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The Neuropeptide Y Family: Molecular Characterization of the Multi-Ligand / Multi-Receptor System

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Chiara Cabrele

Dottore in Chimica
Universita' degli Studi di Padova

Born June 6th, 1969
Citizen of Italy

Accepted on the recommendation of

Prof. Dr. A. G. Beck-Sickinger, examiner
Prof. Dr. M. Mutter, co-examiner
Prof. Dr. G. Folkers, co-examiner

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ABBREVIATIONS

Ahx, 6-aminohexanoic acid

Aib, aminoisobutyric acid

BHK, baby hamster kidney

Boc, *tert*-butyloxycarbonyl

Bpa, *p*-benzoylphenylalanine

BSA, bovine serum albumin

cAMP, cyclic adenosyl monophosphate

CD, circular dichroism

Cha, cyclohexylalanine

DEAD, diethyl azodicarboxylate

DIAD, diisopropyl azodicarboxylate

DIC, *N*,*N*-diisopropylcarbodiimide

DIEA, *N*,*N*-diisopropylethylamine

DMEM, Dulbecco's modified Eagle's medium

DMF, *N*,*N*-dimethylformamide

2D-NMR, two-dimensional nuclear magnetic resonance

Dpr, 2,3-diamiopropionic acid

E1-3, extracellular loops 1-3

EDTA, ethylenediaminetetraacetic acid

ELISA, enzyme linked immunosorbent assay

ESI-MS, electrospray ionization mass spectrometry

FCS, fetal calf serum

FITC, 4(5)-fluoresceinisothiocyanate

Fmoc, *N*-(9-fluorenyl)methoxycarbonyl

GABA, γ-amino butyric acid

HMPB, 4-hydroxymethyl-3-methoxyphenoxybutyric acid

HOBt, 1-hydroxy-benzotriazole

HPLC, high performance liquid chromatography

Hyp, 4-trans-hydroxyproline

Ig, immunoglobulin

IPTG, isopropyl-β-D-thiogalactopyranoside

KLH, keyhole limpet hemocyanin

MBHA, 4-methylbenzhydrylamine

MEM, minimum essential medium

M.W., molecular weight

Nal, β-naftyl-alanine

NOE, nuclear Overhauser effect

NPY, neuropeptide Y (h, human; p, porcine)

NTS, nucleus tractus solitarius

Orn, ornithine

Pac, 1-phenyl-2-aminomethyl-cyclopropanoic acid

PBS, phosphate buffered saline

Pmc, *2,2,5,7,8*-pentamethylchroman-6-sulfonyl

PP, pancreatic polypeptide (a, avian; c, chicken; g, goose; h, human; p, porcine; r, rat)

PYY, peptide YY

TBTU, 1H-benzotriazolium,1-[bis(dimethylamino)-methylene]-,tetrafuoroborate (1-),3-oxide

tBu, *tert*-butyl

TFA, trifluoroacetic acid

TASP, template assisted synthetic protein

Tic, tetrahydroisoquinoline-3-carboxylic acid

Trt, trityl

SUMMARY

Neuropeptide Y (NPY), pancreatic polypeptide (PP) and peptide YY (PYY) are members of the NPY hormone family. They consist of 36 amino acids, are C-terminally amidated and show high sequence homology, which is 69% between NPY and PYY, and 50% between NPY and PP. The X-ray structure of avian PP reveals a hairpin-like fold of a type II polyproline helix (residues 1-8) and of an α-helix (residues 14-31). The C-terminal pentapeptide adopts a flexible turn projecting away from the hairpin loop. NPY is one of the most abundant neuropeptides in the central and peripheral nervous systems, and is present in high concentrations in the brain, especially in the cortical areas, hippocampus and hypothalamus. The most relevant biological actions of NPY are vasoconstriction, stimulation of food intake, increase in memory retention, inhibition of the sexual behavior, anxiolysis, regulation of neurotransmitter release, and modulation of ethanol consumption. These effects are transmitted by at least five different receptor subtypes which belong to the large superfamily of the G-protein coupled receptors; they are referred to as the Y_1-, Y_2-, Y_4-, Y_5- and y_6-receptors. The affinity of NPY and PYY is in the subnanomolar range for the receptors Y_1, Y_2 and Y_5 (IC_{50} 0.04-0.8 nM), while their Y_4-receptor affinity is in the nanomolar range (IC_{50} 5.5 nM for NPY). In contrast to NPY and PYY, the third member of the NPY family, PP, binds to the Y_4-receptor with subnanomolar affinity (IC_{50} 0.04 nM), while to the other receptors only with nanomolar (Y_5) and micromolar (Y_1 and Y_2) affinity.

In order to characterize each receptor subtype individually on the structural as well as on the biological level, highly potent and selective compounds are required, which would recognize only one receptor in a specific manner. A combination of different approaches that leads to the identification of the structural and functional features which are responsible for the pharmacological profile of the native ligand has been applied. To gain insight into the most important requirements of the primary structure, systematic alanine-scanning, N-/C-terminal and central truncation of NPY were performed (chapter 1). Based on the results of these investigations, it was concluded that the C-terminal pentapeptide is essential for the binding to all Y-receptors, while the behavior of N-terminally and centrally truncated peptides was different with respect to each receptor (chapter 1). Among the alanine-monosubstituted NPY analogs, [Ala^{13}]-NPY and [Ala^{27}]-NPY turned out to be Y_2-receptor selective (75- to 370-fold relative to the other receptors; chapter 2). Because of

the flexibility of the peptide conformation, it is generally required to introduce constraints into the molecule, in order to limit the number of the conformations energetically accessible to the peptide. Thus, with the aim of inducing a more rigid conformation of the C-terminus of NPY, we designed analogs containing the NPY segments 1-4 and 33-36 connected by a spacer between the residues 4 and 33, and head to tail cyclized through a second spacer (chapter 3). For the preparation of these peptides, a method was developed that allows not only to carry out the chain assembly on the solid phase but also to couple the N- and C-termini while the peptide is still bound to the solid support. This was achieved by the attachment of the phenolic group of tyrosine methyl ester, representing Tyr^{36} of NPY, to the resin. The amino function of this amino acid was used to elongate the peptide chain, while its carboxy function was submitted to cyclization after hydrolysis of the methyl ester. Fluorenylmethoxycarbonyl/*tert*-butyl chemistry was chosen for the chain assembly, and the recovery of the free phenolic group was smoothly accomplished with trifluoroacetic acid in the presence of suitable scavengers. The cyclic peptides showed circular dichroism (CD) spectra characteristic of β-turns. The binding affinity of the compounds was tested at the Y_1-, Y_2- and Y_5-receptor systems: although with moderate affinity, they turned out to be Y_1-receptor preferring ligands. These results showed that only eight amino acids which correspond to the N- and C-termini of NPY are sufficient to displace some native peptide from the Y_1-receptor binding site (chapter 3).

The binding properties of the members of the NPY family at the Y-receptors were modulated by the design of chimeric analogs of NPY, PYY and PP. In general, the introduction of PP segments into NPY led to a decrease in affinity at all receptors and also to a destabilization of the helical structure in solution. In contrast, the presence of NPY positions in human PP (hPP) did not affect the high affinity at the Y_4-receptor, but significantly increased the affinity at the other receptors: in particular, the chimeric analog of hPP containing the NPY segments 1-7 and 19-23 turned out to be as potent as NPY at the Y_1-receptor and > five-fold more potent at the Y_5-receptor (IC_{50} 0.1 nM). All hPP analogs maintained the highly helical character of hPP, however their tertiary structure was more similar to that of NPY than of hPP, as suggested by their CD profiles (chapter 4).

In the search for selective NPY ligands, the conformationally constrained aminoisobutyric acid (Aib) was used to design a compound that selectively bound to the Y_5-receptor: [Ala^{31}, Aib^{32}]-NPY (IC_{50} 5 nM). This analog was found to act as an agonist of NPY *in vitro* and to stimulate food intake in rats. The solution structure of [Ala^{31}, Aib^{32}]-

NPY was investigated by CD, 2D-NMR and molecular dynamics. Comparison with the structure of the native NPY revealed a conformational change in the C-terminal part of the peptide: while the analog adopted a 3_{10}-helical turn in the region 28-31, followed by a not well defined structure, NPY showed an α-helix extending to the C-terminal end. Furthermore, in contrast to NPY, for the Aib-containing analog no dimerization was observed under the NMR conditions (chapter 5).

To investigate the ability of the motif Ala-Aib to induce Y_5-receptor selectivity, we introduced it into the chimeric analogs of hPP with the highest affinity at the Y_5-receptor. Accordingly, the new [Ala^{31}, Aib^{32}]-containing chimera turned out to be Y_5-receptor selective with subnanomolar affinity (IC_{50} up to 0.2 nM). They were tested *in vitro* and *in vivo* and were found to be agonists of NPY and to increase food intake in rats (chapter 5).

Further [Ala^{31}, Aib^{32}]-containing analogs of NPY and of the PP/NPY chimera were synthesized: all peptides were Y_5-receptor selective. This supported the hypothesis that the motif Ala-Aib is a key motif for selectivity at the Y_5-receptor. To better understand the structural role of Aib at position 32, other amino acids were introduced at this position, like proline, D-proline and hydroxyproline (Hyp): while [Ala^{31}, Pro^{32}]-NPY and [Ala^{31}, Hyp^{32}]-NPY selectively bound to the Y_5-receptor with a good affinity (12 nM and 40 nM, respectively), the introduction of D-proline led to a drastic loss of affinity. These results suggested that the presence of a turn-inducing motif at position 32 of NPY may favor the binding at the Y_5-receptor in a specific way that is not tolerated at the other Y-receptors.

In conclusion, structure-affinity and structure-activity relationship studies led to the development of the first class of potent Y_5-receptor selective agonists: the administration of these selective molecules *in vivo* induced increase in food intake in a dose-dependent manner. This observation suggests that the orexigenic function of NPY may be transmitted by the Y_5-receptor. Therefore, the structural and biological characterization of this receptor is of major importance for the understanding of the complex mechanism that regulates feeding and for the development of anti-obesity drugs. To this aim, the selective Aib-containing analogs provide a very important and promising tool.

