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Review

Molecular Characterization of the Ligand–Receptor Interaction of the Neuropeptide Y Family

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Abstract: Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) belong to the NPY hormone family and activate a class of receptors called the Y-receptors, and also belong to the large superfamily of the G-protein coupled receptors. Structure–affinity and structure–activity relationship studies of peptide analogs, combined with studies based on site-directed mutagenesis and anti-receptor antibodies, have given insight into the individual characterization of each receptor subtype relative to its interaction with the ligand, as well as to its biological function. A number of selective antagonists at the Y_1-receptor are available whose structures resemble that of the *C*-terminus of NPY. Some of these compounds, like BIBP3226, BIBO3304 and GW1229, have recently been used for *in vivo* investigations of the NPY-induced increase in food intake. Y_2-receptor selective agonists are the analog cyclo-(28/32)-Ac-[Lys^{28}-Glu^{32}]-(25–36)-pNPY and the TASP molecule containing two units of the NPY segment 21–36. Now the first antagonist with nanomolar affinity for the Y_2-receptor is also known, BIIE0246. So far, the native peptide PP has been shown to be the most potent ligand at the Y_4-receptor. However, by the design of PP/NPY chimera, some analogs have been found that bind not only to the Y_4-, but also to the Y_5-receptor with subnanomolar affinities, and are as potent as NPY at the Y_1-receptor. For the characterization of the Y_5-receptor *in vitro* and *in vivo*, a new class of highly selective agonists is now available. This consists of analogs of NPY and of PP/NPY chimera which all contain the motif Ala^{31}-Aib^{32}. This motif has been shown to induce a 3_{10}-helical turn in the region 28–31 of NPY and is suggested to be the key motif for high Y_5-receptor selectivity. The results of feeding experiments in rats treated with the first highly specific Y_5-receptor agonists support the hypothesis that this receptor plays a role in the NPY-induced stimulation of food intake. In conclusion, the selective compounds for the different Y-receptor subtypes known so far are promising tools for a better understanding of the physiological properties of the hormones of the NPY family and related receptors. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: NPY; PYY; PP; Y-receptors; structure–affinity relationship; receptor selectivity; food intake

Abbreviations: Ahx, 6-amino-hexanoic acid; Bpa, *p*-benzoyl-L-phenylalanaine; cAMP, cyclic adenosyl monophosphate; Cha, cyclohexylalanine; Dpr, 2,3-diaminopropionic acid; GABA, gamma-aminobutyric acid; Nal, beta-naftylalanine; NPY, neuropeptide Y (h, human; p, porcine); NTS, Nucleus Tractus Solitarius; Pac, 1-phenyl-2-aminomethyl-cyclopropanoic acid; PYY, peptide YY; PP, pancreatic polypeptide (a, avian; c, chicken; r, rat); TASP, Template Assembled Synthetic Proteins; Tic, tetrahydroisoquinoline-3-carboxylic acid.

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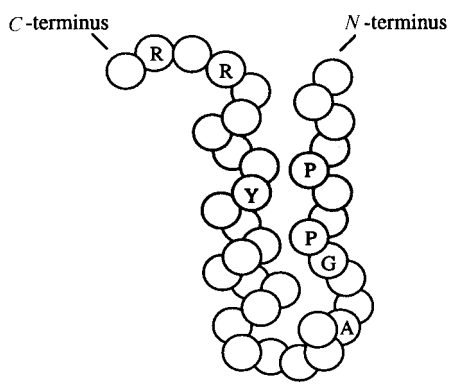
THE NEUROPEPTIDE Y FAMILY

Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) are the members of a peptide hormone family, called the NPY family. The three peptides consist of 36 residues and are *C*-terminally amidated. PP was first isolated from chicken pancreas [1]. At present, more than 30 PP sequences are known: all of them have been identified in tetrapods and share at least 20% identity. PYY was found for the first time in porcine upper small intestinal tissues [2]. So far, PYY sequences have been identified from 20 different species of vertebrates, which show a minimal identity of 42% [3]. Finally, NPY was first isolated from extracts of porcine brain [4], and more than 15 species are known at present. NPY is the most conserved peptide during evolution with at least 61% identity. Seven positions are constant among all species of NPY, PYY and PP; these are Pro^{5}, Pro^{8}, Gly^{9}, Ala^{12}, Tyr^{27}, Arg^{33} and Arg^{35} (Figure 1) [3]. Further highly conserved positions are Pro^{2}, Tyr^{20}, Thr^{32} and Tyr^{36}.

The three-dimensional structure of avian PP (aPP) was determined by X-ray crystallography and consists of an extended type II polyproline helix (residues 1–8) followed by a turn (residues 9–13) and an amphipathic α-helix (residues 14–31). The tertiary structure is characterized by a hairpin-like fold, also referred to as the PP-fold [5] (Figure 1, top). The *C*-terminal end is a flexible turn projecting away from the hairpin loop. It is assumed that the PP-fold is the structural feature common to the whole NPY family. The solution structure of NPY has been investigated by circular dichroism (CD) and 2D-NMR. Darbon and co-workers [6] suggested the following conformation for human NPY in water at pH 3.2: a polyproline stretch (residues 1–10) connected to two short α-helices (residues 15–26 and 28–35) by a tight hairpin (residues 11–14). The peptide was a monomer with a hydrophobic core that kept the *N*- and *C*-terminal ends very close to each other. A dimer of NPY consisting of an antiparallel, hydrophobic packing of the two helical units (each one extending over the residues 11–36 or 13–36) was observed by Cowley and co-workers [7] and Monks and co-workers [8]. The *N*-terminal residues adopted an unordered conformation. A recent work has demonstrated that the monomer and the dimer of NPY are both present under NMR conditions in equilibrium [9].



pNPY YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY

pPYY YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY

hPP APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY

Figure 1 Amino acid sequences of pNPY and pPYY, and of hPP. For each peptide the constant positions among all species investigated are underlined. On the top, the characteristic PP-fold is shown and the seven constant positions between NPY, PYY and PP are indicated.

NPY is widely distributed within the peripheral and central nervous systems and is one of the most abundant neuropeptides in the brain. PYY [10] and PP [11] are synthesized and released by the intestinal and pancreatic endocrine cells. NPY has neurotransmitter properties [12], while PYY and PP act as hormones in an endo- and exocrine fashion, i.e. by regulating pancreatic and gastric secretion [13]. Central effects of NPY and PYY are stimulation of feeding, luteinizing hormone, adrenocorticotrophic hormone and insulin secretion, reduction of growth hormone release, anxiolysis, thermogenesis and temperature regulation [14]. NPY, like PYY, causes long-lasting vasoconstriction in skeletal muscle [15], heart [16], kidney [17] and brain [18], whereas it has been shown to reduce local blood flow in a variety of vascular beds in different species [19]. Presynaptically, NPY inhibits its own release as well as the release of noradrenaline and ATP, and suppresses synaptic inhibition mediated by GABA receptors [20]. In addition, NPY and PYY enhance memory retention [21], and NPY is involved in the modulation of ethanol consumption and resistance [22].

THE Y-RECEPTORS

The effects induced by NPY, PYY and PP are mediated by at least six different receptor subtypes. They belong to the large superfamily of G-protein coupled receptors and are denoted as the Y_1-, Y_2-, Y_3-, Y_4-,

Y_5- and y_6-receptors (Table 1) [23]. In particular, the Y-receptors act via pertussis toxin-sensitive G-proteins, like members of the G_i and G_o family. Therefore, their activation leads to the inhibition of adenylyl cyclase and, consequently, to the inhibition of cAMP accumulation in tissues and cells. In addition, inhibition and stimulation of K^+ and Ca^{2+} channels have been observed in neurons [24] and in the vasculature [25], respectively.

Receptor sequence alignment revealed a high homology of the Y_1-receptor to the Y_4- (42%) and y_6-receptors (51%), but it revealed a low homology to the Y_2- (31%) and Y_5-receptors (35%) [26]. The Y_1-receptor is expressed in blood vessels, cerebral cortex, thalamus and amygdala. The most important Y_1-receptor mediated effects of NPY are vasoconstriction [27,28] and anxiolysis [29]. In addition, this receptor seems to play an important role in the feeding behavior, together with the Y_5-receptor [30]. The pharmacological profile of the Y_1-receptor is characterized by high affinity for NPY, PYY and the corresponding analogs containing Pro^{34}, and low affinity for the *N*-terminally truncated analogs and for PP [31–33].

The Y_2-receptor is expressed in sympathetic and parasympathetic nerve fibres, hippocampus, intestine and certain blood vessels. The effects associated with this receptor are suppression of neurotransmitter release [27,34], enhanced memory retention [35], suppression of noradrenaline [36] and glutamate [37] release. NPY, PYY and *C*-terminal fragments are potent Y_2-receptor ligands, while the analogs containing Pro^{34} and PP bind to the receptor only poorly [38–40].

The Y_3-receptor is localized in the brainstem. Some effects mediated by this receptor are inhibition of catecholamine release [41] and modulation of the arterial blood pressure [42,43]. Recently, it has been found that Y_3-receptors are present in a group of neurons in the nucleus tractus solitarius (NTS) [44], the central termination site for visceral afferens. This suggests that NPY-induced effects in the NTS, like bradycardia, hypotension, bluting of the baroflex and a blocking of the local effects of glutamate, are mediated by the Y_3-receptor. The Y_3-receptor binds NPY and its analog containing Pro^{34}, but is insensitive to PYY and PP [42,43].

The Y_4-receptor is expressed in peripheral tissues, such as heart, intestine, colon and pancreas. Its activation induces inhibition of pancreatic secretion and gall bladder contraction [45]. PP binds to the Y_4-receptor in the picomolar range, while NPY, PYY and the corresponding analogs with Pro^{34} have nanomolar affinities [46,47].

The Y_5-receptor is expressed in the hypothalamus, where it has been proposed to induce food intake [39]. NPY, PYY, the [Pro^{34}]-substituted analogs and the large *N*-terminally truncated analogs, like NPY (2–36) and (3–36), bind to the Y_5-receptor with nanomolar affinity, while the shorter *C*-terminal fragments and PP show a reduced affinity [39,48].

The y_6-receptor has been found in mice and rabbits, but not in primates, although its mRNA is present in various tissues. The pharmacological profile of this receptor is still controversial, as one study reported an order of ligand affinity similar to that for the Y_1-receptor [49], whereas another study suggested an order of potency closer to that for the Y_4-receptor [50]. Since no physiologically relevant actions have been attributed to the y_6-receptor so far, it has been denoted with a lower case y, according to IUPHAR recommendations [23].

