Structure–Affinity Relationships of C-terminal Cyclic Analogue of Neuropeptide Y for the Y₁-Receptor

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Received June 22, 2000; accepted September 5, 2000

We previously reported that a cyclic octapeptide amide, c[D-Cys²⁹, Cys³⁴]NPY Ac-29–36 (YM-42454) showed a high affinity for Y₁-receptors in SK-N-MC cells ($K_i=0.047\ \mu\text{M}$) but not for Y₂-receptors in the porcine hippocampus membranes ($K_i>10\ \mu\text{M}$). To explore the critical residues of this unique cyclic peptide for Y₁-binding activity, the structure–affinity relationships were investigated by means of amino acid replacement. The results indicated that the hydrophobic side-chains of Leu³⁰ and Ile³¹, the guanidinium groups of Arg³³ and Arg³⁵, and the C-terminal amide are critical for the binding affinity of YM-42454 to the Y₁-receptor. On the other hand, Thr³² in YM-42454 might not be critical for the Y₁-binding affinity. ¹H-NMR studies for YM-42454 and its derivatives have suggested that the critical residues are involved in the direct interaction with a Y₁-receptor rather than in maintaining the bioactive conformation.

Key words neuropeptide Y; Y₁-receptor; cyclic peptide; structure–affinity relationship; binding affinity

Neuropeptide Y (NPY) is a 36-amino-acid C-terminally amidated peptide initially isolated from porcine brain by Tatemoto and co-workers.²⁾ It is a member of the pancreatic polypeptide family (PP-family) and is one of the most abundant neuropeptides in the mammalian central and peripheral nervous systems. It has various biological functions such as vasoconstriction,^{3,4)} stimulation of food intake^{5,6)} and anxiolysis.⁷⁾

The three-dimensional structure of one member of the PP-family, avian PP (aPP), has been determined by X-ray crystallography.⁸⁾ It consists of a polyproline type II-like helix (residues 1–8), a β -turn (residues 9–13), an α -helix (residues 14–32), and a flexible tail (residues 33–36). The polyproline helix and α -helix pack with hydrophobic interactions and form the hairpin-like structure. A modeling study based on the aPP structure predicted that NPY would also have the hairpin-like structure.⁹⁾ This has been supported by NMR investigations,^{10–12)} although both N- and C-terminals are not well defined.

Several receptor subtypes were proposed based on the various biological responses by NPY and its analogues.^{13–16)} At present, five receptor subtypes (Y₁, Y₂, Y₄, Y₅, Y₆) have been cloned.^{17–21)} The Y₁-receptor mainly mediates vasoconstriction, anxiolysis, sedation, and food intake. The Y₂-receptor is related to the suppression of transmitter release.²²⁾ It was previously thought that the entire NPY molecule was required to activate the Y₁-receptor whereas C-terminal fragments such as NPY13–36 can activate the Y₂-receptor.^{13,23)} Structure–activity relationship studies of NPY including L-Ala and D-amino-acid scans have revealed that Arg³³, Arg³⁵, and Tyr³⁶-amide are the most crucial residues for binding to the both Y₁ and Y₂-receptor subtypes.^{24–27)} Several NPY analogues—such as truncated, branched, and cyclic peptide derivatives—have been reported to have various biological activities and specificities.^{28–33)}

We previously reported that a C-terminal cyclic peptide of

NPY, c[D-Cys²⁹, Cys³⁴]NPY Ac-29–36 (YM-42454), showed a high affinity and selectivity for Y₁-receptors in SK-N-MC cells ($K_i=0.047\ \mu\text{M}$) despite the lack of the N-terminal sequence of NPY.³⁴⁾ The affinity was about 200-fold higher than the corresponding C-terminal linear peptide, NPY Ac-28–36 ($K_i=8.7\ \mu\text{M}$), and about 1/500 of porcine NPY (pNPY, $K_i=0.092\ \text{nM}$). YM-42454 was designed to stabilize the hypothetical bioactive conformation of the C-terminal octapeptide of human NPY (hNPY) based on the homology modeling with the crystal structure of aPP.