ZUSAMMENFASSUNG

Neuropeptid Y (NPY), pankreatisches Polypeptid (PP) und Peptid YY (PYY) sind Hormone, die zu der NPY-Familie gehören. Sie bestehen aus 36 Aminosäuren, sind C-terminal amidiert und zeigen hohe strukturelle Homologie, die 69% zwischen NPY und PYY, und 50% zwischen NPY und PP beträgt. Die Röntgenstruktur von Vogel-PP zeigt eine antiparallele Faltung einer Polyprolin Helix II (Reste 1-8) und einer α-Helix (Reste 14-31). Das C-terminale Pentapeptid ist ungeordnet. NPY ist eines der am häufigsten vorhandenen Neuropeptide in den zentralen und peripheren Nervensystemen, und ist in hohen Konzentrationen im Gehirn, besonders in den kortikalen Bereichen, im Hippocampus und im Hypothalamus anwesend. Die relevantesten biologischen Funktionen von NPY sind Gefässverengung, Stimulation des Nahrungsaufnahme, Steigerung der Gedächtnisleistung, Hemmung des sexuellen Verhaltens, Anxiolyse, Regelung der Neurotransmitterausschüttung und Modulation des Äthanolkonsums. Diese Effekte werden durch mindestens fünf unterschiedliche Rezeptoren übertragen, die zu der Familie der G-Protein gekoppelter Rezeptoren gehören: Y_1-, Y_2-, Y_4-, Y_5- und y_6-Rezeptor. Die Affinität von NPY und PYY liegt im subnanomolaren Bereich für die Rezeptoren Y_1, Y_2 und Y_5 (IC_{50} 0.04-0.8 nM), während die Affinität am Y_4-Rezeptor im nanomolaren Bereich (IC_{50} 5.5 nM für NPY) liegt. Im Gegensatz zu NPY und PYY bindet das Peptid PP an den Y_4-Rezeptor mit subnanomolar Affinität (IC_{50} 0.04 nM), während es an den anderen Rezeptoren nur mit nanomolarer (Y_5) und micromolarer (Y_1 und Y_2) Affinität bindet.

Um jeden Rezeptorsubtyp strukturell sowie biologisch zu charakterisieren, werden Moleküle benötigt, die nur einen einzigen Rezeptor in einer spezifischen Weise erkennen. Unterschiedliche Verfahren, die zu der Erkenntnis der strukturellen und Funktionseigenschaften führen, welche für das pharmakologische Profil des natürlichen Liganden verantwortlich sind, wurden im Rahmen dieser Arbeit kombiniert. Um Einblick in die wichtigsten Anforderungen der Primärstruktur zu bekommen, wurde ein systematischer Alanin-Austausch und N-/C-terminal und zentrale Verkürzung von NPY durchgeführt (Kapitel 1). Gestützt auf die Resultate dieser Untersuchungen wurde gefolgert, daß das C-terminale Pentapeptid für die Bindung an alle Y-Rezeptoren notwendig ist, während das Verhalten von N-terminal und von zentral verkürzten Peptiden in Bezug auf jeden Rezeptor unterschiedlich ist (Kapitel 1). Unter den Alanin-

monosubstituirten Analoga von NPY waren [Ala^{13}]-NPY und [Ala^{27}]-NPY selektiv für den Y_2-Rezeptor (75- bzw. 370-fach im Verhältnis zu den anderen Rezeptoren; Kapitel 2).

Aufgrund der Flexibilität der Peptidkette ist es im Allgemeinen notwendig, konformationelle Einschränkungen in dem Molekül vorzunehmen, um die Zahl der Freiheitsgrade zu beschränken, die dem Peptid energisch zugänglich sind. Mit dem Ziel, eine rigidere Konformation des C-Terminus von NPY zu erhalten, entwarfen wir Analoga von NPY, welche die Segmente 1-4 und 33-36 verbunden durch ein Spacer zwischen den Resten 4 und 33 enthalten, und die durch einen zweiten Spacer zwischen den N- und C-Resten zyklisiert sind (Kapitel 3). Für die Herstellung dieser Peptide entwickelten wir eine Methode, die nicht nur die Verlängerung der Peptidkette auf dem festen Polymerträger erlaubt, sondern die Zyklisierung der N- und C-Termini ermöglicht, während sich das Peptid noch am festen Support befindet. Dies wurde durch das Anknüpfen der Phenolgruppe von Tyrosinmethylester, welcher Tyr^{36} von NPY darstellt, an das Harz erzielt. Dessen Aminofunktion wurde verwendet, um die Peptidkette zu verlängern, während die Carboxyfunktion für die Zyklisierung nach Hydrolyse des Methylesters aktiviert wurde. Die Fluorenylmethoxycarbonyl/*tert*-butyl Strategie wurde für die Peptidsynthese gewählt, und die Abspaltung der Phenolgruppe vom Harz erfolgte glatt mit Trifluoressigsäure in Anwesenheit von geeigneten Abfängern. Die Zirkular-Dichroismus (CD) Spektren der zyklischen Peptide zeigten die Anwesenheit von β-Turns. Die Verbindungen wurden an den Y_1-, Y_2- und Y_5-Rezeptor Systemen getestet: obwohl nur mit mäßiger Affinität bevorzugten sie den Y_1-Rezeptor. Diese Resultate zeigten, daß nur acht Aminosäuren, die den N- und C-terminal Segmente von NPY entsprechen, ausreichend sind, das am Y_1-Rezeptor gebundene natürliche Peptid von der Bindungsstelle zum Teil zu verdrängen (Kapitel 3).

Die Bindungsaffinitäten der Peptide der NPY-Familie an den Y-Rezeptoren wurden durch das Design von Chimär-Analoga von NPY, PYY und PP moduliert. Im allgemeinen führte die Einführung der PP-Segmente in NPY zu einer Abnahme an der Affinität an allen Rezeptoren und auch zu einer Destabilisierung der helikalen Struktur in Lösung. Demgegenüber beeinflußte das Vorhandensein der NPY-Positionen in menschlichem PP (hPP) die hohe Affinität am Y_4-Rezeptor nicht, und erhöht zudem erheblich die Affinität an den anderen Rezeptoren: insbesondere war die Bindung des Chimär-Analogon von hPP, welches die NPY-Segmente 1-7 und 19-23 enthält, am Y_1-Rezeptor (IC_{50} 0.1 nM) so stark affin wie bei NPY und am Y_5-Rezeptor sogar > fünf-fach stärker. Alle hPP-Analoga behielten die stabile helikale Konformation von hPP, obwohl ihre tertiäre Struktur

ähnlicher der von NPY als der von hPP war, was durch ihre CD-Profile nahegelegt wurde (Kapitel 4).

In der Suche nach selektiven NPY-Liganden wurde die konformativ eingeschränkte Aminoisobuttersäure (Aib) benutzt. Es wurde das Molekül [Ala^{31}, Aib^{32}]-NPY synthetisiert, das selektiv an den Y_5-Rezeptor bindet (IC_{50} 5 nM). Dieses Analogon wirkte als ein Agonist von NPY *in vitro* und erhöht die Nahrungsaufnahme in Ratten. Die Struktur in Lösung von [Ala^{31}, Aib^{32}]-NPY wurde durch CD, 2D-NMR und molekulare Dynamik bestimmt. Der Vergleich mit der Struktur des natürlichen NPY zeigte eine Konformationsänderung im C-terminalen Bereich des Peptids: während das Analogon einen 3_{10}-helikalen Turn von den Resten 28-31, gefolgt von einer nicht sehr gut definierten Struktur, aufweist, hat NPY eine α-Helix in diesem Bereich, die sich bis zum C-Terminus fortsetzt. Im Gegensatz zu NPY wurde außerdem für das Aib-enthaltende Analogon keine Dimerisierung unter NMR Bedingungen beobachtet (Kapitel 5).

Um die Fähigkeit des Motivs Ala-Aib, am Y_5-Rezeptor Selektivität zu induzieren, besser zu verstehen, führten wir dieses in Chimär-Analoga von hPP ein, die die höchste Affinität am Y_5-Rezeptor aufwiesen. Dementsprechend resultierten neue, Y_5-Rezeptor selektive [Ala^{31}, Aib^{32}]-enthaltende Chimära mit subnanomolarer Affinität (IC_{50} bis zu 0.2 nM). Sie wurden *in vitro* und *in vivo* getestet und wirkten als Agonisten von NPY an Zellen sowie stimulierten sie die Nahrungsaufnahme in Ratten (Kapitel 5).

Weitere [Ala^{31}, Aib^{32}]-enthaltende Analoga von NPY und der PP/NPY Chimära wurden synthetisiert: alle Peptide waren selektiv für den Y_5-Rezeptor. Diese Resultate unterstützten die Hypothese, daß das Motiv Ala-Aib ein Schlüsselmotiv für die Selektivität am Y_5-Rezeptor ist (Kapitel 6).

Um die strukturelle Rolle von Aib in der Position 32 besser zu verstehen, wurden andere Aminosäuren, wie Prolin, D-Prolin und Hydroxyprolin (Hyp) in dieser Position eingeführt: während [Ala^{31}, Pro^{32}]-NPY und [Ala^{31}, Hyp^{32}]-NPY an den Y_5-Rezeptor mit guter Affinität selektiv binden (12 nM bzw. 40 nM), führte die Einführung von D-Prolin zu einem drastischen Verlust der Affinität. Diese Resultate legen nahe, daß das Vorhandensein eines Turns, den das Element in Position 32 von NPY verursacht, die Bindung an den Y_5-Rezeptor in einer spezifischen Weise begünstigen kann, die nicht durch die anderen Y-Rezeptoren zugelassen wird (Kapitel 6).

Zusammenfassend kann gefolgert werden, daß Studien über Struktur-Affinität und Struktur-Aktivität zu der Entwicklung der ersten hoch affinen und selektiven Agonisten am Y_5-Rezeptor führten: diese selektiven Moleküle verursachten eine Zunahme der Nahrungsaufnahme in Ratten in einer dosisabhängigen Weise. Diese Beobachtung legt

nahe, daß die Funktion von NPY, die Nahrungsaufnahme zu stimulieren, durch den Y_5-Rezeptor übertragen werden kann. Folglich sind die strukturellen und biologischen Eigenschaften dieses Rezeptors für das Verständnis des komplizierten Mechanismus, der die Nahrungsaufnahme regelt, und für die Entwicklung von Arzneimittel gegen die Fettsucht von entscheidender Bedeutung. Für dieses Ziel stellen die selektiven Aib-enthaltenden Analoga einen sehr wichtigen und vielversprechenden Ansatz dar.

CHAPTER 1

Molecular Characterization of the Ligand-Receptor Interaction of the Neuropeptide Y Family (Review)

Chiara Cabrele and Annette G. Beck-Sickinger

Department of Pharmacy, Swiss Federal Institute of Technology, Zurich, Switzerland

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1.1 Abstract

Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) belong to the NPY hormone family and activate a class of receptors which are called the Y-receptors and 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 with respect to its interaction with the ligands 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 C-terminal segments, cyclopeptides, like cyclo-(28/32)-Ac-[Lys^{28}-Glu^{32}]-(25-36)-pNPY, and the TASP molecule that contains two units of the NPY segment 21-36. Now, the first selective antagonist with nanomolar affinity for the Y_2-receptor (named BIIE0246) has been described. 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}. The results of the feeding experiments in rats treated with the first highly specific analogs for the Y_5-receptor support the hypothesis that this receptor plays a role in the NPY-induced stimulation of food intake. In conclusion, selective compounds for the different Y-receptor subtypes known so far are promising tools to better understand the physiological properties of the hormones of the NPY family and related receptors.