STRUCTURE–AFFINITY AND STRUCTURE–ACTIVITY RELATIONSHIP STUDIES

In order to investigate the individual structural features that characterize each Y-receptor subtype–ligand complex, the design of a variety of peptides has been required based on the modifcation of the primary and, consequently, of the secondary and tertiary structure of the natural ligands. The successive analysis of their affinity and activity and their conformation has given insight into the receptor binding properties. First, it is important to understand the function of each part of the ligand, which is best performed by the single exchange of each residue with L-Ala [51] or with the corresponding D-isomer [52], in order to evaluate the importance of each position, i.e. of the chemical properties of each side-chain as well as of its orientation. Secondly, the length of the natural ligand can be modified by *N*- and *C*-terminal truncation or by deletion of amino acids along the sequence. Obviously, the structure–affinity and structure–activity relationship study is complicated by the existence of multiple receptor subtypes: for example, NPY and PYY bind equally potently to the receptors Y_1, Y_2 and Y_5. Only PP shows selectivity for the Y_4-receptor (IC_{50} in the picomolar range). The ability of NPY and PYY to bind to three different receptor subtypes with high potency is probably due to their conformational flexibility, which makes the peptides suitable to adopt more than one energetically favorable structure induced by the receptors. To unequivocally characterize one receptor subtype with respect to the others, it is

Table 1 Characterization of the Y-Receptor Subtypes

Receptor	Y_1	Y_2	Y_3	Y_4	Y_5	y_6
Ligands	NPY, [P^{34}]-NPY, PYY $\gg$ NPY/PYY fragments, PP	PYY, NPY, NPY (2–36) $\gg$ [P^{34}]-NPY, PP	NPY $\geq$ [P^{34}]-NPY $\geq$ NPY (13–36) $\gg$ PYY, PP	PP $\gg$ PYY $>$ NPY $\geq$ [P^{34}]-NPY $\gg$ NPY/PYY fragments	NPY, PYY, [P^{34}]-NPY, NPY (2–36), NPY (3–36) $>$ PP	NPY, PYY $>$ PP
Amino acids	384	381	Not yet cloned	375	455	371
Signal transduction	cAMP inhibition Ca^{2+} mobilization	cAMP inhibition Ca^{2+} mobilization	cAMP inhibition Ca^{2+} mobilization	cAMP inhibition Ca^{2+} mobilization	cAMP inhibition Ca^{2+} mobilization	
Major occurrence	Periphery, hypothalamus	Brain, hippocampus	Brainstem	Intestine, colon	Hypothalamus	Not in human
Related action	Vasoconstriction, anxiolysis, food intake?	Memory, epilepsy, secretion	Inhibition of catecholamine release	Gastro-intestinal regulation	Food intake?, epilepsy?	No activity known

essential to develop selective ligands which can be used as tools for structural as well as biological investigations. To limit the conformational space available to a peptide chain, constraints are introduced by means of special amino acid units, spacer templates or cyclization [53]. The increased rigidity will lead to receptor selectivity if the induced or stabilized conformation is similar to that adopted from the native ligand when interacting with one specific receptor; if not, there will be loss of affinity at all receptor subtypes. The aim of a structure–activity relationship study is to identify the bioactive conformation of a ligand in order to develop potent and selective non-peptide drugs. So far, efforts to obtain potent and selctive non-peptide agonists for G-protein coupled receptors have been more difficult than the efforts to obtain potent and selective antagonists [54]. One reason might be that if the ligand is too small, it can not induce the receptor to change from the inactive to the active conformation because of the lack of contact points between the ligand and the receptor.

An alternative approach to the use of modified ligands is the site-directed mutagenesis of the receptor, in order to identify the positions that are important for the ligand binding and for its own activation [55–57]. A further method is the application of selective anti-receptor antibodies: these can be used either in competion binding studies with the native ligand [58] or in receptor localization studies in the membrane, as well as in intact cells (Eckard CP, Cabrele C, Wieland HA, Beck-Sickinger AG, submitted).

THE Y_1-RECEPTOR

N-Terminally Truncated Analogs

The most striking feature of the Y_1-receptor compared to the other subtypes is its low affinity for analogs of NPY and PYY lacking the *N*-terminal part [59] (Table 2): NPY (2–36) showed a 75-fold decrease in affinity and a reduced activity (8.1%) in comparison with NPY. The affinity of the shorter sequences 3–36, 13–36 and 18–36 was in the micromolar range.

Single Amino Acid Replacements

The contribution of each amino acid side-chain of NPY to the receptor binding was investigated by the systematic single exchange of each residue of NPY by L-Ala [51]. The four natural Ala residues at positions 12, 14, 18 and 23 were substituted by Gly. This study revealed that the most sensitive positions are the following: Pro^2, Pro^5, Arg^{19}, Tyr^{20} and the *C*-terminal positions 27–36 (Table 3 and Figure 2). The substitution of Pro^2 by Ala led to a more than 500-fold loss of affinity. This can be explained by the lack of either a turn motif or of hydrophobicity. On the one hand, the increase in hydrophobicity by the incorporation of Leu or Phe led to a moderate

Table 2 Binding Affinity of Analogs of NPY at the Y_1- and Y_2-Receptor Subtypes

Peptide	Y_1 IC_{50} [nM]	Y_2 IC_{50} [nM]	Cyclic peptide	Y_2 K_i [nM]
pNPY	0.2	0.04	cyclo-(27/31) Ac-[Glu^{27}, Lys^{31}]-(25–36)-pNPY	0.8
pNPY-(2–36)	15	0.06	cyclo-(27/31) Ac-[Lys^{27}, Glu^{31}]-(25–36)-pNPY	5.5
pNPY-(3–36)	810	0.06	cyclo-(27/31) Ac-[Orn^{27}, Asp^{31}]-(25–36)-pNPY	153
pNPY-(13–36)	780	0.32	cyclo-(28/32) Ac-[Lys^{28}, Glu^{32}]-(25–36)-pNPY	0.6
pNPY-(18–36)	2700	0.25	cyclo-(28/32) Ac-[Orn^{28}, Asp^{32}]-(25–36)-pNPY	>3900
pNPY-(22–36)	6600	0.41	cyclo-(28/34) Ac-[Lys^{28}, Glu^{34}]-(25–36)-pNPY	>1000
Ac-(25–36)-pNPY	35 000	160		
Ac-[Cha^{30}]-(25–36)-pNPY	>38 000	18		
Ac-[Cha^{31}]-(25–36)-pNPY	>44 000	16		
Ac-[$Cha^{30,31}$]-(25–36)-pNPY	>25 000	8		
Ac-[Nal^{30}]-(25–36)-pNPY	>50 000	2000		
Ac-[Nal^{31}]-(25–36)-pNPY	3800	51		
Ac-[$Nal^{30,31}$]-(25–36)-pNPY	3800	51		
Ac-[Tic^{30}]-(25–36)-pNPY	>10 000	1000		
Ac-[Tic^{31}]-(25–36)-pNPY	38 000	225		
Ac-[$Tic^{30,31}$]-(25–36)-pNPY	>45 000	650		

Table 3 Effect of the Single Amino Acid Replacement on the Binding Affinity of NPY at the Y-Receptors

Position	NPY residue	Replacement	Y_1 IC_{50} [nM]	Y_2 IC_{50} [nM]	Y_4 IC_{50} [nM]	Y_5 IC_{50} [nM]
1	Tyr	Ala	21 ± 14	0.17 ± 0.04	5.8	2.2
		Bpa	15			
2	Pro	Ala	114 ± 39	0.34 ± 0.04	7.8	5.5
		Leu	14 ± 0	0.19 ± 0.14		
		Phe	72 ± 1	0.28 ± 0.03		
		His	664 ± 304			
5	Pro	Ala	228 ± 103	24 ± 8	25	32
		Leu	19			
		Phe	33			
8	Pro	Ala	32 ± 16	0.8 ± 0.4	60	55
11	Asp	Ala	8 ± 3	0.17 ± 0.05	3.1	0.5
19	Arg	Ala	282 ± 48	1.6 ± 0.5	4.1	1.4
		Lys	4			
20	Tyr	Ala	90 ± 0	1.2 ± 0.6	161	19
		Bpa	6	0.7		
21	Tyr	Ala	6 ± 2	0.24 ± 0.12	66	32
		Bpa	0.5	0.35 ± 0.15		
25	Arg	Ala	11 ± 3	0.67 ± 0.19	201	80
27	Tyr	Ala	250 ± 70	1.4 ± 0.1	340	370
		Phe	5.0 ± 2.4	0.35 ± 0.10		
		Bpa	6	0.2		
28	Ile	Ala	64 ± 26	0.12 ± 0.04		
29	Asn	Ala	58 ± 13	1.4 ± 0.7		
		Gln	11 ± 3	0.91 ± 0.06		
30	Leu	Ala	26 ± 0	0.10 ± 0.05		
		Phe	77			
		D-Trp	570 ± 30	3.9 ± 3.2		
31	Ile	Ala	365 ± 45	0.28 ± 0.06		
		D-Trp	925 ± 75	4.0 ± 1.6		
32	Thr	Ala	723 ± 69	45 ± 30	380	7.7
		D-Trp	955 ± 45	36.5 ± 16.4		
33	Arg	Ala	7000 ± 0	54 ± 21	>1000	94
34	Gln	Ala	65 ± 48	6.0 ± 0.1	7.4	1.3
		Pro	0.5	29		
		D-Pro	266		271	156
		Leu			0.3	1.8
35	Arg	Ala	13 000 ± 0	3000 ± 0	>1000	>1000
36	Tyr	Ala	970 ± 30	780 ± 0	141	68
		Phe	2.6 ± 0.4	0.42 ± 0.23		
		Bpa	118	0.3		
		His	470			

