Ahx$^{9-17}$]NPY, which also shows high Y_1 affinity.

A further interesting analogue was developed by Fretz *et al.* [26]: (Pro^{34})[des-AA$^{7-9}$, des-AA$^{15-21}$]NPY. In this case the pentapeptide NPY(10–14) of the loop region of the hormone was used for linkage. The Gln in position 34 was replaced by the turn-inducing Pro which is well tolerated in the Y_1 mediated system [27]. The high Y_1 binding capacity of this analogue indicates that the complete loop is not essential for Y_1 receptor recognition, but the choice of the linker plays an important role. These results are in agreement with our studies. We can confirm the great significance of the N-terminal segment NPY(1–6) and of the C-terminal part NPY(22–36). Additionally, it seems that further amino acids around the spacer (Ahx) are not directly involved in receptor recognition. Further studies will show whether the centrally truncated segments are still able to transduce signals and act as full or partial agonists or as antagonists.

In summary, linear truncated analogues of NPY exhibiting high Y_1 affinity have been systematically developed. Binding studies on human neuroblastoma cell line SK-N-MC (Y_1) showed that for a given N-terminus there exists only one optimal length for the C-terminal segment. A long N-terminus requires a short C-terminus and vice versa: this is clearly demonstrated by the two analogues [Ahx$^{7-19}$]NPY and [Ahx$^{8-20}$]NPY. Obviously no definite amino acid of the loop region is essential, but the 3D arrangement of the C- and N-terminal segments seems to be important for receptor interaction. We conclude that the Y_1 receptor either recognizes a discontinuous binding site, comprising segments of the N-terminal backbone and parts of the C-terminal helix, or the N-terminal part is able to stabilize the active conformation of the C-terminus only by its definite conformation.

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