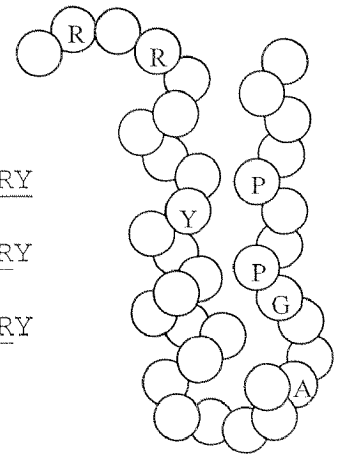
Keywords: NPY, PYY, PP, Y-receptors, structure-affinity relationship, receptor selectivity, food intake.

1.2 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 only 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 christallography 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, right). 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 found 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]

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.



pNPY YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY

pPYY YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY

hPP APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY

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

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]

1.3 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 the 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 vascular bed,[25] respectively.

Receptor sequence alignment revealed a high identity of the Y_1-receptor to the Y_4- (42%) and y_6-receptor (51%), while a decreased one to the Y_2- (31%) and Y_5-receptor (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 block 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 of 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

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>>NPY/PYY-fragments, PP	PYY, NPY, NPY (2-36)>>[P^{34}]-NPY, PP	NPY≥[P^{34}]-NPY≥ NPY (13-36)>> PYY, PP	PP>>PYY>NPY≥ [P^{34}]-NPY>>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?	no activity known

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 low y, according to the IUPHAR recommendations.[23]

1.4 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 modification of the primary and, consequently, of the secondary and tertiary structure of the natural ligands. The successive analysis of their affinity and activity and of 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 high 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 essential to develop selective ligands which can be used as tools for structural as well as for 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 which 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.[59]

1.5 The Y_1-Receptor

1.5.1 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[60] (Table 2): NPY (2-36) shows 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 is in the micromolar range.

1.5.2 Single Amino Acid Replacements

The contribution of each 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

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)	2 700	0.25	cyclo-(28/32) Ac-[Orn^{28}, Asp^{32}]-(25-36)-pNPY	> 3 900
pNPY-(22-36)	6 600	0.41	cyclo-(28/34) Ac-[Lys^{28}, Glu^{34}]-(25-36)-pNPY	> 1 000
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	2 000		
Ac-[Nal^{31}]-(25-36)-pNPY	3 800	51		
Ac-[$Nal^{30, 31}$]-(25-36)-pNPY	3 800	51		
Ac-[Tic^{30}]-(25-36)-pNPY	> 10 000	1 000		
Ac-[Tic^{31}]-(25-36)-pNPY	38 000	225		
Ac-[$Tic^{30, 31}$]-(25-36)-pNPY	> 45 000	650		

positions 27 to 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 recovery of affinity; on the other hand, the presence of His decreased the affinity dramatically (> 3 000-fold).[51] The replacement of Pro^5 reduced the affinity more than 1 000-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 > 1 000-fold lower affinity, which should be attributed to the lack of the basic side-chain, as the substitution of Arg by 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*-benzoylphenylalanine) reduced the affinity only 30-fold.[61] 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 of Tyr with Ala led to a decrease in affinity, especially

at position 27 (> 1 000-fold), while the introduction of Phe or Bpa still resulted in good affinity.[51, 61] 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 residue 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 the IC_{50} value of 570 nM.[61] 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.[61] The lack of hydrophilicity at position 32 after the replacement of Thr with Ala was accompanied by a dramatic reduction of affinity (> 3 000-fold). After the incorporation of D-Trp, the affinity was very low (IC_{50} 955 nM).[62] Arg^{33} and Arg^{35} turned out to be the most important residues for the Y_1-receptor binding: in fact, both analogs containing Ala^{33} or Ala^{35} bound to the receptor with an affinity of 7 000 nM and 13 000 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).[63] 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.[61] 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 nM and 21 nM, respectively, versus 0.2 nM for NPY).[61] 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 a hydrogen bond, intramolecularly or with the receptor. 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 conclusion, the C-terminal decapeptide 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.

1.5.3 PP/NPY Chimera

NPY and PP reveal a completely different affinity at the Y_1-receptor, which is 0.2 nM and > 1 000 nM, respectively. The substitution of the pNPY sequence 19-23 RYYSA by the corresponding h/rPP QYAAD/QYETQ led to a decrease in affinity (Table 4). Structurally, this exchange was characterized by a helix destabilization.[64] 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 hPP segments 1-7 or 1-17, both analogs were still potent, with an affinity of 3 nM and 1.9 nM, respectively.[64] [hPP$^{1-7}$]-pNPY showed a CD spectrum that was very similar to that of pNPY, and [hPP$^{1-17}$]-pNPY was characterized by a high helical content (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 segments 1-7 or 19-23, or both of them.[64] [$pNPY^{1-7, 19-23}$]-hPP turned out to be as potent as NPY itself (IC_{50} 0.22 nM). These hPP analogs are all as helical as the unmodified hPP, however they probably adopt a different tertiary structure which might be more similar to the structure of NPY than of hPP. This observation suggests that the NPY segments 1-7 and 19-23 drive the formation of the bioactive conformation of the ligand.

1.5.4 C-Terminally Modified Analogs of NPY

The importance of the tyrosine amide at position 36 of NPY for the 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.[65] The free carboxylic group led to a complete loss of affinity (> 10 000 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 oxigen atom of the carbonyl group was 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 is important for the binding and also for the activation of the receptor.

1.5.5 Centrally Truncated Analogs

Although the results of the Ala-scan showed that the central positions of NPY are not essential for the 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 a moderate affinity (Table 5). The shortest peptide [Ahx^{5-24}]-pNPY did not bind to the Y_1-receptor (IC_{50} > 4 000).[66] Rist and co-workers[67] 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 to 22 for the first three sets and 17 to 22 for the last one. They found that the analogs corresponding to x = 22 were very poor ligands (IC_{50} 940 nM, 280 nM, 260 nM 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

Table 3. Effect of the single amino acid replacement on the binding affinity of NPY at the Y-receptors, according to Refs. 51, 59 and 61.

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	7 000 ±0	54 ±21	> 1 000	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	3 000 ±0	> 1 000	> 1 000
36	Tyr	Ala	970 ±30	780 ±0	141	68
		Phe	2.6 ±0.4	0.42 ±0.23		
		Bpa	118	0.3		
		His	470			

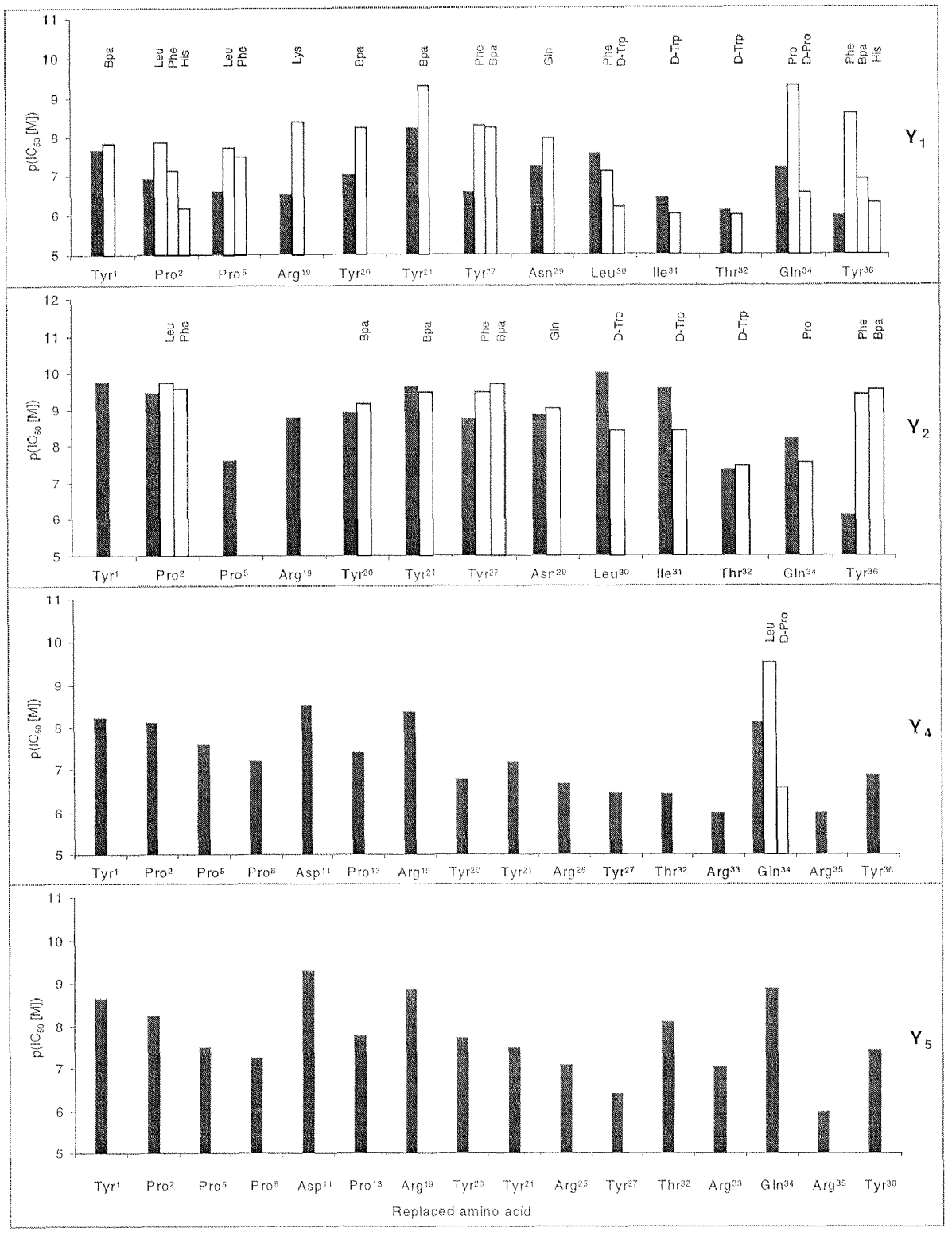


Figure 2. Single amino acid replacement of NPY analogs and their affinity to the receptors Y_1, Y_2, Y_4 and Y_5. The black bars are relative to the L-Ala-substitution, while the white bars are relative to the replacement with the amino acid that is indicated on the top. For the complete Ala-scanning of NPY at the Y_1- and Y_2-receptors, see Ref. 51.

Table 4. Binding affinity of pNPY, hPP and some PP/NPY chimera at the Y-receptors.