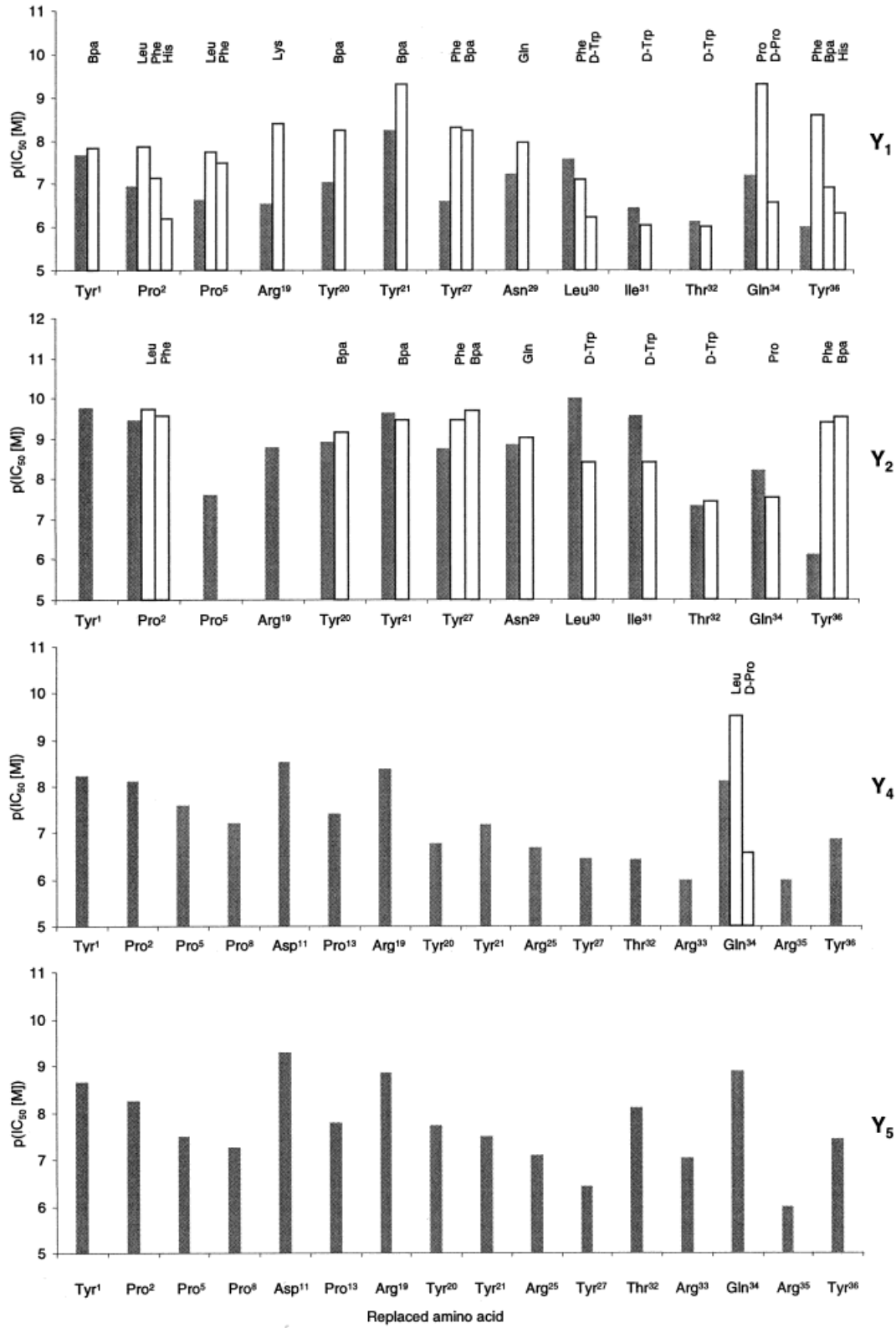


Figure 2 Single amino acid replacement of NPY and related affinity at the human receptors Y_1, Y_2, Y_4 and Y_5. The black bars are relative to the Ala-substitution, while the white bars are relative to the replacement with the amino acids which are reported on the top. For the complete Ala-scanning of NPY at the Y_1- and Y_2-receptors, see Ref. [51].

recovery of affinity; on the other hand, the presence of His decreased the affinity dramatically (>3000-fold) [51]. The replacement of Pro^5 reduced the affinity by more than 1000-fold. Also at this position the presence of a turn-inducing residue seems to be required, as the more hydrophobic side-chain of Leu or Phe improved the binding only partially in comparison with Ala [51]. The exchange of Arg^{19} by Ala corresponded to a >1000-fold lower affinity, which should be attributed to the lack of the basic side-chain, as the substitution of Arg by another basic residue like Lys led to an affinity that was reduced only 20-fold [51]. The mutation Tyr20Ala led to a ligand which had 450-fold less affinity than NPY, while Bpa^{20} (*p*-benzoyl-phenylalanine) reduced the affinity only 30-fold (Beck-Sickinger AG, unpublished results). This suggests that the hydrophobic character of the side-chain at this position is important for the ligand to adopt the bioactive conformation. Hydrophobicity is required also at positions 21 and 27, because the replacement Tyr/Ala led to a decrease in affinity, especially at position 27 (>1000-fold), while the introduction of Phe or Bpa still resulted in good affinity [51]. A more than 300-fold decrease in affinity corresponded to the decrease in hydrophobicity after the substitution of Ile by Ala at position 28. The introduction of Ala at position 29 in place of Asn resulted in 290-fold lower affinity, while the replacement with the homolog Gln led to an affinity that was 55-fold lower than that of NPY [51]. This suggests that the Asn side-chain might play a role in interacting with the receptor or in stabilizing the bioactive conformation of the ligand. Ala^{30} in place of Leu gave an affinity of 26 nM, while the aromatic residue Phe further reduced the binding potency to 77 nM [51]. The presence of D-Trp resulted in an affinity of 570 nM (Beck-Sickinger AG, unpublished results). The results of these three substitutions at position 30 suggest that the chemistry, size and orientation of the side-chain are all of major importance for the binding. Also, Ala^{31} was poorly tolerated and it led to an affinity of 365 nM. The introduction of D-Trp reduced the affinity to 925 nM (Beck-Sickinger AG, unpublished results). The lack of hydrophilicity at position 32 after the replacement of Thr with Ala was accompanied by a dramatic reduction of affinity (>3600-fold). After the incorporation of D-Trp, the affinity was totally lost (IC_{50} 955 nM) (Cabrele C, Langer M, Bader R, Wieland HA, Doods HN, Zerbe O, Beck-Sickinger AG, submitted (a)). Arg^{33} and Arg^{35} turned out to be the most important residues for Y_1-receptor binding: in fact, both analogs containing Ala^{33} or Ala^{35} bound to the receptor with an affinity of 7000 and 13000 nM, respectively. The substitution of Gln^{34} by Ala corresponded to >300-fold lower affinity. However, the introduction of Pro gave a ligand which was as potent as NPY (IC_{50} 0.5 nM) [51]. This suggests that the turn-inducing residue favors the bioactive conformation of the peptide. Furthermore, the orientation of the *C*-terminal turn turned out to be important, as the analog containing D-Pro bound with an affinity of only 266 nM [51]. At position 36, the substitution of Tyr by Ala led to an affinity of 970 nM, while the substitution by a more similar residue like Phe limited the loss of affinity (IC_{50} 2.6 nM) [51]. However, the incorporation of the large, highly hydrophobic residue Bpa corresponded to an affinity of 118 nM (Beck-Sickinger AG, unpublished results). Also, the imidazole ring of His was poorly tolerated (IC_{50} 470 nM) [51]. Therefore, a hydrophobic side-chain seems to be favored at the *C*-terminus of NPY, but its size is determinant as well. Interestingly, the NPY analog containing Bpa at position 1 in place of Tyr resulted in the binding of the Y_1-receptor with the same reduced affinity as the Ala-substituted analog (IC_{50} 15 and 21 nM, respectively, versus 0.2 nM for NPY) (Beck-Sickinger AG, unpublished results). This suggests that the loss of affinity might be due to the lack of the phenolic group of Tyr^1, which might be involved in the formation of an intramolecular hydrogen bond. However, the still good affinity of [Bpa^1]-NPY is also indicative of the flexibility and spatial availability of the *N*-terminus of NPY.

The D-amino acid scan of NPY performed by Kirby and co-workers [52] showed an affinity profile which was very similar to that obtained by the Ala-scan [51]. This suggests that the most important positions are sensitive not only to the exchange of the side-chain but also to the orientation of the side-chain itself. In summary, the *C*-terminal decapeptide of NPY was found to be of major importance for the Y_1-receptor binding. Furthermore, the importance of the Pro residues 2 and 5 and of the Tyr residues 20 and 27 is probably due to their role in stabilizing the hairpin-like structure of the hormone by means of a hydrophobic core.

PP/NPY Chimera

NPY and PP reveal a completely different affinity at the Y_1-receptor, which is 0.2 and >100 nM, respectively. The substitution of the pig NPY (pNPY) sequence 19–23 RYYSA by the corresponding h/rPP QYAAD/QYETQ led to a decrease in affinity

Table 4 Binding Affinity of pNPY, hPP and Some PP/NPY Chimera at the Human Y-Receptors

Peptide	Y_1 IC_{50} [nM]	Y_2 IC_{50} [nM]	Y_4 IC_{50} [nM]	Y_5 IC_{50} [nM]
pNPY	0.2	0.04	5.5	0.6
hPP	>100	>1000	0.04	27[a]
[E^4, P^{34}]-pNPY	6.6		2	12[a]
[hPP^{19-23}]-pNPY	10.5	1.0	>1000	191[a]
[hPP^{19-23}, P^{34}]-pNPY	4.2	1000	15	62
[hPP^{19-23}, H^{34}]-pNPY	43	1000	133	113
[rPP^{19-23}]-pNPY	70	12	21	265
[rPP^{19-23}, P^{34}]-pNPY	4.8	1000	46	32
[rPP^{19-23}, H^{34}]-pNPY	112	1000	460	308
[hPP^{1-7}]-pNPY	3		3.7	2.2
[hPP^{1-17}]-pNPY	1.9		0.3	0.45
[hPP^{1-17}, H^{34}]-pNPY	1.4		0.15	1
[$pNPY^{1-7}$]-hPP	4.2		4.2	3
[$pNPY^{1-7,19-23}$]-hPP	0.22	12	0.08	0.11
[cPP^{1-7}, $pNPY^{19-23}$]-hPP	0.4	28	0.02	0.07
[cPP^{1-7}, NPY^{19-23}, H^{34}]-hPP	5.7	22	0.06	0.04

[a] IC_{50} is relative to rat Y_5-receptor.

(Table 4). Structurally, this exchange was characterized by a helix destabilization (Cabrele C, Wieland HA, Langer M, Stidsen C, Beck-Sickinger AG, submitted (b)). Interestingly, some of the lost affinity was recovered by the introduction of Pro at position 34 in place of Gln, indicating that the presence of a turn-inducing element at the *C*-terminus may favor the binding, according to the result of the single amino acid replacement [51]. In contrast, when His was incorporated in place of Gln, the affinity was drastically lost. When pNPY was modified by the introduction of the human PP (hPP) segment 1–7 or 1–17, both analogs were still potent, with an affinity of 3 and 1.9 nM, respectively (Cabrele *et al.*, submitted (b)). [hPP^{1-7}]-pNPY showed a CD spectrum that was very similar to that of pNPY, and [hPP^{1-17}]-NPY was characterized by a highly stable helix (64% versus 18% found for NPY). These results support the hypothesis that the *N*-terminal part of the molecule is important for the stabilization of the *C*-terminal helix, especially by interdigitation of the Pro residues at positions 2 and 5 with the Tyr side-chains at positions 20 and 27. The hPP sequence 1–17 seems to be more suitable for the intramolecular stabilization of the helix than the corresponding NPY one. The pNPY sequence 19–23 has been shown to be an important structural motif as well, maybe by playing a role in the formation and orientation of the *C*-terminal helix with respect to the *N*-terminus.

The affinity of hPP at the Y_1-receptor was increased by the incorporation of the pNPY segment 1–7 or 19–23, or both of them (Cabrele *et al.*, submitted (b)). [$pNPY^{1-7,19-23}$]-hPP turned out to be as potent as NPY itself (IC_{50} 0.22 nM). The hPP analogs are all as highly helical as the unmodified hPP, however they probably adopt a different tertiary structure, which may be more similar to that of NPY than to that of hPP. This observation suggests that the NPY segments 1–7 and 19–23 drive the formation of the bioactive conformation of the ligand.