In this report, the structure–affinity relationships of YM-42454 for the Y₁-receptor were investigated by means of amino acid replacement and ¹H-NMR experiments.

Results and Discussion

YM-42454 is an interesting Y₁-receptor ligand that has only a region corresponding to the C-terminal residues of NPY and does not show Y₂-binding activity.³⁴⁾ To investigate which residues of YM-42454 were critical for Y₁-binding activity, structure–activity relationships were studied with a total of eighteen derivatives (Table 1). In general, the crucial residues are considered to play an important role in the direct interaction with the Y₁-receptor and/or in maintaining the bioactive conformation. To explore the role of the crucial residues, ¹H-NMR studies were carried out on YM-42454 and some of its derivatives in aqueous solution. In following discussions, the numbering of the residue positions in YM-42454 is according to that of NPY.

Structure–Affinity Relationships of YM-42454 The first set of experiments involved replacing Arg³³ or Arg³⁵ of YM-42454 with Nle (norleucine), Asn, His and Lys (1–7). The corresponding Arg residues in NPY are essential for its Y₁-binding activity.^{24–27)} All the substitutions drastically decreased the receptor binding ability although peptide **2** partially retained Y₁-binding affinity ($K_i=4.2\ \mu\text{M}$). These results suggest that both Arg residues are also important for the

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Table 1. Binding Affinity to SK-N-MC Cells (Y_1) and Analytical Data of YM-42454 Derivatives

Peptide	Amino acid sequence ^{a)}	SK-N-MC (Y_1) ^{b)} K_i (μ M)	RP-HPLC ^{c)} t_R ^{d)} (min) (purity, %)	(M+H) ⁺ Calcd ^{e)}	Obsd ^{f)}
YM-42454	Ac-cys-Leu-Ile-Thr-Arg-Cys-Arg-Tyr-NH ₂	0.047	7.2 (97)	1066.5	1066.2
NPY Ac-28—36	Ac-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH ₂	8.7	7.2 (99)	1217.7	1217.2
1	Ac-cys-Leu-Ile-Thr-Nle-Cys-Arg-Tyr-NH ₂	>10	26.8 (99)	1023.5	1023.5
2	Ac-cys-Leu-Ile-Thr-Lys-Cys-Arg-Tyr-NH ₂	4.2	6.7 (99)	1038.5	1038.5
3	Ac-cys-Leu-Ile-Thr-Asn-Cys-Arg-Tyr-NH ₂	>10	8.1 (99)	1024.5	1024.6
4	Ac-cys-Leu-Ile-Thr-His-Cys-Arg-Tyr-NH ₂	>10	6.7 (97)	1047.5	1047.6
5	Ac-cys-Leu-Ile-Thr-Arg-Cys-Nle-Tyr-NH ₂	>10	16.8 (99)	1023.5	1023.7
6	Ac-cys-Leu-Ile-Thr-Arg-Cys-Lys-Tyr-NH ₂	>10	6.9 (97)	1038.5	1038.7
7	Ac-cys-Leu-Ile-Thr-Arg-Cys-His-Tyr-NH ₂	>10	7.1 (99)	1047.5	1047.7
8	Ac-cys-Leu-Ile-Thr-Arg-Cys-Arg-Nal-NH ₂	1.5	26.3 (100)	1100.6	1100.7
9	Ac-cys-Leu-Ile-Thr-Arg-Cys-Arg-Tyr-NHCH ₃	>10	8.1 (96)	1080.5	1080.5
10	Ac-cys-Ala-Ile-Thr-Arg-Cys-Arg-Tyr-NH ₂	3.2	4.2 (99)	1024.5	1024.5
11	Ac-cys-Leu-Ala-Thr-Arg-Cys-Arg-Tyr-NH ₂	5.0	4.1 (99)	1024.5	1024.6
12	Ac-cys-Leu-Pro-Thr-Arg-Cys-Arg-Tyr-NH ₂	>10	4.2 (99)	1050.5	1050.6
13	Ac-cys-Leu-pro-Thr-Arg-Cys-Arg-Tyr-NH ₂	>10	4.4 (100)	1050.5	1050.7
14	Ac-cys-Leu-Ile-Ala-Arg-Cys-Arg-Tyr-NH ₂	0.68	6.4 (97)	1036.5	1036.7
15	Ac-cys-Leu-Ile-Val-Arg-Cys-Arg-Tyr-NH ₂	0.23	12.2 (99)	1064.6	1064.7
16	Ac-cys-Leu-Ile-Trp-Arg-Cys-Arg-Tyr-NH ₂	0.49	16.6 (98)	1151.6	1151.6
17	Ac-cys-Leu-Ile-thr-Arg-Cys-Arg-Tyr-NH ₂	1.9	6.6 (99)	1066.5	1066.7
18	Ac-cys-Leu-Ile-trp-Arg-Cys-Arg-Tyr-NH ₂	5.3	17.3 (100)	1151.6	1151.6