Peptide	Y_1	Y_2	Y_4	Y_5
	IC_{50} [nM]	IC_{50} [nM]	IC_{50} [nM]	IC_{50} [nM]
pNPY	0.2	0.04	5.5	0.8
hPP	> 1 000	> 1 000	0.04	57
[E^4, P^{34}]-pNPY	6.6		2	12
[hPP^{19-23}]-pNPY	10.5	1.0	> 1 000	191
[hPP^{19-23}, P^{34}]-pNPY	4.2	1 000	15	62
[hPP^{19-23}, H^{34}]-pNPY	43	1 000	133	113
[rPP^{19-23}]-pNPY	70	12	21	265
[rPP^{19-23}, P^{34}]-pNPY	4.8	1 000	46	32
[rPP^{19-23}, H^{34}]-pNPY	112	1 000	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	0.6	28	0.02	0.07
[cPP^{1-7}, $pNPY^{19-23}$, H^{34}]-hPP	5.7	22	0.06	0.04

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 nM 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[68] observed that high Y_1-receptor affinity was obtained only when the number of the centrally truncated residues was limited to 8 (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.

Table 5. Binding affinity of some centrally truncated analogs of NPY and hPP at the Y-receptors.

Peptide	Y_1	Y_2	Y_4	Y_5
	IC_{50} [nM]	IC_{50} [nM]	IC_{50} [nM]	IC_{50} [nM]
[Ahx$^{5-24}$]-pNPY	> 4 000	1.4	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	7 000	1		
[Pac$^{5-24}$]-pNPY	4 500	1		
cyclo-(2/30)-[Glu^{2}, Ahx$^{5-24}$, Lys^{30}]-pNPY	> 10 000	5.7		
[Ahx$^{5-24}$]-hPP	> 500	> 1 000	144	> 1 000
[Ahx$^{5-20}$]-hPP	> 1 500	> 1 000	216	> 7 000
[Tyr$^{5-20}$]-hPP	> 500	> 1 000	27	> 5 000

1.5.6 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-induced increase in cytosolic Ca^{2+} in HEL cells[69] (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 compounds at the Y_1-receptor was determined to be in the range 2-10 nM. The high potency 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 nonapeptides described above, 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,[70] and binds to the Y_1-receptor in the picomolar range (K_i 0.041 nM). However, GW1229 has been found to be a potent agonist at the Y_4-receptor as well, with a K_i value of 0.3 nM, which limits its use as a pharmacological tool.[71-73]

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 carboxy group by the 4-hydroxyphenylmethyl moiety.[74] 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 two hours) 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.[75]

Table 6. Binding affinity and activity of N-terminally truncated analogs of NPY.

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)-Phe	5	520
INPIXRLRY	X = (2,6-Cl_2-Bzl)-Tyr	2	50
INPXYRLRY	X = Aib	9	260
INXIYRLRY	X = (3,4-dehydro)-Pro	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. 69 and 92).

Two even 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,[76] and LY357897 (K_i 0.75 nM), a trisubstituted indole.[77]

The antagonist binding site at the Y_1-receptor has been investigated by the combination of site-directed mutagenesis and molecular modeling studies (Figure 4).[57, 78] 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}. As 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 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, like tachykinin,[79, 80] angiotensin,[81] cholecystokinin/gastrin[82] or *k*-opioid receptors,[83] the two domains were found to be different. However, an overlap in the agonist and the antagonist binding domains was reported for the endothelin-1 receptor as well.[84]

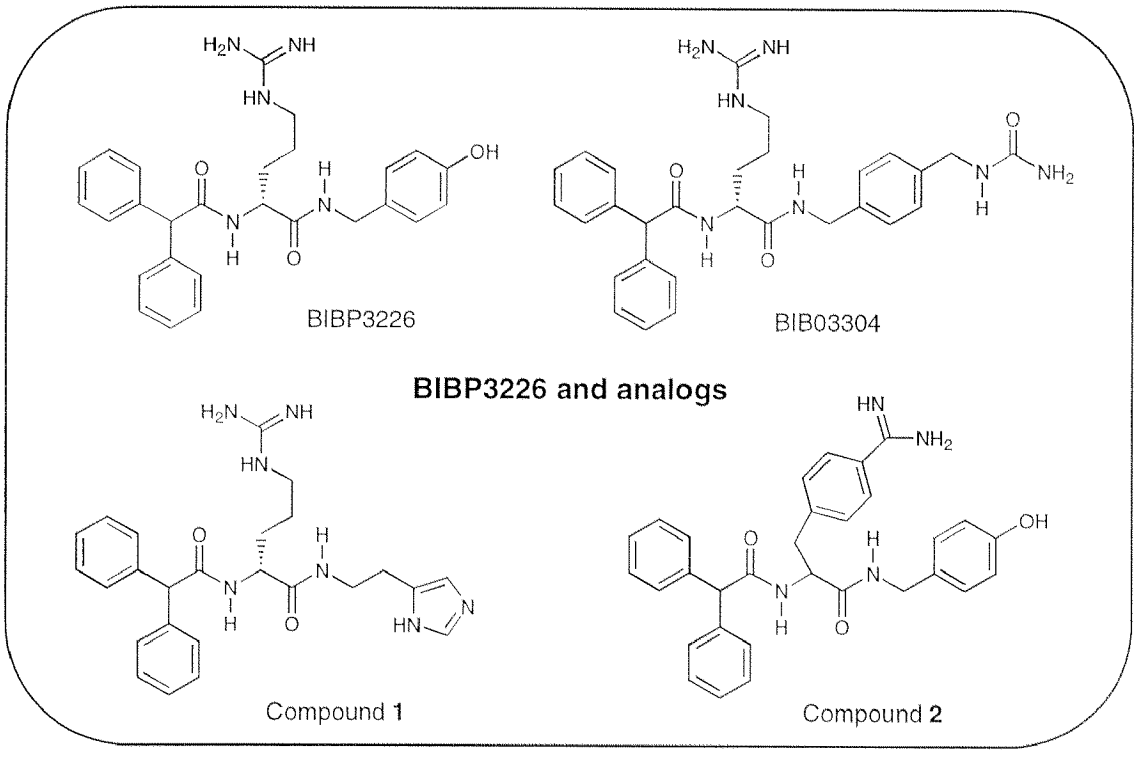
Although NPY and BIBP3226 share some overlapping regions at the Y_1-receptor, they probably stabilize two different conformations of the receptor. This would explain why the first one acts as an agonist, while the second one as an antagonist.

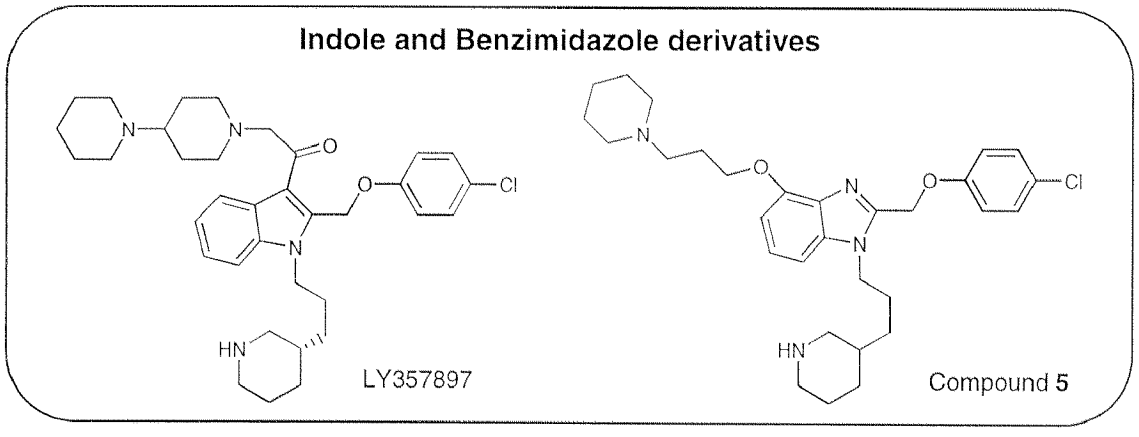
Structure-activity relationship studies on analogs of BIBP3226 led to the following observations:[85, 86] (1) the substitution of the 4-hydroxy-benzyl group of BIBP3226 by the imidazolylethyl group led to 1 000-fold lower activity (compound **1**, Figure 3), probably because of the lack of a hydrogen 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 the 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).

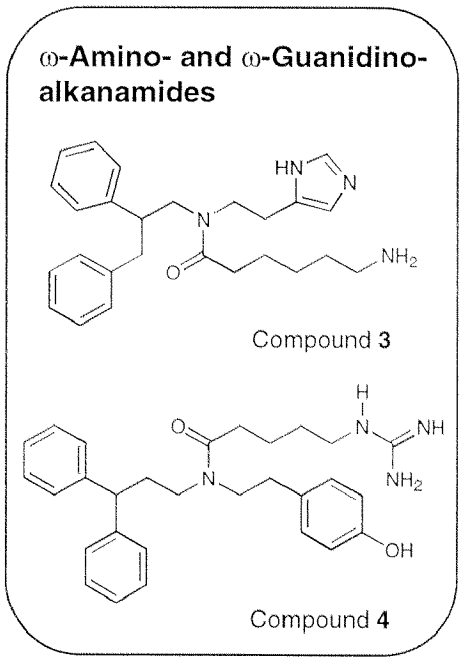
Müller and co-workers[87] carried out structure-activity relationship studies on *N,N*-disubstituted ω-amino- and ω-guanidinoalkanamides that resemble the structure of BIBP3226 and contain an imidazole or a phenolic group. It was found that in the imidazole series the presence of an amino group was preferred with respect 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 an overlapping region with respect to their diarylalkyl moiety.

Starting from the compound LY357897,[77] the possibility to replace 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.[88] 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.







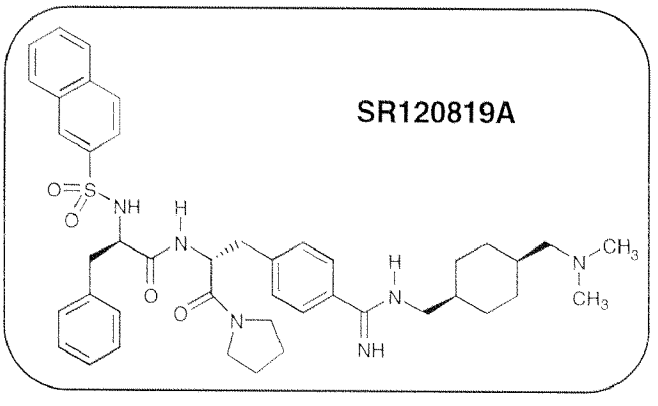


Figure 3. Y_1-receptor selective antagonists. For their pharmacological properties, see text and following Refs.: 74 for BIBP3226; 76 for BIBO3304; 75 for SR120819A, 77 for LY357897; 85 and 86 for the compounds **1** and **2**; 87 for the compounds **3** and **4**; 88 for the compound **5**.