C-terminally Modified Analogs of NPY

The importance of the tyrosine amide at position 36 of NPY for binding to the Y_1-receptor was confirmed by the investigation of the affinity of NPY analogs containing different chemical modifications at the *C*-terminus [60]. The free carboxylic group led to a complete loss of affinity (>10000 nM). This observation suggests that a negatively-charged *C*-terminal end might be electrostatically unfavorable. The presence of tyrosine methyl ester at the carboxy end gave an affinity of 715 nM, while that of tyrosinol led to an affinity of 101 nM. After the conversion of the amide group to thioamide, the affinity was 9 nM, indicating that the substitution of the oxygen atom of the carbonyl group is still tolerated. Interestingly, NPY (1–35)-tyramide was found to bind to the

Y_1-receptor with an affinity of 149 nM and to act as an antagonist. This indicates that the *C*-terminal end of NPY plays an important role in binding to and also activating the receptor.

Centrally Truncated Analogs

Although the results of the Ala-scan showed that the central positions of NPY are not essential for binding to the Y_1-receptor [51], the analogs containing the *N*- and *C*-terminal NPY segments connected through a spacer, i.e. 6-amino hexanoic acid (Ahx), showed only moderate affinity (Table 5). The shortest peptide [Ahx^{5-24}]-pNPY did not bind to the Y_1-receptor ($IC_{50} > 4000$) [61]. Rist and co-workers [62] synthesized the four sets of analogs [Ahx^{6-x}]-NPY, [Ahx^{7-x}]-NPY, [Ahx^{8-x}]-NPY, [Ahx^{9-x}]-NPY, where x was 18–22 for the first three sets and 17–22 for the last one. They found that the analogs corresponding to $x = 22$ were very poor ligands (IC_{50} 940, 280, 260 and 780 nM). This might be explained by the fact that the *C*-terminal helix consisting of only 13 residues was too short. Moreover, the length of the *N*-terminal part seems to be an important parameter to stabilize the bioactive conformation; accordingly, the pentapeptide 1–5 was probably too short (940 nM), whereas the octapeptide 1–8 was probably too long (780 nM). In the two sets [Ahx^{6-x}]-NPY and [Ahx^{7-x}]-NPY, the most potent analogs corresponded to $x = 19$, with an affinity of 140 and 55 nM, respectively. For the set [Ahx^{8-x}]-NPY the best ligand was obtained with $x = 20$ (IC_{50} 28 nM), while for the series [Ahx^{9-x}]-NPY it corresponded to $x = 17$ (IC_{50} 13 nM). Therefore, among all centrally truncated analogs the most potent ligand was that containing the longest *N*- and *C*-terminal segments, consisting of 8 and 19 residues, respectively.

By the synthesis of a series of centrally truncated and conformationally constrained analogs, Kirby and co-workers [63] observed that high Y_1-receptor affinity was obtained only when the number of the centrally truncated residues was limited to eight (deletion of the residues 10–17). Moreover, the location of the disulfide bridge and the chirality of the Cys residues influenced the Y_1-receptor affinity. The best Y_1-receptor ligand turned out to be the analog cyclo-(7/21)-des-AA^{10-17}[$Cys^{7,21}$]-NPY which was almost as potent as NPY itself.

Antagonists

Although short *N*-terminally truncated analogs of NPY are poor ligands at the Y_1-receptor, a new class of small peptides was found to antagonize the NPY receptor into the increase of cytosolic Ca^{2+} in HEL cells [64] (Table 6). These peptides contain nine amino acids and correspond to the *C*-terminal nonapeptide of NPY, modified at positions 30 and 34 by the introduction of Pro in place of Leu and Leu in place of Gln, respectively, and at position 32 by the substitution of Thr with aromatic amino acids, like Tyr, Phe, (4-Ph)-Phe or (2,6-dichloro-benzyl)-Tyr. One analog contains the additional exchange Ile31Aib, and another one contains (3,4-dehydro)-Pro at position 30. The antagonistic activity of the

Table 5 Binding Affinity of Some Centrally Truncated Analogs of NPY and hPP at the Y-Receptors

Peptide	Y_1 IC_{50} [nM]	Y_2 IC_{50} [nM]	Y_4 IC_{50} [nM]	Y_5 IC_{50} [nM]
[Ahx^{5-24}]-pNPY	>4000	2	600	795
[Ahx^{6-22}]-pNPY	940			
[Ahx^{6-19}]-pNPY	140			
[Ahx^{7-22}]-pNPY	280			
[Ahx^{7-19}]-pNPY	55			
[Ahx^{8-22}]-pNPY	260			
[Ahx^{8-20}]-pNPY	28		67	31
[Ahx^{9-22}]-pNPY	780			
[Ahx^{9-17}]-pNPY	13		45	11
[Tic^1, Ahx^{5-24}]-pNPY	7000	1		
[Pac^{5-24}]-pNPY	4500	1		
cyclo-(2/30)-[Glu^2, Ahx^{5-24}, Lys^{30}]-pNPY	>10 000	5.7		
[Ahx^{5-24}]-hPP	>500	>1000	144	>1000
[Ahx^{5-20}]-hPP	>1500	>1000	216	>7000
[Tyr^{5-20}]-hPP	>500	>1000	27	>5000

Table 6 Binding Affinity and Activity of *N*-Terminally Truncated Analogs of NPY at the Y-Receptor Subtypes

Sequence		Y_1 IC_{50} [nM][a]	Y_2 IC_{50} [nM][b]
RHYINLIYRLRY			12
HYINLIYRLRY			12
YINLIYRLRY			8
INLIYRLRY			40
NLIYRLRY			300
INPIYRLRY		9	170
INPIFRLRY		10	480
INPIXRLRY	X = (4-Ph)-F	5	520
INPIXRLRY	X = (2,6-Cl_2-Bzl)-Y	2	50
INPXYRLRY	X = Aib	9	260
INXIYRLRY	X = (3,4-dehydro)-P	2	40

[a] Inhibition of NPY-induced increase in cytosolic calcium in HEL cells.
[b] Displacement of [^{3}H]-NPY from rat brain membranes (for more details, see Refs. [64,87]).

compounds at the Y_1-receptor was determined to be in the range 2–10 nM. The high activity of the analogs compared with the corresponding unmodified NPY segment (28–36) was correlated to their ability to adopt a stable helix which is initiated by the turn-inducing sequence Asn^{29}-Pro^{30}. Surprisingly, the dimers of some of the nonapetides described above, which were covalently linked by lactam or disulfide bridges, bound to the receptor with subnanomolar affinity. One example is the dimer of Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-NH_2 that contains two interchain lactam bridges between Glu and Dpr (2,3-diaminopropionic acid). This compound is known as GW1229, GR231118 and 1229U91 [65], and binds to the Y_1-receptor in the picomolar range (K_i 0.041 nM). However, GW1229 has been found also to be a potent agonist at the Y_4-receptor, with a K_i value of 0.3 nM, which limits its use as a pharmacological tool [66–68].

Based on the finding that the *C*-terminal part of NPY is directly involved in the interaction with the receptor and in its activation, a number of Y_1-receptor selective non-peptide antagonists have been developed in the past few years (Figure 3). The first one, BIBP3226, consists of an arginine amide in the D configuration, derivatized at the amino group by the diphenyl acetyl moiety and at the carbonyl group by the 4-hydroxyphenylmethyl moiety [69]. This compound bound to the Y_1-receptor with high affinity (its K_i was of 7 nM versus the K_i of 0.2 nM for NPY) and also reduced the NPY-induced increase in intracellular calcium as well as the pressor response *in vitro* and *in vivo*. The relatively short duration of action (within 2 h) and the lack of oral bioavailability represent two limitations of BIBP3226. The first orally-active Y_1-receptor selective antagonist, known as SR120819A, showed a K_i value of 15 nM [70]. Two further more potent antagonists have recently been developed, which are characterized by a subnanomolar affinity: BIBO3304 (IC_{50} 0.38 nM), an analog of BIBP3226 where the hydroxy group has been replaced with the methylurea moiety [71], and LY357897 (K_i 0.75 nM), a trisubstituted indole [72].

The antagonist binding site at the Y_1-receptor has been investigated by the combination of site-directed mutagenesis and molecular modeling studies (Plate 1) [57,73]. The finding that a large number of mutants maintained affinity for both NPY and BIBP3226, or lost it for both of them, suggests the presence of an overlapping binding site of the agonist and the antagonist. Four points were found to be important for the binding of the native ligand, but not of the antagonist: Asp^{104}, Tyr^{100}, His^{298} and Trp^{288}. Since Asp^{104} and Asp^{287} are believed to interact with the two Arg residues at positions 33 and 35 of NPY, it was expected that only one of the two mutations had to lead to loss of affinity for the antagonist, because only one Arg is present in BIBP3226. This was found to be true for the mutation Asp287Ala. Tyr^{100} is suggested to interact with the *C*-terminal amide group of NPY; as the amide moiety is not present in the antagonist, the mutation Tyr100Ala was obviously not important for the binding of BIBP3226. A hydrogen bond is supposed to exist between His^{298} and the phenolic group of Tyr^{36} of NPY; the corresponding group of BIBP3226