a) Amino acids are expressed by 3 letter codes. Codes starting with capital letters refer to L-isomers, whereas the others refer to D-isomers. Cysteines are cross-linked by an intramolecular disulfide bond. b) K_i value of porcine NPY in SK-N-MC was 0.092 nM. c) CAPCELL PAK C₁₈ SG300 S5 (250 mm×4.6 mm), 0.1% aqueous Trifluoroacetic acid (TFA)/CH₃CN 75/25, flow rate 1 ml/min, detection at 220 nm. d) Retention time. e) Theoretical values calculated with monoisotopic mass. f) Data obtained by MALDI-TOF-MS.

binding of YM-42454 to the Y_1 -receptor. However, it is interesting that the replacement of Arg³³ with Lys resulted in weak affinity to the Y_1 -receptor, while only Arg was permissible at position 35. The guanidinium group of Arg is not only positively charged but also has the ability to form plural hydrogen bonds. In addition, it has a pseudo-aromatic character that is a consequence of its planar nature and π -electron system. These versatile properties of Arg in YM-42454 may play an important role for receptor recognition, especially at position 35.

The replacement of Tyr³⁶ with 2-naphthyl-Ala (Nal) decreased the binding affinity about 30-fold. In the case of NPY, the replacement of Tyr³⁶ with Phe showed a small reduction of Y_1 -binding affinity while the replacement of Tyr³⁶ with Ala significantly reduced its binding ability.²⁷⁾ The moderate reduction of peptide **8** might be due to the lack of a hydroxyl group and/or a somewhat larger aromatic ring than that of Tyr residue.

When the C-terminal amide of YM-42454 was converted to the N-methyl-amide, this derivative (**9**) showed a drastically reduced affinity to the Y_1 -receptor. This result indicated that the C-terminal carbamoyl group is essential for Y_1 -binding affinity in YM-42454. The deamidated derivative of NPY showed complete loss of affinity to the Y_1 -receptor, indicating that the C-terminal amide of NPY is also essential for receptor binding.^{14,35)}

The derivatives in which Leu³⁰ or Ile³¹ were replaced with Ala (**10**, **11**) exhibited a distinct reduction of Y_1 -binding affinity (K_i values of 3.3 and 5.0 μ M, respectively). These results suggest that the hydrophobic side-chains of Leu³⁰ and Ile³¹ are important for Y_1 -binding. In the case of NPY, the replacement of Ile³¹ with Ala resulted in a significantly reduced affinity for the Y_1 -receptor, while the replacement of Leu³⁰ with Ala resulted in a less reduced affinity for the Y_1 -receptor.²⁷⁾ The modeling and NMR studies of NPY suggest that