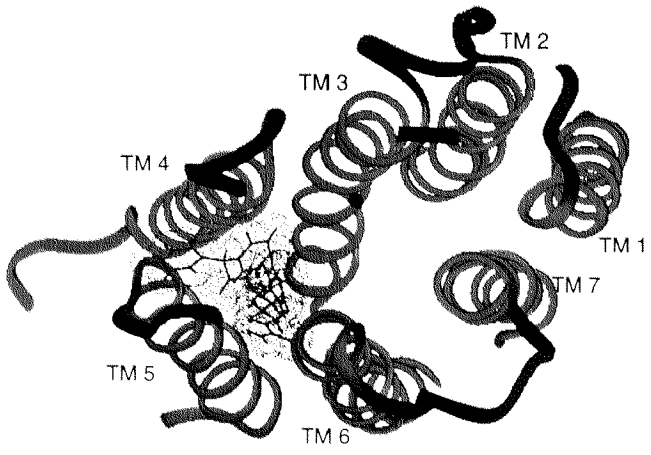


Figure 4. Model of the binding of the antagonist BIBP3226 (green) to the human Y_1-receptor. View down the axis of the transmembrane helices (TM, in red). The extracellular loops are shown in blue.

1.5.7 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 which 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 PP the affinity is even increased.

The development of selective antagonists has given insight into the structural requirements for the 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 crosslinking 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.[59, 89]

1.6 The Y_2-Receptor

1.6.1 N-Terminally Truncated Analogs

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[60] (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).[60] The acetylated dodecapeptide NPY (25-36) showed a minor affinity of 160 nM.[60] However, by the introduction of special amino acids with hydrophobic and conformationally restricted side-chains, the binding potency was enhanced up to 20-fold.[90, 91] The substitution of Leu^{30} or Ile^{31} by cyclohexylalanine (Cha) yielded two molecules with an IC_{50} of 18 nM 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} 2 000 nM). Surprisingly, the double substituted peptide was as potent as the [Nal^{31}]-substituted one. A similar behavior was observed for the three analogs containing tetrahydroisoquinoline-3-carboxylic acid (Tic): Tic^{30} led to an affinity of 1 000 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 the 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[69, 92] (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. In contrast, the presence of (3,4-dehydro)-Pro at this position was better tolerated (IC_{50} 40 nM versus 170 nM). Furthermore, 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 co-workers[93] 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 nM 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 was 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 6 000-fold. The series of cyclic dodecapeptides, where position 34 was submitted to 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.[94] 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 5).

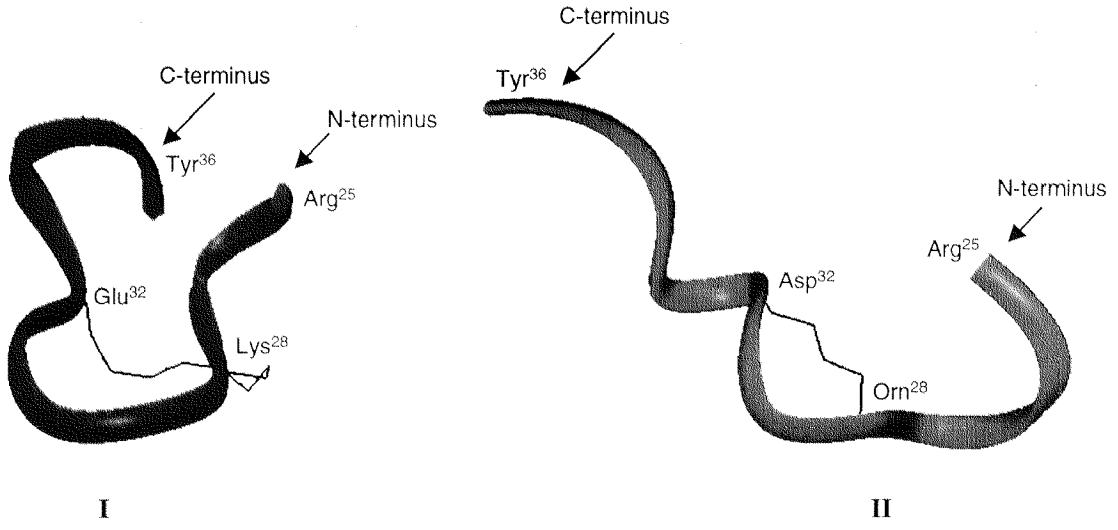


Figure 5. Ribbon representation of the NMR mean structure of cyclo-(28/32) Ac-[Lys^{28}, Glu^{32}]-(25-36)-pNPY (**I**) and of cyclo-(28/32) Ac-[Orn^{28}, Asp^{32}]-(25-36)-pNPY (**II**). The lactam bridge trace is shown as a thin line.

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 low affinity.[93] 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 to each other and oriented in a well defined way.

Based on the concept that the structure and the biological action of a peptide or a protein can be modulated by the assembly of peptide elements on a synthetic template (the so called TASP concept),[95] 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, selectively bound 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*.[96] Previously, Grouzmann and co-workers[97] 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 moderate affinity, but not to the Y_1-receptor, and was reported to reduce the NPY-induced mobilization of intracellular calcium.

1.6.2 Single Amino Acid Replacements

The Ala-scan affected the Y_2-receptor binding to a minor extent compared with the Y_1-receptor binding.[51] In general, the loss of affinity was in the range two- 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 positions 33-36 (Table 3 and Figure 2). In the N-terminal region, only the substitution of Pro^5 by Ala led to 600-fold lower affinity. 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 a 18-fold loss of affinity, which indicates that the presence of an aromatic residue is favored.[61] The same behavior was observed at position 27, where the incorporation of Ala led to a decrease in affinity, while Phe or Bpa gave a better affinity.[51, 61] 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 1 000-fold decrease in affinity. Unexpectedly, the more drastic change by D-Trp led to almost the same loss of affinity as Ala.[62] The substitution of Arg at position 33 or 35 was associated with a decrease in affinity of 1 350- or 75 000-fold. Ala^{34} induced 150-fold lower affinity, and Pro at this position further reduced the binding potency more than 700-fold.[63] At position 36 the Ala-replacement was very poorly tolerated, as the affinity was reduced 19 500-fold. As already observed for Tyr^{20} and Tyr^{27}, the introduction of Phe or Bpa led to a minor loss of affinity (eight- to ten-fold).[51, 61]
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 to that of NPY ([Ala^3]-NPY and [Ala^{30}]-NPY) or up to 100-fold lower.[98]

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 >

3 300-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]

1.6.3 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) (Table 4). 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.

1.6.4 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 (1.4 nM).[66] The binding potency of this ligand was slightly increased by introducing the hydrophobic residue Tic at position 1 (1 nM), or by substituting Ahx with 1-phenyl-2-aminomethyl-cyclopropanoic acid (Pac) (1 nM)[100] (Table 5). The analog cyclo-(2/30)-[Glu^{2}, Ahx$^{5-24}$, Lys^{30}]-NPY[101] showed a slightly lower affinity than the linear peptide [Ahx$^{5-24}$]-NPY 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 a 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.

1.6.5 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: this is denoted as BIIE0246.[102] The structure of BIIE0246 is shown in Figure 6: in contrast to the Y_1-receptor selective antagonist BIBP3226, BIIE0246 contains an Arg residue in the L configuration. The rest of the molecule is highly hydrophobic. This molecule selectively bound to the Y_2-receptor with nanomolar affinity (IC_{50} 3.3 nM) and was found to antagonize NPY in the rat vas deferens assay.

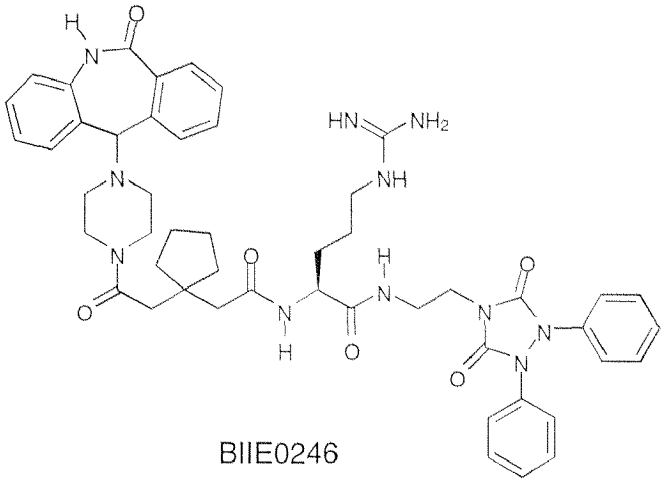


Figure 6. Structure of the Y_2-receptor selective antagonist BIIE0246.

1.6.6 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 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.

1.7 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,[103] 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.[104] In contrast, [Leu^{31}, Pro^{34}]-NPY, PP and two other chimera, [NPY^{1-7}]-PYY and [NPY^{1-3}]-PYY, showed no effect. The different behavior of the four NPY/PYY chimera suggests that the residues 13 and 14 of NPY play an important role for 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.[105] 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.[103]

1.8 The Y_4-receptor

One feature of the Y_4-receptor is that it binds PP with high affinity provided that receptor and ligand derive both from the same species.[46] NPY and PYY showed a > 100-fold lower affinity than hPP at the hY_4-receptor (IC_{50} 5.5 nM for NPY versus 0.04 nM for hPP).[59]

1.8.1 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 > 1 000 nM[59] (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. 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 the 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 the binding to the Y_4-receptor as well.

1.8.2 Centrally Truncated Analogs

The highly Y_2-receptor selective analog [Ahx^{5-24}]-NPY bound to the Y_4-receptor only with low affinity (600 nM).[59] The elongation of the N- and C-terminal segments 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 and its replacement with the spacer Ahx led to an affinity of 144 nM, 3 600-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 nM to 27 nM[59] (Table 5). These results suggest that the central part of hPP is important to induce and stabilize the bioactive conformation.

1.8.3 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).[64] 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.[64] 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 hPP segment 19-23 could be partially recovered by the additional exchange of Gln^{34} by Pro.[64] 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 helical content, suggests that the highly 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 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).[64] 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.[64] 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.

1.8.4 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.

1.9 The Y_5-Receptor

Great interest has been focused on the most recently cloned receptor subtype,[39, 48] as it has been speculated to play an important role in the feeding behavior, together with the Y_1-receptor.[72, 106-111] Therefore, structure-affinity and structure-activity relationship studies are 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.