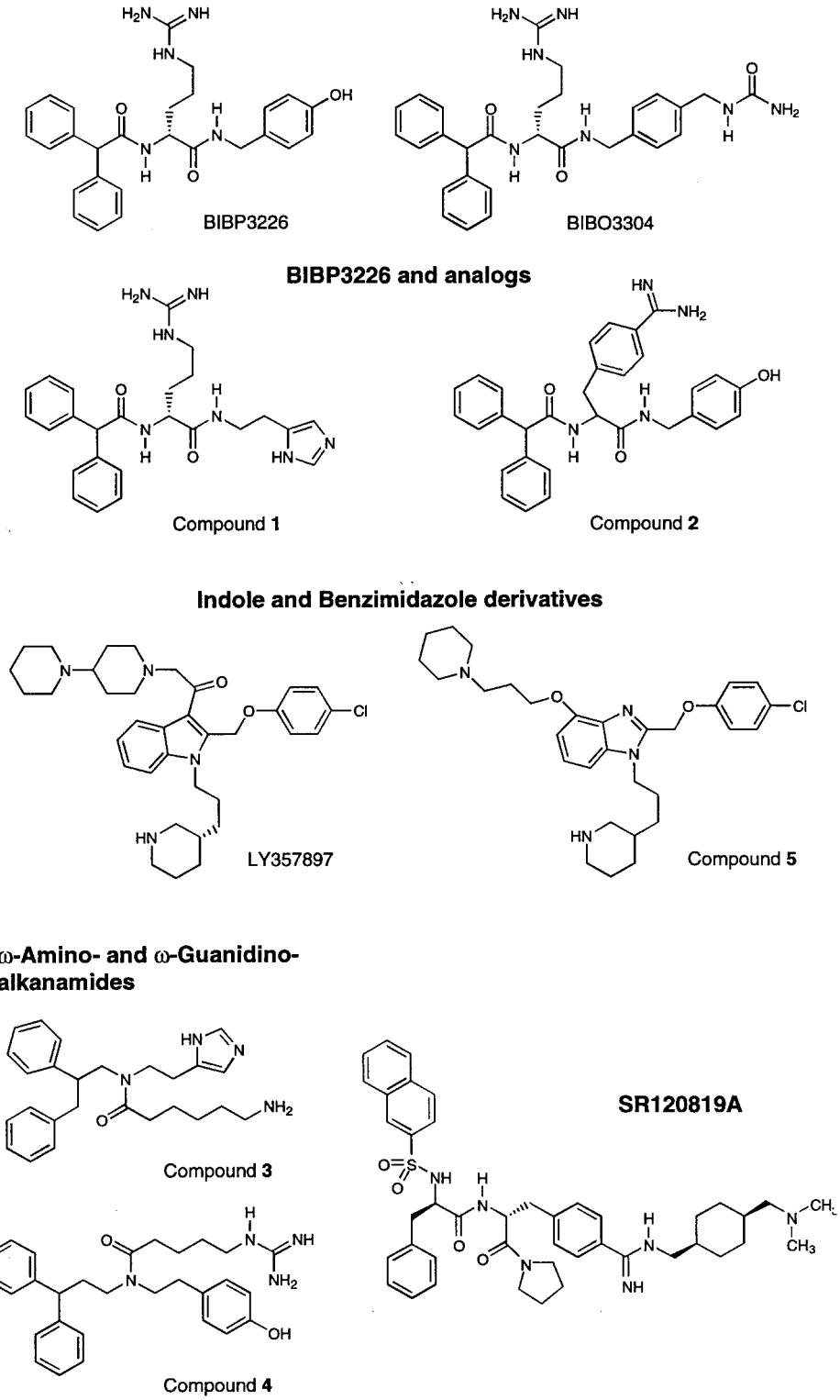


Figure 3 Y_1-receptor selective antagonists. For their pharmacological properties, see text and following Refs.: [69] for BIBP3226; [71] for BIBO3304; [70] for SR120819A, [72] for LY357897; [80,81] for compounds **1** and **2**; [82] for compounds **3** and **4**; [83] for compound **5**.

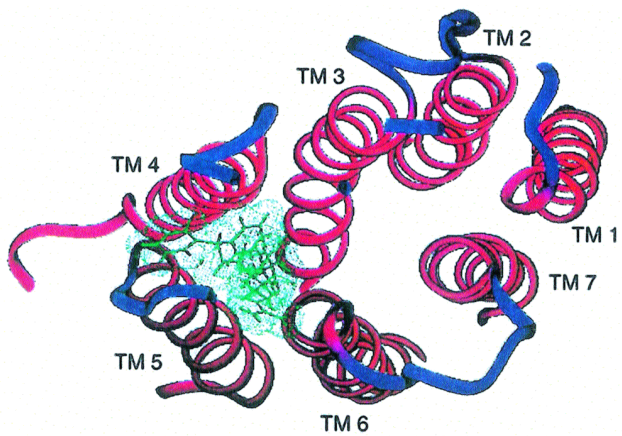


Plate 1 Model of the binding of the antagonist BIBP3226 (green) to the human Y_1-receptor. View down the axis of the transmembrane helices (TM, in red). The extracellular loops are shown in blue.

was hypothesized to be oriented in a different way and to be too far away from His^{298} because of the absence of one methylene group. Interestingly, only the mutant Tyr211Ala was found still to bind the native ligand, but not the antagonist: this seems to be due to an interaction of the Tyr residue with the diphenylmethyl moiety of BIBP3226, a moiety that is absent in NPY. In conclusion, the agonist and antagonist binding domains share common contact points within the transmembrane segments 4–6. Obviously the native ligand interacts with the receptor to a larger extent, thus further important points have been identified for NPY at the top of the transmembrane segment 2, in the first and third extracellular loop. The overlap between the agonist and the antagonist binding sites for NPY and BIBP3226 at the Y_1-receptor was surprising, as in many other systems such as tachykinin [74,75], angiotensin [76], cholecystokinin/gastrin [77] or *k*-opioid receptors [78], the two domains were found to be different. An overlapping between the agonist and the antagonist domains was reported for the endothelin-1 receptor [79].

Although NPY and BIBP3226 share some overlapping regions at the Y_1-receptor, they probably induce two different conformations of the receptor. This would explain why the first one acts as an agonist, while the second one as an antagonist.

Structure–activity relationship studies on analogs of BIBP3226 led to the following observations [80,81]: (1) the substitution of the 4-hydroxybenzyl group of BIBP3226 by the imidazolylethyl group led to 1000-fold lower activity (compound **1**, Figure 3), probably because of the lack of a hydrogen bond to the side-chain of Gln^{219} [57]. (2) Additionally, when Arg was replaced by Orn the compound turned out to be inactive. (3) The methylation of the arginine amide of BIBP3226 did not change the activity significantly, while the lack of a phenolic group resulted in six-fold lower activity. (4) The introduction of conformationally constrained Arg side-chains led to low activity or inactivity (compound **2**, Figure 3).

Müller and co-workers [82] carried out structure–activity relationship studies on *N*,*N*-disubstituted ω-amino- and ω-guanidinoalkanamides that resemble the structure of BIBP3226 and contain an imidazole or a phenolic group. It was found that in the imidazole series the presence of an amino group was preferred to that of a guanidinium group (compound **3**, Figure 3). In contrast, the compounds of the phenol series containing a guanidinium group showed higher activity than the corresponding homologs containing an amino group (compound **4**, Figure 3).

Accordingly, these findings lead to the conclusion that Y_1-receptor antagonists with one (BIBP3226 and the compounds of the phenol series) and two (compounds of the imidazole series) basic groups seem to have different binding sites, but have an overlapping region with respect to their diarylalkyl moiety.

Starting from the compound LY357897 [72], the possibility of replacing the indole core with a benzimidazole core was investigated and structure–affinity and structure–activity relationship studies were carried out on a series of trisubstituted benzimidazoles [83]. The results suggest that at least two basic amine functionalities are required for high affinity, which may mimic the two Arg residues at positions 33 and 35 of NPY. The ligand with the highest affinity in the benzimidazole series (K_i 1.7 nM; compound **5**, Figure 3) is similar to LY357897: an overlapping binding site of the two compounds is represented by the common 3-(3-piperidinyl)-propyl moiety at position 1 of the indole or benzimidazole core, while the second amino function is different and also differently oriented in the two molecules.

Conclusions

For high affinity at the Y_1-receptor, the *C*-terminal part of the ligand requires the two Arg residues at positions 33 and 35. The bioactive conformation of the *C*-terminus is induced and stabilized by the rest of the molecule. NMR studies on NPY indicate the presence of a *C*-terminal helix that starts in the central region (around position 15); therefore, it is obvious that the central part of the peptide plays a crucial role in inducing the helical motif with the proper features (i.e. amphipaticity, hydrophobic moment and axis orientation), as suggested by the observation that the binding potency of NPY and PP can be strongly modulated by exchanging the segment 19–23 between the two peptides (Table 4). The importance of the central region of NPY is also suggested by the fact that the analogs that lack this region are poor ligands. Furthermore, the *N*-terminal truncation of NPY is not tolerated, which seems to be due to the lack of intramolecular interactions between the *N*- and *C*-termini, by which the tertiary structure of the ligand can be stabilized. Instead, the exchange of the *N*-terminus between NPY and PP is well tolerated, and in the case of the PP analogs the affinity is even increased.

The development of selective antagonists has given insight into the structural requirements for receptor binding: at least one amino group resembling one of the two Arg residues of the *C*-terminus is necessary. Molecular modeling studies on the complex of BIBP3226 with the Y_1-receptor suggest an overlapping region of the binding sites of the agonist and the antagonist, which is located within the transmembranes 4–6.

Studies based on receptor-ligand cross-linking and anti-receptor antibodies have suggested that the *C*-terminal helix of NPY may be close to the second and third extracellular loop of the receptor [58]. Furthermore, selective anti-receptor antibodies have been used for receptor localization and molecular weight determination [84].

THE Y_2-RECEPTOR

N-Terminally Truncated Segments

NPY analogs lacking Tyr^1 or the dipeptide Tyr^1-Pro^2 were found to be as potent as NPY with respect to the binding to the Y_2-receptor [59] (IC_{50} 0.06 nM for the analogs versus 0.04 nM for NPY) (Table 2). Even shorter fragments (13–36, 18–36 and 22–36) bound to this receptor with subnanomolar affinity (IC_{50} 0.25–0.41 nM) [59]. The acetylated dodecapeptide NPY (25–36) showed a minor affinity of 160 nM [59]. However, by the introduction of special amino acids with hydrophobic and conformationally restricted side-chains, the binding potency was enhanced up to 20-fold [85,86]. The substitution of Leu^{30} or Ile^{31} by cyclohexylalanine (Cha) yielded two molecules with IC_{50} values of 18 and 16 nM, respectively. The double substituted analog, Ac-[$Cha^{30,31}$]-(25–36)-NPY, bound two-fold better than the monosubstituted analogs. The larger side-chain of β-naftyl-alanine (Nal) was tolerated at position 31 (IC_{50} 51 nM) but not at position 30 (IC_{50} 2000 nM). Surprisingly, the double substituted peptide turned out to be as potent as the [Nal^{31}]-substituted one. Similar behavior was observed for the three analogs containing tetrahydroisoquinoline-3-carboxylic acid (Tic): Tic^{30} led to an affinity of 1000 nM, Tic^{31} to one of 225 nM, and their combination to an IC_{50} of 650 nM. All these compounds were also able to strongly activate the receptor in the rat vas deferens assay. On the other hand, the Ala/Gly-substituted analogs at positions 28–32 were found to be very poor Y_2-receptor ligands as well as poor activators. These results suggest that large and hydrophobic side-chains in the region 28–32 favor Y_2-receptor binding as well as its activation. This hypothesis is supported by the observation that the dodecapeptide [Tyr^{32}, Leu^{34}]-(25–36)-NPY bound to the Y_2-receptor with a 12 nM affinity [64,87] (Table 6). The successive shortening of the analog to 11 and 10 residues slightly improved the binding, as the decapeptide bound to the receptor with an affinity of 8 nM. Further *N*-terminal truncations led first to a slight loss (five-fold for the nonapeptide) and then to a significant loss of affinity (38-fold for the octapeptide). Accordingly, the *C*-terminal nonapeptide turned out to be the minimal sequence required for the binding. Interestingly, the series of the nonapeptides containing Pro at position 30 in place of Leu had in general a lesser affinity. This suggests that a turn-inducing residue at the *N*-terminus does not favor Y_2-receptor binding. Instead, a phenolic group at position 32 seems to be preferred in comparison with a phenyl ring (IC_{50} 170 nM versus 480 nM).

In order to stabilize the conformation of the *C*-terminal dodecapeptide 25–36 of NPY, Rist and coworkers [88] introduced a lactam bridge of the type $i-i+4$ and varied its position along the sequence (from $i=25$ to $i=28$) as well as its orientation (CO → NH or NH → CO) and size (Lys/Glu or Orn/Asp) (Table 2). The two most potent ligands at the Y_2-receptor, corresponding to the lactamizations Glu^{27}-Lys^{31} and Lys^{28}-Glu^{32}, showed an affinity of 0.8 and 0.6 nM, respectively. By changing the orientation of the amide bond in the first peptide to Lys^{27}-Glu^{31}, a loss of affinity occurred (seven-fold), which turned out to be even more drastic after the shortening of the bridge by the cyclization between Orn^{27} and Asp^{31} (191-fold lower affinity). In the second peptide, the shortening of the length of the lactam bridge by two methylene groups by side-chain cyclization of the residues Orn^{28}-Asp^{32} also reduced the affinity more than 5000-fold. The series of cyclic dodecapeptides, where position 34 was involved in lactamization, showed partial to complete loss of affinity. These results suggest that the binding depends on the conformation of the *C*-terminus and that modifications have to be introduced with caution.