these hydrophobic residues interact with the N-terminal polyproline-like helix and that this interaction stabilizes the hairpin-like structure.^{9,10)} Since YM-42454 does not have the N-terminal portion of NPY, these side-chains are probably exposed and could be involved in the direct binding interaction rather than in maintaining the binding conformation. The derivatives with L-Pro and D-Pro at position 31 (**12**, **13**) showed drastically reduced binding affinities. As D- and L-Pro are structurally restricted amino acids, their introduction might have an adverse effect on the conformation responsible for the Y_1 -receptor binding. Conformational aspects of peptides **10** and **13** are described below in the ¹H-NMR study section.

The replacements of Thr³² with Ala, Val and Trp (**14**—**16**) retained the Y_1 -binding ability better than other substitutions (K_i values of 0.68, 0.23 and 0.49 μ M, respectively). These results suggest that position 32 does not appear to be crucial for the binding of YM-42454 to the Y_1 -receptor. They are interesting results because the substitution of Thr³² in NPY with Ala significantly reduced the Y_1 -binding affinity.²⁷⁾ On the other hand, the conversion of the residues at position 32 to D-amino acids (**17**, **18**) reduced the Y_1 -binding affinity compared to that of the original L-amino acid derivatives (YM-42454, **16**, respectively). A D-amino acid has the same side-chain as the corresponding L-amino acid but the orientation of its side-chain is different. To investigate the effect of the replacement with D-amino acid at position 32 on the overall conformation, a ¹H-NMR study was performed for the peptide **17** (see next section).

¹H-NMR Study of YM-42454 and Its Derivatives A ¹H-NMR study was carried out on YM-42454, NPY Ac-28—36, and six of the derivatives (**2**, **6**, **9**, **10**, **13**, **17**) in aqueous solution. All the investigated peptide analogues gave only one set of signals in each ¹H-NMR spectrum. Chemical shifts, temperature coefficients and ³J_{NH α} coupling constants

Table 2. Amide Proton Chemical Shifts of YM-42454 and Its Related Peptides^{a)} (ppm)

Peptide	Position ^{b)}								
	28	29	30	31	32	33	34	35	36
YM-42454		8.48	8.76	8.18	7.83	8.38	8.63	8.35	8.29
NPY Ac-28—36	8.32	8.65	8.29	8.21	8.24	8.38	8.45	8.46	8.32
2		8.49	8.76	8.21	7.81	8.32	8.63	8.35	8.30
6		8.48	8.75	8.19	7.82	8.38	8.64	8.47	8.27
9		8.48	8.76	8.18	7.82	8.37	8.63	8.37	8.33
10		8.53	8.88	8.11	7.86	8.36	8.54	8.29	8.30
13		8.42	8.07	—	8.48	8.44	7.96	8.33	8.23
17		8.61	8.68	8.08	8.34	8.68	8.33	8.42	8.30

a) Measured in aqueous solution (90% H₂O/10% D₂O) at 283 K, pH 4.5. b) The numbering of the positions is according to that of NPY.

Table 3. ³J_{NH α} Coupling Constants of YM-42454 and Its Related Peptides^{a)} (Hz)

Peptide	Position ^{b)}								
	28	29	30	31	32	33	34	35	36
YM-42454		6.9	6.3	5.8	6.2	5.9	6.6	6.9	7.8
NPY Ac-28-36	^{c)}	7.4	6.5	7.5	7.4	6.8	^{c)}	^{c)}	^{c)}
2		6.9	6.2	5.7	6.6	6.0	6.8	6.9	7.8
6		6.9	6.3	5.6	6.9	6.3	6.6	7.1	7.9
9		6.6	5.9	6.0	6.0	5.9	6.6	6.9	7.4
10		7.1	4.6	5.8	7.5	6.4	6.9	^{c)}	^{c)}
13	^{c)}		8.8	—	7.0	^{c)}	6.3	6.8	6.8
17		6.3	7.8	8.7	^{c)}	6.9	^{c)}	6.9	7.8

a) Measured in aqueous solution (90% H₂O/10% D₂O) at 283 K, pH 4.5. b) The numbering of the positions is according to that of NPY. c) Coupling constant could not be measured due to overlapping.