1.9.1 Single Amino Acid Replacements

From the Ala-scan study[59] it has been shown that the importance of the Pro residues increased from position 2 to position 8, with a loss of affinity in the range seven- to 70-fold (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 more than 400-fold. Tyr^{36} was sensitive to Ala-substitution as well, and its replacement was characterized by 85-fold loss of affinity. The substitution of Arg^{19} only slightly decreased the binding potency (IC_{50} 1.4 nM versus 0.8 nM for NPY), the replacement of Arg^{25} or

Arg^{33} resulted in a loss of affinity that was more than 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 reduced the affinity of up to two-fold, whereas the presence of D-Pro at this position was poorly tolerated.[59] 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 0.8 nM for NPY).

1.9.2 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[59] (Table 5).

1.9.3 PP/NPY Chimera

The substitution of the pNPY segment 19-23 with the corresponding hPP or rPP segment induced a decrease in affinity up to 300-fold,[64] 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 the 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.[64] Interestingly, the combination of hPP (1-17) and NPY (18-36) led to a slightly higher affinity (IC_{50} 0.45 nM versus 0.8 nM for NPY). This suggests that the stabilization of the NPY helix favors the binding of the Y_5-receptor. Accordingly, the helical content of this chimeric peptide was determined to be 64% from its CD curve.[64]

The affinity of hPP for the Y_5-receptor could not be increased by single exchange at the N-terminus; however, the introduction of the whole segment 1-7 of pNPY led to a significant increase in affinity (IC_{50} 3 nM).[64] 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 the multiple replacement of the hPP positions 19-23 with the corresponding pNPY residues led to good affinity (IC_{50} 1-2 nM).[64] 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.[64]. Additionally, the mutation Pro34His led to the analog [cPP^{1-7}, $pNPY^{19-23}$, His^{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 as well as 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 conformational prerequisite, however, it is not sufficient for subnanomolar affinity at the Y_5-receptor.

1.9.4 Selective Analogs

A class of highly selective ligands has recently been developed. These compounds 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.[62, 112] Among the series of the (Ala-Aib)-substituted NPY analogs, the most potent ligand was simply the double substituted analog [Ala^{31}, Aib^{32}]-pNPY with an affinity of 5 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.[112] Interestingly, the analog [Ahx^{5-24}, Ala^{31}, Aib^{32}]-NPY had higher affinity (40-fold) 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 an affinity of 16 nM.[112] 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.

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 over the residues 28-31, followed by an apparently not-well defined structure of the last five residues (Figure 7, right).[62]

Surprisingly, the inverse motif Aib-Ala was found to significantly improve the binding potency of NPY at the Y_5-receptor (K_i 0.5 nM).[113] 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 feeding studies *in vivo*: the data showed that it can stimulate food intake in rats in a dose-dependent manner.[62] Accordingly, it can be concluded that the Y_5-receptor is involved in food intake regulation (Figure 7, left).

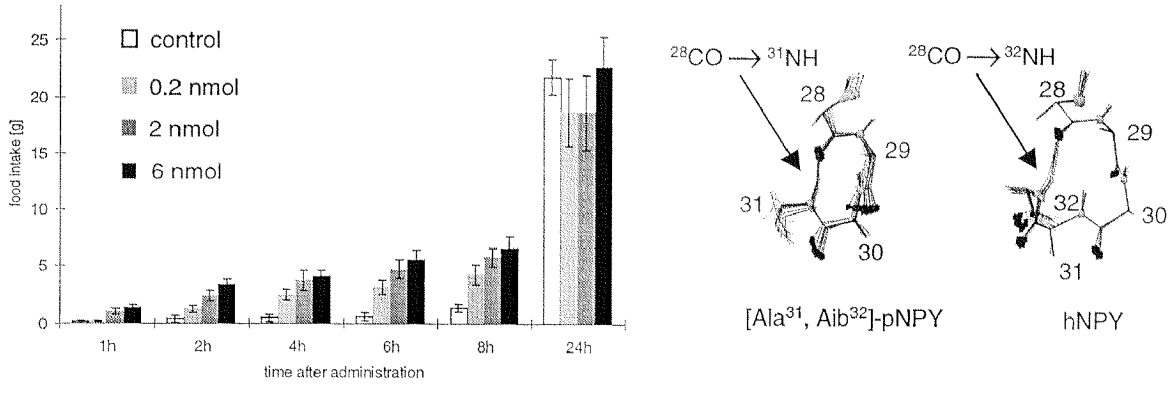
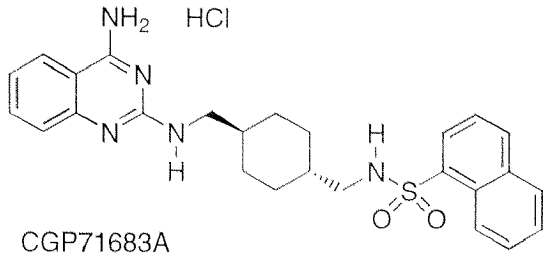


Figure 7. Structure and biological action of the Y_5-receptor selective agonist [Ala^{31}, Aib^{32}]-pNPY. Dose-dependent increase in food intake in rats after treatment with the Y_5-receptor selective agonist (on the left). Hydrogen bond pattern over the residues 28-32 of [Ala^{31}, Aib^{32}]-pNPY and hNPY[62] (on the right). The structure of hNPY is according to the NMR data of Monks and co-workers.[8]

1.9.5 Antagonist

The recently developed antagonist CGP71683A is characterized by an IC_{50} value of 1.4 nM at the Y_5-receptor, and by a micromolar affinity at the other receptors (Figure 8). This

antagonist has been used to investigate the role of the Y_5-receptor in the NPY-induced increase in food intake:[106] the data have shown inhibitory effects either in lean or obese rats, supporting the hypothesis that the Y_5-receptor has a role in the feeding behavior.



CGP71683A

Figure 8. Structure of the Y_5-receptor selective antagonist CG71683A.

1.9.6 Conclusions

A stable α-helix is an important structural requirement for the binding of NPY analogs to the Y_5-receptor. However, this is not sufficient for high affinity, as demonstrated by the moderate affinity of the highly helical PP which showed an affinity of 57 nM at the 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.

1.10 Concluding Remarks

The emerging strong evidence for a multifunctional role of NPY (and related peptides), especially as analgesic, anxiolytic, antihypertensive and orexigenic neuropeptide, 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 activity transmitted by 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 concernig NPY and feeding behavior: are both the receptors Y_1 and Y_5 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, 114]

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.

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1.11 References

1. Kimmel JR, Hayden LJ, Pollock HG. Isolation and characterization of a new pancreatic polypeptide hormone. *J. Biol. Chem.* 1975; **250**: 9369-9376.

2. Tatemoto K, Mutt V. Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. *Nature* 1980; **285**: 417-418.

3. Larhammar D. Evolution of neuropeptide Y, peptide YY and pancreatic polypeptide. *Regul. Pept.* 1996; **62**: 1-11.

4. Tatemoto K. Neuropeptide Y: complete amino acid sequence of the brain peptide. *Proc. Natl. Acad. Sci. USA* 1982; **79**: 5485-5489.

5. Blundell TL, Pitts JE, Tickle IJ, Wood SP, Wu C-W. X-ray analysis (1.4 Å resolution) of avian pancreatic polypeptide: small globular protein hormone. *Proc. Natl. Acad. Sci. USA* 1981; **78**: 4175-4179.

6. Darbon H, Bernassau JM, Deleuze C, Chenu J, Roussel A, Cambillau C. Solution conformation of human neuropeptide Y by 1H nuclear magnetic resonance and restrained molecular dynamics. *Eur. J. Biochem.* 1992; **209**: 765-771.

7. Cowley DJ, Hoflack JM, Pelton JT, Saudek V. Structure of neuropeptide Y dimer in solution. *Eur. J. Biochem.* 1992; **205**: 1099-1106.

8. Monks SA, Karagianis G, Howlett GJ, Norton RS. Solution structure of human neuropeptide Y. *J. Biomol. NMR* 1996; **8**: 379-390.

9. Nordmann A, Blommers MJ, Fretz H, Arvinte T, Drake AF. Aspects of the molecular structure and dynamics of neuropeptide Y. *Eur. J. Biochem.* 1999; **261**: 216-226.

10. Lundberg JM, Tatemoto K, Terenius L, Hellstrom PM, Mutt V, Hökfelt T, Hamberger B. Localization of peptide YY (PYY) in gastrointestinal endocrine cells and effects on intestinal blood flow and motility. *Proc. Natl. Acad. Sci. USA* 1982; **79**: 4471-4475.

11. El-Salhy M, Wilander E, Grimelius L, Terenius L, Lundberg JM, Tatemoto K. The distribution of polypeptide YY (PYY) - and pancreatic polypeptide (PP) - immunoreactive cells in the domestic fowl. *Histochemistry* 1982; **75**: 25-30.

12. Lundberg JM. Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. *Pharmacol. Rev.* 1996; **48**: 113-178.

13. Sundler F, Bottcher G, Eckblad E, Håkanson R in: *The biology of neuropeptide Y and related peptides*. Colmers WF, Wahlestedt C, Ed., pp. 157-196, Humana Press, Totowa, NJ, 1993.

14. Turton MD, O'Shea D, Bloom SR in: *Neuropeptide Y and drug development*. Grundemar L, Bloom SR, Ed., pp. 15-39, Academic Press, 1997.

15. Pernow J, Lundberg JM, Kaijser L. Vasoconstrictor effects in vivo and plasma disappearance rate of neuropeptide Y in man. *Life Sci.* 1987; **40**: 47-54.

16. Maturi MF, Greene R, Speir E, Burrus C, Dorsey LM, Markle DR, Maxwell M, Schmidt W, Goldstein SR, Patterson RE. Neuropeptide Y. A peptide found in human coronary arteries constricts primarily small coronary arteries to produce myocardial ischemia in dogs. *J. Clin. Invest.* 1989; **83**: 1217-1224.

17. Ahlborg G, Weitzberg E, Sollevi A, Lundberg JM. Splanchnic and renal vasoconstrictor and metabolic responses to neuropeptide Y in resting and exercising man. *Acta Physiol. Scand.* 1992; **145**: 139-149.

18. Edvinsson L, Emson P, McCulloch J, Tatemoto K, Uddman R. Neuropeptide Y: immunocytochemical localization to and effect upon feline pial arteries and veins in vitro and in situ. *Acta Physiol. Scand.* 1984; **122**: 155-163.

19. Rudehill A, Olcen M, Sollevi A, Hamberger B, Lundberg JM. Release of neuropeptide Y upon haemorrhagic hypovolaemia in relation to vasoconstrictor effects in the pig. *Acta Physiol. Scand.* 1987; **131**: 517-523.

20. Obrietan K, van den Pol AN. Neuropeptide Y depresses GABA-mediated calcium transients in developing suprachiasmatic nucleus neurons: a novel form of calcium long-term depression. *J. Neurosci.* 1996; **16**: 3521-3533.