The analog cyclo-(28/32) Ac-[Lys^{28}, Glu^{32}]-(25–36)-NPY revealed full agonistic properties at the Y_2-receptor, modulating the calcium channel current in the human neuroblastoma cell line SH-SY5Y [89]. The solution structure of this cyclopeptide was investigated by 2D-NMR and molecular dynamics. This study showed the presence of a short helix over

the residues 29–34 ending with a turn at the *C*-terminus and facing to the *N*-terminal fragment as in a hairpin-like structure (Figure 4). Molecular modeling on the set of peptides cyclized by a lactam bridge between the residues $i-i+4$ was performed by using the NMR data available for cyclo-(28/32) Ac-[Lys^{28}, Glu^{32}]-(25–36)-NPY. It was concluded that high affinity is correlated with a tight hairpin, where the *N*- and *C*-terminal ends are very close to each other. In contrast, a more open conformation is characterized by minor affinity [88]. This suggests that the bioactive conformation of the ligand at the Y_2-receptor should consist of a tightly closed structure in which the *N*- and *C*-terminal parts are close and oriented to each other in a well-defined way.

Based on the concept that the structure and the biological action of a peptide and protein can be modulated by the assembly of peptide elements by means of a synthetic template (the so called TASP concept) [90], Mutter and co-workers synthesized a TASP molecule that consists of two units of the *C*-terminal NPY fragment 21–36 attached via chemoselective ligation to a cyclic template: this molecule, denoted as TASP-V, bound selectively to the Y_2-receptor relative to the Y_1-receptor and acted as an agonist of NPY by inhibiting the cAMP accumulation *in vitro* and by reducing the nasal and bronchial obstruction evoked by histamine *in vivo* [91]. Previously, Grouzmann and co-workers [92] reported a TASP molecule containing four units of the *C*-terminal tetrapeptide of NPY 33-36; the compound, referred to as T_4-[NPY(33-36)]$_4$, bound to the Y_2-receptor with some affinity, but not to the Y_1-receptor, and was reported to reduce the NPY-induced mobilization of intracellular calcium.

Single Amino Acid Replacements

The Ala-scan of NPY affected Y_2-receptor binding to a minor extent compared with Y_1-receptor binding [51]. In general, the loss of affinity was in the range 2- to 20-fold (corresponding to IC_{50} values in the range 0.07–0.8 nM). Exceptions were the following positions: Pro^5, Arg^{19}, Tyr^{20}, Tyr^{27}, Asn^{29}, Thr^{32} and the positions 33–36 (Table 3 and Figure 2). In the *N*-terminal region, only the substitution of Pro^5 by Ala significantly reduced the affinity (600-fold). Ala^{19} gave a 40-fold reduced affinity. The substitution of Tyr^{20} by Ala was characterized by a 30-fold reduction of affinity, while the introduction of Bpa caused only an 18-fold loss of affinity, which indicates that the presence of an aromatic residue is favored (Beck-Sickinger AG, unpublished results). The same behavior was observed at positions 21 and 27, where the incorporation of Ala led to a decrease in affinity, while Phe or Bpa gave a better affinity [51]. Ala^{29} in place of Asn also reduced the affinity 35-fold, however, after the introduction of Gln, homolog of the natural residue, the affinity was partially recovered [51]. A more dramatic loss of affinity was caused by the substitution of the *C*-terminal pentapeptide. Replacement of Thr^{32} with Ala led to a more than 1000-fold decrease in affinity. Unexpectedly, the more drastic change by D-Trp led to almost the same loss of affinity as Ala (Cabrele *et al.*, submitted (a)). The substitution of Arg at position 33 or 35 was associated with a decrease in affinity of 1350- or 75000-fold. Ala^{34} induced 150-fold lower affinity, and Pro at this position further reduced the binding potency more than 700-fold [51]. At position 36 the Ala-replacement

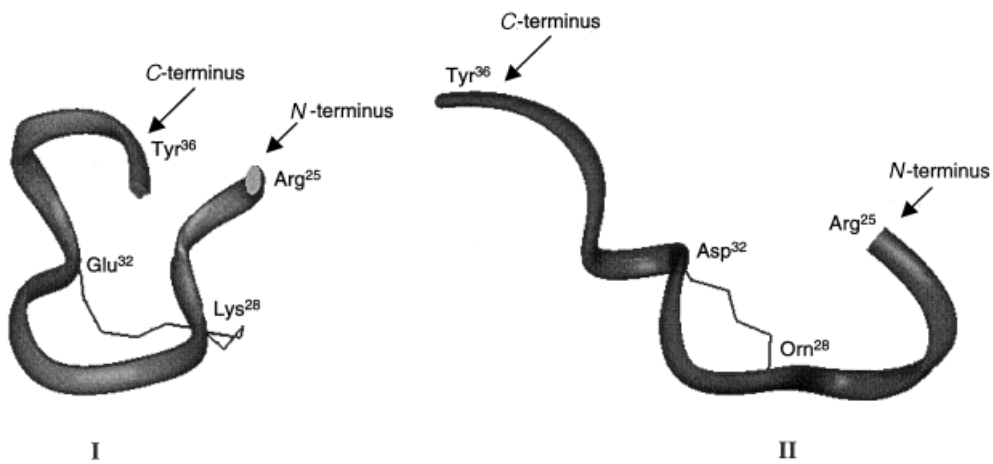


Figure 4 Ribbon representation of the NMR mean structure of cyclo-(28/32) Ac-[Lys^{28}, Glu^{32}]-(25–36)-pNPY (**I**) and of cyclo-(28/32) Ac-[Orn^{28}, Asp^{32}]-(25–36)-pNPY (**II**). The lactam bridge trace is shown as a thin line.

was very poorly tolerated, as the affinity was reduced 19500-fold. As already observed for Tyr^{20}, Tyr^{21} and Tyr^{27}, the introduction of Phe or Bpa led to a minor loss of affinity (eight- to ten-fold) [51].

The activity of the Ala-monosubstituted NPY analogs was tested in mucosal preparations of rat jejunum, where NPY exerts an antisecretory function: all analogs turned out to be agonists with the exception of [Ala^{34}]-NPY that was inactive. Their potency was comparable with that of NPY ([Ala^{3}]-NPY and [Ala^{30}]-NPY) or up to 100-fold lower [93].

The replacement of each amino acid with the corresponding D-isomer [52] significantly reduced the affinity only in the region 30–35, which was 62-fold lower for D-Leu^{30} and >3300-fold lower for D-Arg^{33} and D-Gln^{34}. The stereochemistry turned out to be less important at position 36, where the D-isomer led to a minor reduction (eight-fold) in affinity.

The results of the Ala- and D-amino acid-scan showed that the most important part of the ligand consists of the *C*-terminal fragment, where the side-chains play a role in stabilizing the bioactive conformation of the ligand or in interacting directly with the receptor. Interestingly, an aromatic side-chain (Tyr, Bpa or Phe) at position 36 is required for high affinity, but its orientation is not very important, as suggested by the still high affinity of the analog [D-Tyr^{36}]-NPY (K_i 2.4 nM) [52].

PP/NPY Chimera

PP has very low affinity at the Y_2-receptor (in the micromolar range). By the introduction of the pNPY segment 19–23 into hPP, it was possible to achieve a receptor affinity in the nanomolar range (IC_{50} 23 nM) (Cabrele *et al.*, submitted (b)). Moreover, the additional replacement of Pro^{34} with Gln led to a further increase in affinity, as expected by the knowledge that Pro^{34} is poorly tolerated at the Y_2-receptor.

Centrally Truncated Analogs

In contrast to the Y_1-receptor, the Y_2-receptor is able to bind the centrally truncated analog [Ahx^{5-24}]-NPY with high affinity (2 nM) [61]. The binding potency of this ligand was slightly increased by introducing the hydrophobic residue Tic at position 1 (1 nM), or by substituting Ahx by 1-phenyl-2-aminomethyl-cyclopropanoic acid (Pac) (1 nM) [94] (Table 5). The analog cyclo-(2/30)-[Ahx^{5-24}, Glu^{2}, Lys^{30}]-NPY [95] showed a slightly lower affinity than the linear peptide but a higher activity in inhibiting the accumulation of cAMP via the Y_2-receptor. Furthermore, the cyclopeptide was unable to increase blood pressure in rats via the Y_1-receptor. Thus, the cyclization led to a ligand with higher Y_2-receptor specificity. This suggests that the *N*-terminal part of the molecule induces the right orientation of the *C*-terminus, which is required to activate the Y_2- but not the Y_1-receptor subtype.

Antagonist

Efforts to develop potent and selective antagonists for the Y_2-receptor have been difficult so far. Recently, the first non-peptidic Y_2-receptor antagonist with high affinity and selectivity has been developed, denoted as BIIE0246 (Doods HN, Gaida W, Wieland HA, Dollinger H, Schnorrenberg H, Esser F, Engel W, Eberlein W, Rudolf K, submitted). The structure of BIIE0246 is shown in the Figure 5: in contrast to the Y_1-receptor selective antagonist BIBP3226, it contains an Arg residue in the L-configuration. The rest of the molecule is highly hydrophobic. This molecule bound to the Y_2-receptor with nanomolar affinity (IC_{50} 3.3 nM) and was found to antagonize the NPY receptor in the rat vas deferens assay.

Conclusions

Based on the solution structure of the selective analog cyclo-(28/32) Ac-[Lys^{28}, Glu^{32}]-(25–36)-pNPY and on the high affinity and selectivity of the centrally truncated analog [Ahx^{5-24}]-pNPY and related peptides, it is suggested that the bioactive conformation of the ligand at the Y_2-receptor may consist of a hairpin-like structure where the *N*- and

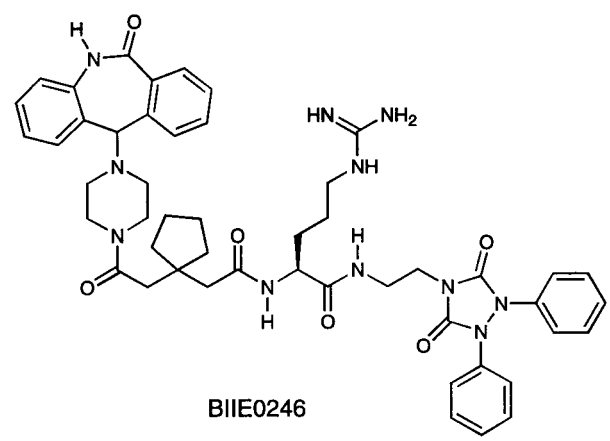


Figure 5 Structure of the Y_2-receptor selective antagonist BIIE0246.