Table 4. Amide Temperature Coefficients of YM-42454 and Its Related Peptides^{a)} ($-\Delta\delta/\Delta T$, ppb/K)

Peptide	Position ^{b)}								
	28	29	30	31	32	33	34	35	36
YM-42454		7.4	8.0	9.7	3.3	7.6	9.8	8.6	10.3
NPY Ac-28-36	8.9	7.7	8.4	7.5	8.7	9.1	8.8	9.5	10.0
2		7.8	8.3	10.0	3.0	7.5	10.3	8.9	10.6
6		7.8	8.3	10.3	3.1	7.6	10.3	9.3	10.3
10		7.8	9.4	8.8	3.0	7.8	7.8	6.9	10.7
13	^{c)}		7.2	—	6.0	^{c)}	0.7	8.2	8.3
17		5.1	11.0	12.2	10.4	11.0	7.9	8.8	9.8

a) Temperature coefficients were measured in aqueous solution (90% H₂O/10% D₂O) from 277 K to 303 K at pH 4.5. b) The numbering of the positions is according to that of NPY. c) Temperature coefficient could not be measured due to overlapping.

for the amide protons of these peptide analogues are shown in Tables 2—4.

In the linear peptide, NPY Ac-28—36, all of the ¹H-NMR parameters were in the motionally averaged range, suggesting that this peptide is very flexible in aqueous solution. In contrast, some spectral features of YM-42454 indicated that the conformation of this peptide, at least its cyclic portion, should be restricted. The amide proton signals for the cyclic portion of YM-42454 were somewhat broad compared to those of exocyclic moiety. The amide proton of Thr³² showed a relatively small temperature coefficient (3.3 ppb/K). In the case of a small peptide, a small temperature coefficient is generally considered to indicate that this amide proton is somewhat shielded from the solvent.³⁶⁾ The relatively small temperature coefficient of Thr³² amide proton in YM-42454 suggests that this amide proton could be involved in the hy-

drogen bond. This amide proton also had a somewhat higher field chemical shift (7.83 ppm) than that of other residues (8.18—8.76 ppm), which is probably due to the conformational feature of YM-42454. Moreover, the reasonable number and intensity of inter-residue nuclear Overhauser effects (NOEs) were observed for YM-42454 in the two-dimensional (2D) NOE spectroscopy (NOESY) spectrum, while few and weak inter-residue NOEs were observed for NPY Ac-28—36 (data not shown). This restricted conformation of YM-42454 should be suitable for the Y₁-binding since the affinity of YM-42454 for the Y₁-receptor was 185-fold higher than that of its corresponding linear peptide, NPY Ac-28—36, which might be flexible in solution. The solution structure of YM-42454 based on the NOEs will be reported in detail elsewhere.

The NMR parameters of peptides **2**, **6**, **9** and **10** are similar

to those of YM-42454, indicating that these substitutions did not cause a notable conformational change. The results suggest that the reduction of Y_1 -binding affinity for these derivatives is due to the loss of the crucial interactions in the Y_1 -receptor binding rather than to the overall conformational change. Therefore, the hydrophobic side-chain of Leu³⁰, the guanidinium groups of Arg³³ and Arg³⁵, and C-terminal amide of YM-42454 are involved in the direct interaction with the Y_1 -receptor rather than in maintaining the bioactive conformation.