21. Flood JF, Baker ML, Hernandez EN, Morley JE. Modulation of memory processing by neuropeptide Y varies with brain injection site. *Brain Res.* 1989; **503**: 73-82.

22. Thiele TE, Marsh DJ, Ste. Marie L, Bernstein IL, Palmiter RD. Ethanol consumption and resistance are inversely related to neuropeptide Y levels. *Nature* 1998; **396**: 366-369.

23. Michel MC, Beck-Sickinger AG, Cox H, Doods HN, Herzog H, Larhammar D, Quirion R, Schwartz T, Westfall T. XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol. Rev.* 1998; **50**: 143-150.

24. Ewald DA, Sternweis PC, Miller RJ. Guanine nucleotide-binding protein Go-induced coupling of neuropeptide Y receptors to Ca2+ channels in sensory neurons. *Proc. Natl. Acad. Sci. USA* 1988; **85**: 3633-3637.

25. Michel MC, Rascher W. Neuropeptide Y: a possible role in hypertension? *J. Hypertens.* 1995; **13**: 385-395.

26. Blomqvist AG, Herzog H. Y-receptor subtypes--how many more? *Trends Neurosci.* 1997; **20**: 294-298.

27. Wahlestedt C, Håkanson R. Effects of neuropeptide Y (NPY) at the sympathetic neuroeffector junction. Can pre- and postjunctional receptors be distinguished? *Med. Biol.* 1986; **64**: 85-88.

28. Grundemar L, Jonas SE, Morner N, Hogestatt ED, Wahlestedt C, Håkanson R. Characterization of vascular neuropeptide Y receptors. *Br. J. Pharmacol.* 1992; **105**: 45-50.

29. Wahlestedt C, Pich EM, Koob GF, Yee F, Heilig M. Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligodeoxynucleotides. *Science* 1993; **259**: 528-531.

30. Inui A. Neuropeptide Y feeding receptors: are multiple subtypes involved? *Trends Pharmacol. Sci.* 1999; **20**: 43-46.

31. Herzog H, Hort YJ, Ball HJ, Hayes G, Shine J, Selbie LA. Cloned human neuropeptide Y receptor couples to two different second messenger systems. *Proc. Natl. Acad. Sci. USA* 1992; **89**: 5794-5798.

32. Larhammar D, Blomqvist AG, Yee F, Jazin E, Yoo H, Wahlestedt C. Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y1 type. *J. Biol. Chem.* 1992; **267**: 10935-10938.

33. Krause J, Eva C, Seeburg PH, Sprengel R. Neuropeptide Y1 subtype pharmacology of a recombinantly expressed neuropeptide receptor. *Mol. Pharmacol.* 1992; **41**: 817-821.

34. Potter EK, Mitchell L, McCloskey MJ, Tseng A, Goodman AE, Shine J, McCloskey DI. Pre- and postjunctional actions of neuropeptide Y and related peptides. *Regul. Pept.* 1989; **25**: 167-177.

35. Flood JF, Morley JE. Dissociation of the effects of neuropeptide Y on feeding and memory: evidence for pre- and postsynaptic mediation. *Peptides* 1989; **10**: 963-966.

36. Illes P, Finta EP, Nieber K. Neuropeptide Y potentiates via Y2-receptors the inhibitory effect of noradrenaline in rat locus coeruleus neurones. *Naunyn Schmiedebergs Arch. Pharmacol.* 1993; **348**: 546-548.

37. Colmers WF, Klapstein GJ, Fournier A, St-Pierre S, Treherne KA. Presynaptic inhibition by neuropeptide Y in rat hippocampal slice in vitro is mediated by a Y2 receptor. *Br. J. Pharmacol.* 1991; **102**: 41-44.

38. Rose PM, Fernandes P, Lynch JS, Frazier ST, Fisher SM, Kodukula K, Kienzle B, Seethala R. Cloning and functional expression of a cDNA encoding a human type 2 neuropeptide Y receptor [published erratum appears in *J. Biol. Chem.* 1995; **270**: 29038]. *J. Biol. Chem.* 1995; **270**: 22661-22664.

39. Gerald C, *et al.* A receptor subtype involved in neuropeptide Y-induced food intake. *Nature* 1996; **382**: 168-171.

40. Gehlert DR, Beavers LS, Johnson D, Gackenheimer SL, Schober DA, Gadski RA. Expression cloning of a human brain neuropeptide Y Y2 receptor. *Mol. Pharmacol.* 1996; **49**: 224-228.

41. Nörenberg W, Bek M, Limberger N, Takeda K, Illes P. Inhibition of nicotinic acetylcholine receptor channels in bovine adrenal chromaffin cells by Y3-type neuropeptide Y receptors via the adenylate cyclase/protein kinase A system. *Naunyn Schmiedebergs Arch. Pharmacol.* 1995; **351**: 337-347.

42. Grundemar L, Wahlestedt C, Reis DJ. Long-lasting inhibition of the cardiovascular responses to glutamate and the baroreceptor reflex elicited by neuropeptide Y injected into the nucleus tractus solitarius of the rat. *Neurosci. Lett.* 1991; **122**: 135-139.

43. Grundemar L, Wahlestedt C, Reis DJ. Neuropeptide Y acts at an atypical receptor to evoke cardiovascular depression and to inhibit glutamate responsiveness in the brainstem. *J. Pharmacol. Exp. Ther.* 1991; **258**: 633-638.

44. Lee CC, Miller RJ. Is there really an NPY Y3 receptor? *Regul. Pept.* 1998; **75-76**: 71-78.

45. Schwartz TW. Pancreatic polypeptide: a hormone under vagal control. *Gastroenterology* 1983; **85**: 1411-1425.

46. Gehlert DR, Schober DA, Beavers L, Gadski R, Hoffman JA, Smiley DL, Chance RE, Lundell I, Larhammar D. Characterization of the peptide binding requirements for the cloned human pancreatic polypeptide-preferring receptor. *Mol. Pharmacol.* 1996; **50**: 112-118.

47. Bard JA, Walker MW, Branchek TA, Weinshank RL. Cloning and functional expression of a human Y4 subtype receptor for pancreatic polypeptide, neuropeptide Y, and peptide YY. *J. Biol. Chem.* 1995; **270**: 26762-26765.

48. Hu Y, Bloomquist BT, Cornfield LJ, DeCarr LB, Flores-Riveros JR, Friedman L, Jiang P, Lewis-Higgins L, Sadlowski Y, Schaefer J, Velazquez N, McCaleb ML. Identification of a novel hypothalamic neuropeptide Y receptor associated with feeding behavior. *J. Biol. Chem.* 1996; **271**: 26315-26319.

49. Weinberg DH, Sirinathsinghji DJ, Tan CP, Shiao LL, Morin N, Rigby MR, Heavens RH, Rapoport DR, Bayne ML, Cascieri MA, Strader CD, Linemeyer DL, MacNeil

DJ. Cloning and expression of a novel neuropeptide Y receptor. *J. Biol. Chem.* 1996; **271**: 16435-16438.

50. Gregor P, Feng Y, DeCarr LB, Cornfield LJ, McCaleb ML. Molecular characterization of a second mouse pancreatic polypeptide receptor and its inactivated human homologue. *J. Biol. Chem.* 1996; **271**: 27776-27781.

51. Beck-Sickinger AG, Wieland HA, Wittneben H, Willim KD, Rudolf K, Jung G. Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur. J. Biochem.* 1994; **225**: 947-958.

52. Kirby DA, Boublik JH, Rivier JE. Neuropeptide Y: Y1 and Y2 affinities of the complete series of analogues with single D-residue substitutions. *J. Med. Chem.* 1993; **36**: 3802-3808.

53. Beck-Sickinger AG in: *Methods in molecular biology, neuropeptide protocols*. Irvine GB, Williams CH, Ed., pp. 61-73, Humana Press, Totowa, NJ, 1997.

54. Liao S, Alfaro-Lopez J, Shenderovich MD, Hosohata K, Lin J, Li X, Stropova D, Davis P, Jernigan KA, Porreca F, Yamamura HI, Hruby VJ. De novo design, synthesis, and biological activities of high-affinity and selective non-peptide agonists of the delta-opioid receptor. *J. Med. Chem.* 1998; **41**: 4767-4776.

55. Walker P, Munoz M, Martinez R, Peitsch MC. Acidic residues in extracellular loops of the human Y1 neuropeptide Y receptor are essential for ligand binding. *J. Biol. Chem.* 1994; **269**: 2863-2869.

56. Schwartz TW. Locating ligand-binding sites in 7TM receptors by protein engineering. *Curr. Opin. Biotechnol.* 1994; **5**: 434-444.

57. Sautel M, Rudolf K, Wittneben H, Herzog H, Martinez R, Munoz M, Eberlein W, Engel W, Walker P, Beck-Sickinger AG. Neuropeptide Y and the nonpeptide antagonist BIBP 3226 share an overlapping binding site at the human Y1 receptor. *Mol. Pharmacol.* 1996; **50**: 285-292.

58. Wieland HA, Eckard CP, Doods HN, Beck-Sickinger AG. Probing of the neuropeptide Y-Y1-receptors interaction with anti- receptor antibodies. *Eur. J. Biochem.* 1998; **255**: 595-603.

59. Eckard CP, Cabrele C, Wieland HA, Beck-Sickinger AG. Manuscript submitted.

60. Beck-Sickinger AG, Jung G. Structure-activity relationships of neuropeptide Y analogues with respect to Y1 and Y2 receptors. *Biopolymers* 1995; **37**: 123-142.

61. Beck-Sickinger AG. Unpublished data.

62. Cabrele C, Langer M, Bader R, Wieland HA, Doods HN, Zerbe O, Beck-Sickinger AG. Mnuscript submitted.

63. Fuhlendorff J, Gether U, Aakerlund L, Langeland-Johansen N, Thogersen H, Melberg SG, Olsen UB, Thastrup O, Schwartz TW. [Leu31, Pro34]neuropeptide Y: a specific Y1 receptor agonist. *Proc. Natl. Acad. Sci. U S A* 1990; **87**: 182-186.

64. Cabrele C, Wieland HA, Langer M, Stidsen C, Beck-Sickinger AG. Manuscript submitted.

65. Hoffmann S, Rist B, Videnov G, Jung G, Beck-Sickinger AG. Structure-affinity studies of C-terminally modified analogs of neuropeptide Y led to a novel class of peptidic Y1 receptor antagonist. *Regul. Pept.* 1996; **65**: 61-70.

66. Beck A, Jung G, Gaida W, Koppen H, Lang R, Schnorrenberg G. Highly potent and small neuropeptide Y agonist obtained by linking NPY 1-4 via spacer to alpha-helical NPY 25-36. *FEBS Lett.* 1989; **244**: 119-122.