C-termini are very close to each other. Probably, the *C*-terminus represents the functional part of the molecule which is directly involved in the interaction with the receptor, while the *N*-terminal region plays a structural role: in fact, it seems to be important for the stabilization as well as for the proper orientation of the *C*-terminus.

THE Y_3-RECEPTOR

The Y_3-receptor has not yet been cloned and its pharmacology is still controversial. One feature of this receptor subtype is the lack of affinity for PYY. While the Y_3-receptors found in the bovine chromaffin cells were activated by [Leu^{31}, Pro^{34}]-NPY [96], those present in the NTS were not [42]. Furthermore, the Y_3-receptor-mediated suppression of excitatory and inhibitory currents in the NTS was reported for NPY, [Pro^{34}]-NPY, and for the two NPY/PYY chimera [NPY$^{1-23}$]-PYY and [NPY$^{1-14}$]-PYY [97]. In contrast, [Leu^{31}, Pro^{34}]-NPY, PP and two other chimera, [NPY$^{1-7}$]-PYY and [NPY$^{1-3}$]-PYY, showed no effect. Taking into account that only positions 13 and 14 are different between [NPY$^{1-14}$]-PYY and [NPY$^{1-7}$]-PYY, the different behavior of the NPY/PYY chimera suggests that the residues 13 and 14 of NPY play an important role in the binding to and the activation of the Y_3-receptor. This receptor subtype was also reported to mediate the NPY-induced increase in the number of perivascular carbon deposits, because it was observed that PYY and the weak Y_3-receptor antagonist NPY (18–36) decreased the NPY-induced increase [98]. Therefore, it was concluded that NPY may elevate the vascular permeability in the pulmonary circulation via the Y_3-receptor. It is still unclear, whether the NPY fragment (13–36) is able to activate the Y_3-receptor in the NTS or not [43,44]. However, NPY (13–36) was unable to inhibit the synthesis of catecholamine, which is postulated to be transmitted by the Y_3-receptor [96].

THE Y_4-RECEPTOR

One feature of the Y_4-receptor is that it binds PP with high affinity provided that receptor and ligand both derive from the same species [47]. NPY and PYY showed more than 100-fold lower affinity than hPP at the hY_4-receptor (IC_{50} 5.5 nM for NPY versus 0.04 nM for hPP) (Eckard *et al.*, submitted).

Single Amino Acid Replacements

For high affinity of NPY at the Y_4-receptor, the two Arg residues at positions 33 and 35 were found to be essential, as their replacement with Ala led to an affinity of >1000 nM (Eckard *et al.*, submitted) (Table 3 and Figure 2). When Ala was introduced at positions 1, 2 and 34, the corresponding three analogs were as potent as NPY itself, with an affinity that was in the range 5.8–7.8 nM. Interestingly, the substitution of Gln^{34} by Leu yielded an analog that was 18-fold more potent than NPY (IC_{50} 0.3 nM) and only 7.5-fold less potent than hPP. In contrast, D-Pro^{34} led to a 49-fold decrease in affinity. The substitution of the Pro residues at positions 5 and 8 was characterized by a moderate decrease in affinity (4.5-fold and 11-fold, respectively). The lack of Tyr^{27} led to a 62-fold decreased affinity, while the replacement of Tyr^{20} and Tyr^{36} gave an affinity that was reduced 29- and 26-fold, respectively. Tyr^{21} turned out to be less sensitive to the Ala-substitution, as the affinity only decreased 12-fold. While the replacement of Arg^{25} led to a loss of affinity (37-fold), the replacement of Arg^{19} slightly increased the affinity (IC_{50} 4.1 nM versus 5.5 nM), and Ala^{11} in place of Asp enhanced the affinity to 3.1 nM. The moderate increase in affinity of [Ala^{11}]-NPY and [Ala^{19}]-NPY relative to the native NPY may be attributed to the removal of the charged side-chains of Asp^{11} and Arg^{19}, respectively. Based on the observation that in the hPP sequence the polar but neutral side-chains of Asn and Gln are present at positions 11 and 19, it is suggested that these two positions might be involved in interactions with the receptor and that the presence of charges might be unfavorable because of electrostatic repulsions.

Position 32 was sensitive to Ala-substitution with a 69-fold loss of affinity.

In conclusion, the results of the Ala-scan study indicate that the *C*-terminal region of NPY is mostly important for binding to the Y_4-receptor.

Centrally Truncated Analogs

The highly Y_2-receptor selective analog [Ahx$^{5-24}$]-NPY only bound to the Y_4-receptor with low affinity (600 nM) (Eckard *et al.*, submitted). The shortening of the deleted central segment led to an increase in affinity of up to 45 nM for [Ahx$^{9-17}$]-NPY (Table 5).

The deletion of the central segment 5–24 of hPP led to an affinity of 144 nM, 3600-fold lower than that of the full length peptide. The reduction of the central truncation to the segment 5–20 further decreased the affinity (IC_{50} 216 nM), however, when

the spacer Ahx was substituted by Tyr to give the analog [Tyr$^{5-20}$]-hPP, there was an increase in affinity from 216 to 27 nM (Eckard *et al.*, submitted). These results suggest that the central part of hPP is important to induce and stabilize the bioactive conformation.

PP/NPY Chimera

The NPY analog containing the hPP residues Glu^4 and Pro^{34} turned out to have more affinity than NPY at the Y_4-receptor (IC_{50} 2 nM versus 5.5 nM) (Cabrele *et al.*, submitted (b)). The introduction of the hPP segment 19–23 into pNPY led to a micromolar affinity, while the presence of the hPP segments 1–7 or 1–17 induced an increase in affinity, which corresponded to an IC_{50} of 0.3 nM for the analog [hPP$^{1-17}$]-pNPY. In addition, the replacement of Gln^{34} with His led to an affinity of 0.15 nM (Cabrele *et al.*, submitted (b)). In general, the decrease in affinity of the PP/NPY chimera was accompanied by a decrease in helicity, while the analogs that were found to be more potent than NPY were as helical as NPY or even more. Furthermore, it was observed that the loss of affinity associated with the incorporation of the PP segment 19–23 from rat or human could be partially recovered by the additional exchange of Gln^{34} by Pro (Cabrele *et al.*, submitted (b)). Probably, the foreign central segment induced a different folding of the peptide with an unfavorable orientation of the *C*-terminus, which could be partially corrected by the introduction of Pro at position 34. The finding that the substitution of the *N*-terminal fragment of NPY with the corresponding hPP segment led to an increase in the helix content, suggests that the high helical character of hPP is especially due to the ability of its *N*-terminus to stabilize the *C*-terminal helix by hydrophobic, intramolecular interactions, as suggested by the X-ray structure of aPP.

The introduction of elements of the pNPY sequence into hPP led to analogs that were as potent as hPP or only moderately less potent. While the single exchanges Ala1Tyr, Glu4Lys or Tyr7Asn led to a 25- to 48-fold loss of affinity, the introduction of the whole pNPY segment 1–7 led to a more severe loss of affinity ($>$ 100-fold) (Cabrele *et al.*, submitted (b)). These modifications did not reduce the helix content in comparison with hPP, however, they probably induced a different folding of the peptide backbone. The single replacement of the residues 19–23 did not influence the binding affinity as well, however the substitution of the whole segment led to a 12-fold loss of affinity. The additional exchange of Pro^{34} by Gln allowed the complete recovery of affinity: thus, the analog [pNPY$^{19-23}$, Q^{34}]-hPP was as potent as hPP (Cabrele *et al.*, submitted (b)). Together, these results suggest that both *N*-terminal and central regions of hPP are important structural elements for the PP-fold, and their substitution by the corresponding pNPY regions induces a conformational change that can lead to a significant loss of affinity, as in the case of the analog [NPY$^{1-7}$]-hPP; however the bioactive conformation can be recovered by using the right combination of replacements along the sequence. For example, the combinations (pNPY$^{19-23}$, Q^{34}) and (pNPY$^{1-7,\ 19-23}$) turned out to be favorable and yielded molecules which had a similar affinity to hPP.

Conclusions

The structure–affinity and structure–activity data on the Y_4-receptor available so far suggest that a stable helix of the ligand is required for high affinity: accordingly, PP, the most potent native ligand at this receptor, and the high affinity PP/NPY chimera are all characterized by a high helix content. As already observed for the receptors Y_1 and Y_2, the *C*-terminal part of the ligand is important for high affinity and for receptor activation.

THE Y_5-RECEPTOR

Great interest has been focused on the most recently cloned receptor subtype [39,48], as it has been speculated that it plays an important role in feeding behavior, together with the Y_1-receptor [67,99–104]. Therefore, structure–affinity and structure–activity relationship studies were carried out, in order to gain insight into the biological and structural properties of this receptor subtype relative to the others, especially to the Y_1-receptor.

Single Amino Acid Replacements

From the Ala-scan study (Eckard *et al.*, submitted) it has been shown that the importance of the Pro residues increased from position 2 to position 8, with up to a 90-fold loss of affinity (Table 3). Similar behavior has been observed in the case of the Tyr and Arg residues: while Tyr1Ala led to a three-fold lower affinity, Tyr27Ala reduced the affinity 600-fold. Tyr^{36} was sensitive to Ala-substitution as well, and its replacement was characterized by a 100-fold

loss of affinity. The substitution of Arg^{19} only slightly decreased the binding potency (IC_{50} 1.4 nM versus 0.6 nM for NPY), the replacement of Arg^{25} or Arg^{33} resulted in a loss of affinity of >100-fold, while for the analog that lacked Arg^{35} the affinity dropped to the micromolar range. The introduction of Ala or Leu at position 34 only reduced the affinity two-fold, whereas the presence of D-Pro at this position was poorly tolerated (Eckard *et al.*, submitted). Interestingly, as already observed in the case of the Y_4-receptor, the replacement of Asp^{11} with Ala did not affect the affinity (IC_{50} 0.5 nM versus 0.6 nM for NPY).

Centrally Truncated Analogs

The deletion of central segments of NPY led in general to low affinity. Among the investigated analogs, the one with the highest number of residues at the *N*- and *C*-termini, [Ahx^{9-17}]-pNPY, was the most potent ligand with an IC_{50} of 11 nM (Eckard *et al.*, submitted) (Table 5).

PP/NPY Chimera

The substitution of the pNPY segment 19–23 with the corresponding hPP or rPP segment induced up to a 300-fold decrease in affinity (Cabrele C, Wieland HA, Stidsen C, Beck-Sickinger AG, submitted), which was partially recovered by the introduction of Pro^{34} (Table 4). This behavior has already been observed for the Y_1- and Y_4-receptor subtypes, indicating that this segment is important for the orientation of the *C*-terminal helix and, consequently, of the *C*-terminus, which is known to be essential for binding to all Y-receptors. However, the individual structural features required for each receptor have to be different, as suggested by the different binding potency of the analog [hPP^{19-23}, P^{34}]-pNPY at the three systems: 4.2 nM at the Y_1, 15 nM at the Y_4 and 62 nM at the Y_5 (Cabrele *et al.*, submitted (b)). Interestingly, the coupling of hPP (1–17) with NPY (18–36) led to a slightly higher affinity (IC_{50} 0.45 nM versus 0.6 nM for NPY). This suggests that the stabilization of the NPY helix favors binding at the Y_5-receptor. Accordingly, the helical content of this chimeric peptide was determined to be 64% from its CD curve (Cabrele *et al.*, submitted (b)).