On the contrary, some NMR parameters of peptide **13** and **17** differed from those of YM-42454. The temperature coefficient of the Thr³² amide proton was larger in peptide **13** (6.0 ppb/K) than in YM-42454 (3.3 ppb/K), and this amide proton showed a lower field chemical shift (8.48 ppm) than the corresponding proton in YM-42454 (7.83 ppm). In addition, the amide proton of Cys³⁴ in peptide **13** showed a small temperature coefficient (0.7 ppb/K) and showed a higher field chemical shift (7.96 ppm) than the corresponding proton in YM-42454 (8.63 ppm). And the $^3J_{\text{NH}\alpha}$ coupling constant for the Leu³⁰ in peptide **13** was larger (8.8 Hz) than that for the corresponding residue in YM-42454 (6.3 Hz). As for the peptide **17**, the D-Thr³² amide proton showed a large temperature dependence (10.4 ppb/K) and showed a lower field chemical shift (8.34 ppm) than the corresponding proton in YM-42454 (7.83 ppm). And the $^3J_{\text{NH}\alpha}$ coupling constants for the Leu³⁰ and Ile³¹ in peptide **17** (7.8, 8.7 Hz, respectively), were larger than those of the corresponding amino acids in YM-42454 (6.3, 5.8 Hz, respectively). These results suggest that the solution conformations of peptides **13** and **17** differ from that of YM-42454 and that the reduced binding affinities of these peptides are probably caused by the overall conformational changes. But, since peptide **17** retained a certain degree of Y_1 -binding affinity, its conformational changes might be not so drastic.

Conclusion

The structure-affinity relationships of YM-42454 were investigated by means of amino acid replacement. The guanidinium groups of Arg³³ and Arg³⁵, the hydrophobic side-chains of Leu³⁰ and Ile³¹, and the C-terminal amide in YM-42454 were considered to be crucial for Y_1 -receptor binding. These critical residues nearly agreed with those of NPY although the substitutions at position 32 in YM-42454 influenced Y_1 -binding affinity less than they did in NPY. These results suggest that the binding mode of YM-42454 to the Y_1 -receptor is similar to that of the C-terminal of NPY. ¹H-NMR studies of YM-42454 and some of its derivatives indicated that the crucial residues play an important role in the direct interaction with the Y_1 -receptor rather than in maintaining the conformation. Further investigations of these cyclic peptides, including structural and pharmacological studies, would provide insight into the bioactive conformation of NPY and would lead to the discovery of more potent ligands that are more selective for the Y_1 -receptor.

Experimental

Materials Porcine NPY was purchased from Peptide Institute, Inc. (Osaka, Japan). PYY, [Leu³¹, Pro³⁴]NPY, bacitracin, phenylmethylsulfonyl-fluoride (PMSF) and fraction V bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, U.S.A.). Minimum essential medium (MEM) was obtained from GIBCO Laboratories (Grand Island, NY, U.S.A.)

and fetal bovine serum was obtained from Bioserum (Australia). [¹²⁵I]PYY was obtained from DuPont/NEN Research Products (Wilmington, DE, U.S.A.). All other chemicals were of analytical grade.

Production of the NPY peptide analogues was commissioned to Peptide Institute, Inc. (Osaka, Japan). All peptide analogues were synthesized by solid-phase peptide synthesis using the Boc-strategy on an Applied Biosystems 430A peptide synthesizer (Foster City, CA, U.S.A.).³⁷⁾ After the protected peptide resin was treated with hydrogen fluoride to remove the protecting groups and resin, the intramolecular disulfide bond was formed by a ferricyanide procedure.³⁸⁾ The products were purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) and purity was established (>95%) by analytical RP-HPLC in two diverse systems and by amino acid analysis (AAA). All peptides were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and AAA. Analytical data are shown in Table 1. The structure of YM-42454 was confirmed by 1D and 2D ¹H-NMR. (Data are not shown.)

Cell Cultures Human neuroblastoma cell line SK-N-MC was kindly provided by Dr. June L. Biedler (Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A.). SK-N-MC cells were cultured in MEM supplemented with 10% fetal bovine serum. Penicillin-G (100 IU/ml) and streptomycin (100 µg/ml) were added to the culture media. Cells were grown in a 150-mm tissue culture dish at 37°C in a humidified atmosphere of 95% air-5% CO₂.

Binding Experiments Porcine brains were obtained from local slaughterhouses. The hippocampal tissues³⁹⁾ were homogenized in 5 vol. ice-cold 234 mM sucrose with a motor-driven Teflon-glass homogenizer. The homogenate was then centrifuged at 700×g for 10 min to remove cellular and nuclear debris. The tumor cells⁴⁰⁾ were washed twice with 25 mM Tris buffer (pH 7.4) containing 1 mM PMSF and 1 mg/ml bacitracin, sonicated, and centrifuged at 700×g for 10 min. The supernatant was centrifuged at 100000×g for 20 min at 4°C. The pellets were resuspended in 25 mM Tris buffer and centrifuged again. The resulting membrane fractions were resuspended by homogenization in the same buffer and used for the study. Membrane protein content was determined by Lowry's method,⁴¹⁾ using BSA as a standard.

Binding Assay for the Y_1 -Receptor: All assays were performed at 25°C for 180 min in 0.5 ml of 25 mM Tris buffer (pH 7.4) containing SK-N-MC cell membranes, 50 pM [¹²⁵I]PYY, various concentrations of peptide analogue, 10 mM MgCl₂, 1 mM PMSF, 1 mg/ml bacitracin, and 5 mg/ml BSA. Bound and free peptides were separated by centrifugation at 10000×g for 3 min in a microcentrifuge. Specific binding was calculated as the difference in radioactivity bound in the presence and absence of 1 µM unlabeled NPY. IC₅₀ values were computed by logit-log analysis (Hill plot) and K_i (inhibition constant) values were determined using the Cheng-Prusoff relationship.⁴²⁾

Binding Assay for the Y_2 -Receptor: All assays were performed at 25°C for 120 min in 0.5 ml of 25 mM Tris buffer (pH 7.4) containing porcine hippocampal membranes, 10 pM [¹²⁵I]PYY, various concentrations of peptide analogue, 10 mM MgCl₂, 1 mM PMSF, 1 mg/ml bacitracin, and 5 mg/ml BSA. Bound and free peptides were separated by rapid filtration under vacuum through a GF/B filter treated with 0.1% polyethylenimine. The filter was immediately washed three times with 4 ml of 25 mM Tris buffer containing 10 mM MgCl₂. Nonspecific binding was determined in the presence of 0.2 µM unlabeled NPY. IC₅₀ and K_i values were determined in the same way as in Y_1 -receptor binding assay.

¹H-NMR Experiments All NMR samples were dissolved to a final concentration of 4 mM in 90% H₂O/10% D₂O and the pH was adjusted to 4.5 with dilute NaOH and dilute HCl. ¹H-NMR spectra were acquired at 600 MHz on a Bruker AMX 600 spectrometer immediately after sample preparation. Chemical shift values were given relative to the water signal whose chemical shift was previously determined on the basis of sodium 3-(trimethylsilyl)propionate-2,2,3,3-*d*₄ (TSP) as an internal standard reference at the corresponding temperature. Resonance assignments for the ¹H-NMR spectrum were carried out using double quantum filtered 2D correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY) and NOESY experiments. Water suppression was achieved by a weak presaturation of the water signal during the relaxation delay and, in the case of NOESY experiments, during the mixing time. Coupling constants were measured directly from the 1D spectra using a Gaussian window function. Temperature coefficients of the amide protons were calculated from 1D spectra obtained at six temperatures (277, 283, 288, 293, 298, 303 K).

Acknowledgements We thank Mr. M. Shimizu and Mr. M. Funatsu for analytical support. We also thank Dr. K. Nakajima and Ms. K. Kumagai of Peptide Institute, Inc. for synthesis of peptide analogues and thank Dr. M.

Wälchli of Bruker Japan Co., Ltd. for advice about the ^1H -NMR measurements.

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