The introduction of the segment 1–7 of pNPY into hPP led to an affinity of 3 nM (Cabrele *et al.*, submitted (b)). This modification of the amino acid sequence of hPP changed the folding of the backbone without perturbing the stability of the hPP helix. The single as well as multiple replacement of the hPP positions 19–23 with the corresponding pNPY residues led to good affinity (IC_{50} 1–2 nM) (Cabrele *et al.*, submitted (b)). Surprisingly, the simultaneous introduction of the pNPY segments 1–7 and 19–23 into hPP improved the affinity to 0.11 nM. Furthermore, the incorporation of the segment 1–7 from chicken PP (cPP) instead of that from pNPY gave an IC_{50} of 0.07 nM. Additionally, the mutation Pro34His yielded the analog [cPP^{1-7}, $pNPY^{19-23}$, H^{34}]-hPP that bound to the Y_5-receptor with an affinity of 0.04 nM. These data suggest that the *N*- and *C*-termini and the central region contribute to induce and stabilize the tertiary structure that is adopted from the ligand at the Y_5-receptor. The observation that the most potent ligands were moderately less helical than the other chimera suggests that the helicity is an important structural prerequisite, however, it is not sufficient for subnanomolar affinity at the Y_5-receptor.

Selective Analogs

A class of highly selective ligands has been recently developed. These analogs consist of NPY analogs and PP/NPY chimera containing the common motif Ala^{31}-Aib^{32}. This modification turned out to be exclusively tolerated at the Y_5-receptor and, consequently, it conferred high selectivity for this receptor subtype (Cabrele *et al.*, submitted (b)). Among the series of (Ala-Aib)-substituted NPY analogs, the most potent ligand was simply the double substituted analog [Ala^{31}, Aib^{32}]-pNPY with an affinity of 6 nM, while the most potent PP/NPY chimera was [cPP^{1-7}, $pNPY^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP with an affinity of 0.2 nM. These two newly developed peptides are the first potent Y_5-receptor selective agonists available so far. By the introduction of further Aib residues along the *C*-terminal helix, selectivity and affinity still remained good; i.e. the analog [Aib^{24}, Ala^{31}, Aib^{32}]-pNPY showed an affinity of 14 nM. Interestingly, the analog [Ahx^{5-24}, Ala^{31}, Aib^{32}]-NPY turned out to have >40-fold more affinity than the analog [Ahx^{5-24}]-NPY. Even the short NPY fragment 18–36 containing the motif Ala-Aib bound to the Y_5-receptor with a K_i of 16 nM. These data suggest that the dipeptide Ala-Aib induces the conformation that is favorable for the binding to the Y_5-receptor but not to the other subtypes. The good affinity, even of the centrally truncated analog, indicates that in the absence of the central segment 5–24, the Ala-Aib motif is sufficient to induce and stabilize the

required bioactive conformation of the *C*-terminal part of the ligand.

The solution structure of the analog [Ala^{31}, Aib^{32}]-pNPY in water revealed the presence of a *C*-terminal α-helix ending with a 3_{10}-helical turn of the residues 28–31 (Figure 6, top), followed by an apparently not well-defined structure of the last five residues (Cabrele *et al.*, submitted (a)). Surprisingly, the inverse motif Aib-Ala was found to significantly improve the binding potency of NPY to the Y_5-receptor (K_i 0.5 nM) (Cabrele C, Beck-Sickinger AG, in preparation). However, the selectivity relative to the Y_2-receptor was partially lost (K_i 19 nM). These new data lead to the following observations: (1) the bioactive conformation of the *C*-terminus of NPY at the Y_5-receptor might consist of a specific type of turn structure that is induced and stabilized more correctly by the sequence Aib-Ala rather than by the sequence Ala-Aib at positions 31–32. (2) The Y_2-receptor tolerates the motif Aib-Ala much better than the inverse one, indicating that the presence of Aib-Ala allows the analog to adopt a conformation that can be recognized by this receptor.

The agonist [Ala^{31}, Aib^{32}]-pNPY has been used for *in vivo* feeding studies: the data showed that it can stimulate food intake in rats in a dose-dependent manner (Cabrele *et al.*, submitted (a)). Accordingly, it can be concluded that the Y_5-receptor is involved in food intake regulation (Figure 6, bottom).

Antagonist

The recently developed antagonist CGP71683A is characterized by an IC_{50} of 1.4 nM at the Y_5-receptor, and by a micromolar affinity at the other receptors (Figure 7). This antagonist has been used to investigate the role of the Y_5-receptor in the NPY-induced increase in food intake [99]: the data have shown inhibitory effects either in lean or obese rats, supporting the hypothesis that the Y_5-receptor has a role in feeding behavior.

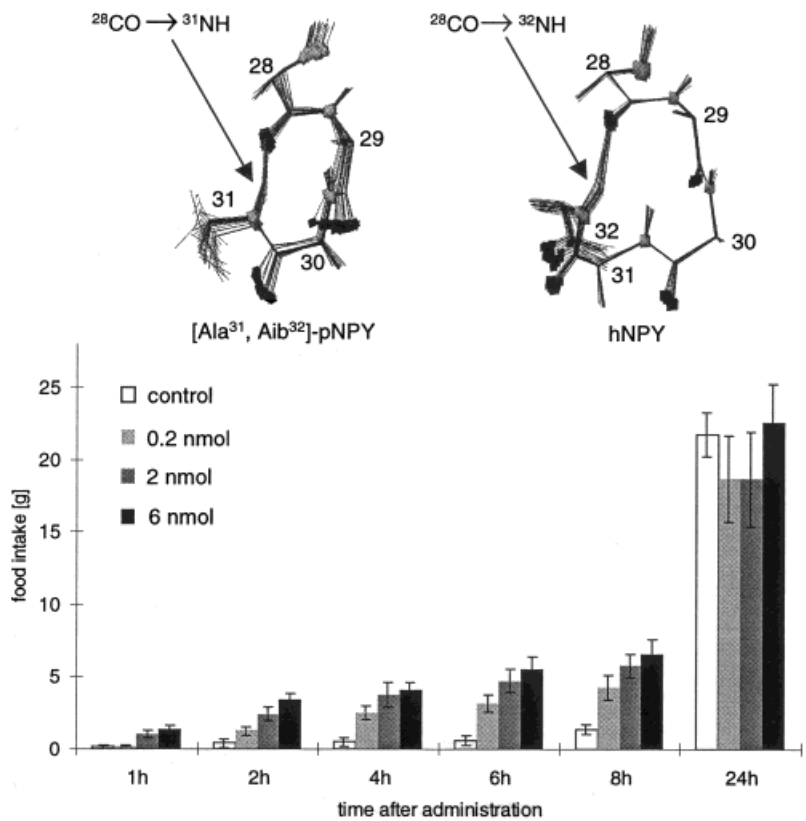
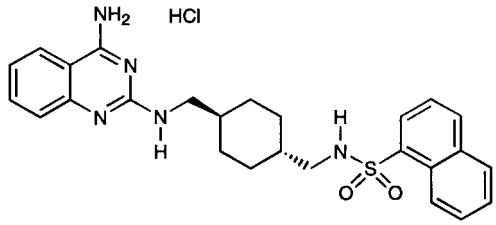


Figure 6 Structure and biological action of the Y_5-receptor selective agonist [Ala^{31}, Aib^{32}]-pNPY. Top: hydrogen bond pattern over the residues 28–32 of [Ala^{31}, Aib^{32}]-pNPY (left) and hNPY (right). The structure of hNPY is according to the NMR data of Monks and co-workers, see Ref. [8]**.** Bottom: dose-dependent increase in food intake in rats after treatment with the Y_5-receptor selective agonist.



CG71683A

Figure 7 Structure of the Y_5-receptor selective antagonist CG71683A.

Conclusions

A stable α-helix is an important structural requirement for binding to the Y_5-receptor, however, it is not sufficient for high affinity, as demonstrated by the moderate affinity of the highly helical PP which showed an affinity of 27 nM at the rat Y_5-receptor. The binding potency of hPP was increased to the subnanomolar range by the incorporation of elements from the primary structure of pNPY and cPP, which induced a change in the global folding of the peptide chain, however, it did not affect the stability of the helix.

The finding that the introduction of the key motif Ala-Aib at positions 31–32 of NPY and of some PP/NPY chimera leads to high selectivity at the Y_5-receptor, has given more insight into the bioactive conformation of the ligand at this receptor subtype: based on the NMR studies of [Ala^{31}, Aib^{32}]-pNPY, the *C*-terminal region of the ligand is suggested to adopt an α-helix ending with a 3_{10}-helical turn. This new structural element is not present in the NMR solution structure of the native NPY, and it may be responsible for the selectivity of the Aib-containing peptide for the Y_5-receptor by inducing a well-defined conformation of the *C*-terminal loop.

CONCLUDING REMARKS

The emerging strong evidence for a multifunctional role of NPY (and related peptides), especially as analgesic, anxiolytic, antihypertensive and orexigen, has increased the necessity of understanding how these neuropeptides exert their actions. To this purpose it is of major importance to characterize the complex system of the multi-receptor subtypes of the members of the NPY family.

In order to answer the question – which effect is transmitted by which receptor – many efforts have been made to develop selective ligands that would be able to act as agonists or antagonists of the native peptides only at one specific subtype. The structure–affinity and structure–activity relationship studies conducted so far have led to the finding of a series of Y_1-receptor selective antagonists which have proved to be useful to clearly identify (e.g. vasoconstriction activity) or at least to suggest (stimulation of feeding) the biological features of this receptor. The Y_2-receptor selective *N*-terminally or centrally truncated, linear or cyclic agonists of NPY and the recently developed antagonist will provide the tools to investigate the function of this subtype. The use of the selective antagonist at the Y_5-receptor has given the first interesting data which have led to the postulation of its role in the NPY-induced increase in food intake. The administration in rats of the first Y_5-receptor selective agonists containing the motif Ala-Aib at the *C*-terminus of NPY and of PP/NPY chimera has stimulated food intake in the animals, supporting the hypothesis that the Y_5-receptor may be related to the orexigenic effect of NPY. However, many questions still have to be answered and some discrepancies have to be cleared up, especially concerning NPY and feeding behavior: are both the Y_1- and Y_5-receptors involved? The evidence suggests a positive answer, however there is no clear explanation how these receptors act and if their responses are related to each other, or if they exert their function separately, under different physiological conditions [30,105].

Thus, it is obvious that a number of specific ligands are needed to distinguish and/or to compare the individual characteristics of each NPY-receptor: for example, an agonist with high selectivity at the Y_1-receptor that might confirm or not the results obtained so far by using selective antagonists is still missing. The better the understanding of the NPY system the higher will be the chance for drug design. In fact, due to the large number of biological functions, NPY and its receptors are potential candidates for drug development.

Acknowledgements

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