67. Rist B, Wieland HA, Willim KD, Beck-Sickinger AG. A rational approach for the development of reduced-size analogues of neuropeptide Y with high affinity to the Y1 receptor. *J. Pept. Sci.* 1995; **1**: 341-348.

68. Kirby DA, Koerber SC, Craig AG, Feinstein RD, Delmas L, Brown MR, Rivier JE. Defining structural requirements for neuropeptide Y receptors using truncated and conformationally restricted analogues. *J. Med. Chem.* 1993; **36**: 385-393.

69. Leban JJ, Heyer D, Landavazo A, Matthews J, Aulabaugh A, Daniels AJ. Novel modified carboxy terminal fragments of neuropeptide Y with high affinity for Y2-type receptors and potent functional antagonism at a Y1-type receptor. *J. Med. Chem.* 1995; **38**: 1150-1157.

70. Daniels AJ, Matthews JE, Slepetis RJ, Jansen M, Viveros OH, Tadepalli A, Harrington W, Heyer D, Landavazo A, Leban JJ, et al. High-affinity neuropeptide Y receptor antagonists. *Proc. Natl. Acad. Sci. USA* 1995; **92**: 9067-9071.

71. Parker EM, Babij CK, Balasubramaniam A, Burrier RE, Guzzi M, Hamud F, Mukhopadhyay G, Rudinski MS, Tao Z, Tice M, Xia L, Mullins DE, Salisbury BG. GR231118 (1229U91) and other analogues of the C-terminus of neuropeptide Y are potent neuropeptide Y Y1 receptor antagonists and neuropeptide Y Y4 receptor agonists. *Eur. J. Pharmacol.* 1998; **349**: 97-105.

72. Kanatani A, Ito J, Ishihara A, Iwaasa H, Fukuroda T, Fukami T, MacNeil DJ, Van der Ploeg LH, Ihara M. NPY-induced feeding involves the action of a Y1-like receptor in rodents. *Regul. Pept.* 1998; **75-76**: 409-415.

73. Schober DA, Van Abbema AM, Smiley DL, Bruns RF, Gehlert DR. The neuropeptide Y Y1 antagonist, 1229U91, a potent agonist for the human pancreatic polypeptide-preferring (NPY Y4) receptor. *Peptides* 1998; **19**: 537-542.

74. Rudolf K, Eberlein W, Engel W, Wieland HA, Willim KD, Entzeroth M, Wienen W, Beck-Sickinger AG, Doods HN. The first highly potent and selective non-peptide neuropeptide Y Y1 receptor antagonist: BIBP3226. *Eur. J. Pharmacol.* 1994; **271**: R11-13.

75. Serradeil-Le Gal C, Valette G, Rouby PE, Pellet A, Oury-Donat F, Brossard G, Lespy L, Marty E, Neliat G, de Cointet P, et al. SR 120819A, an orally-active and selective neuropeptide Y Y1 receptor antagonist. *FEBS Lett.* 1995; **362**: 192-196.

76. Wieland HA, Engel W, Eberlein W, Rudolf K, Doods HN. Subtype selectivity of the novel nonpeptide neuropeptide Y Y1 receptor antagonist BIBO 3304 and its effect on feeding in rodents. *Br. J. Pharmacol.* 1998; **125**: 549-555.

77. Hipskind PA, *et al.* Potent and selective 1,2,3-trisubstituted indole NPY Y-1 antagonists. *J. Med. Chem.* 1997; **40**: 3712-3714.

78. Du P, *et al.* Modeling the G-protein-coupled neuropeptide Y Y1 receptor agonist and antagonist binding sites. *Protein Eng.* 1997; **10**: 109-117.

79. Gether U, Yokota Y, Emonds-Alt X, Breliere JC, Lowe JAd, Snider RM, Nakanishi S, Schwartz TW. Two nonpeptide tachykinin antagonists act through epitopes on corresponding segments of the NK1 and NK2 receptors. *Proc. Natl. Acad. Sci. USA* 1993; **90**: 6194-6198.

80. Fong TM, Cascieri MA, Yu H, Bansal A, Swain C, Strader CD. Amino-aromatic interaction between histidine 197 of the neurokinin-1 receptor and CP 96345. *Nature* 1993; **362**: 350-353.

81. Schambye HT, Hjorth SA, Bergsma DJ, Sathe G, Schwartz TW. Differentiation between binding sites for angiotensin II and nonpeptide antagonists on the angiotensin II type 1 receptors. *Proc. Natl. Acad. Sci. USA* 1994; **91**: 7046-7050.

82. Mantamadiotis T, Baldwin GS. The seventh transmembrane domain of gastrin/CCK receptors contributes to non-peptide antagonist binding. *Biochem. Biophys. Res. Commun.* 1994; **201**: 1382-1389.

83. Wang JB, Johnson PS, Wu JM, Wang WF, Uhl GR. Human kappa opiate receptor second extracellular loop elevates dynorphin's affinity for human mu/kappa chimeras. *J. Biol. Chem.* 1994; **269**: 25966-25969.

84. Lee JA, Brinkmann JA, Longton ED, Peishoff CE, Lago MA, Leber JD, Cousins RD, Gao A, Stadel JM, Kumar CS, et al. Lysine 182 of endothelin B receptor modulates agonist selectivity and antagonist affinity: evidence for the overlap of peptide and non-peptide ligand binding sites. *Biochemistry* 1994; **33**: 14543-14549.

85. Aiglstorfer I, Uffrecht A, Gessele K, Moser C, Schuster A, Merz S, Malawska B, Bernhardt G, Dove S, Buschauer A. NPY Y1 antagonists: structure-activity relationships of arginine derivatives and hybrid compounds with arpromidine-like partial structures. *Regul. Pept.* 1998; **75-76**: 9-21.

86. Rudolf K, Eberlein W, Engel W, Beck-Sickinger AG, Wittneben H, Wieland HA, Doods HN in: *Neuropeptide Y and drug development.* Grundemar L, Bloom SR, Ed., pp. 175-190, Academic Press, 1997.

87. Müller M, Knieps S, Gessele K, Dove S, Bernhardt G, Buschauer A. Synthesis and neuropeptide Y Y1 receptor antagonists of N,N-disubstituted omega-amino and

omega-guanidinoalkanamides. *Arch. Pharm. Pharm. Med. Chem.* 1997; **330**: 333-342.

88. Zarrinmayeh H, Nunes AM, Ornstein PL, Zimmerman DM, Arnold MB, Schober DA, Gackenheimer SL, Bruns RF, Hipskind PA, Britton TC, Cantrell BE, Gehlert DR. Synthesis and evaluation of a series of novel 2-[(4-chlorophenoxy)methyl]benzimidazoles as selective neuropeptide Y Y1 receptor antagonists. *J. Med. Chem.* 1998; **41**: 2709-2719.

89. Eckard CP, Beck-Sickinger AG, Wieland HA. Comparison of antibodies directed against receptor segments of NPY-receptors. *J. Recept. Signal Transduct. Res.* 1999; **19**: 379-394.

90. Hoffmann H, Grouzmann E, Beck-Sickinger AG, Jung G in: *Peptides 1992.* Schneider CH, Eberle AN, Ed., pp. 589-590, Escom, Leiden, 1992.

91. Beck-Sickinger AG, Hoffmann E, Gaida W, Grouzmann E, Dürr H, Jung G. Novel Y2-selective, reduced-size agonists of neuropeptide Y. *Bioorg. Med. Chem. Lett.* 1993; **3**: 937-942.

92. Daniels AJ, Heyer D, Spaltenstein A in: *Neuropeptide Y and drug development.* Grundemar L, Bloom SR, Ed., pp. 127-155, Academic Press, 1997.

93. Rist B, Ingenhoven N, Scapozza L, Schnorrenberg G, Gaida W, Wieland HA, Beck-Sickinger AG. The bioactive conformation of neuropeptide Y analogues at the human Y2- receptor. *Eur. J. Biochem.* 1997; **247**: 1019-1028.

94. Rist B, Zerbe O, Ingenhoven N, Scapozza L, Peers C, Vaughan PF, McDonald RL, Wieland HA, Beck-Sickinger AG. Modified, cyclic dodecapeptide analog of neuropeptide Y is the smallest full agonist at the human Y2 receptor. *FEBS Lett.* 1996; **394**: 169-173.

95. Tuchscherer G, Mutter M. Protein design as a challenge for peptide chemists. *J. Pept. Sci.* 1995; **1**: 3-10.

96. Malis DD, Grouzmann E, Morel DR, Mutter M, Lacroix JS. Influence of TASP-V, a novel neuropeptide Y (NPY) Y2 agonist, on nasal and bronchial responses evoked by

histamine in anaesthetized pigs and in humans. *Br. J. Pharmacol.* 1999; **126**: 989-996.

97. Grouzmann E, Buclin T, Martire M, Cannizzaro C, Dorner B, Razaname A, Mutter M. Characterization of a selective antagonist of neuropeptide Y at the Y2 receptor. Synthesis and pharmacological evaluation of a Y2 antagonist [published erratum appears in *J. Biol. Chem.* 1998; **273**: 27033]. *J. Biol. Chem.* 1997; **272**: 7699-7706.

98. Cox HM, Tough IR, Ingenhoven N, Beck-Sickinger AG. Structure-activity relationships with neuropeptide Y analogues: a comparison of human Y1-, Y2- and rat Y2-like systems. *Regul. Pept.* 1998; **75-76**: 3-8.

99. Fuhlendorff J, Johansen NL, Melberg SG, Thogersen H, Schwartz TW. The antiparallel pancreatic polypeptide fold in the binding of neuropeptide Y to Y1 and Y2 receptors. *J. Biol. Chem.* 1990; **265**: 11706-11712.

100. Beck-Sickinger AG, Hoffmann E, Paulini K, Reissig HU, Willim KD, Wieland HA, Jung G. High-affinity analogues of neuropeptide Y containing conformationally restricted non-proteinogenic amino acids. *Biochem. Soc. Trans.* 1994; **22**: 145-149.

101. Beck-Sickinger AG, Grouzmann E, Hoffmann E, Gaida W, van Meir EG, Waeber B, Jung G. A novel cyclic analog of neuropeptide Y specific for the Y2 receptor. *Eur. J. Biochem.* 1992; **206**: 957-964.

102. Doods HN, Gaida W, Wieland HA, Dollinger H, Schnorrenberg H, Esser F, Engel W, Eberlein W, Rudolf K. Manuscript submitted.

103. McCullough LA, Westfall TC. Neuropeptide Y inhibits depolarization-stimulated catecholamine synthesis in rat pheochromocytoma cells. *Eur. J. Pharmacol.* 1995; **287**: 271-277.

104. Glaum SR, Miller RJ, Rhim H, Maclean D, Georgic LM, MacKenzie RG, Grundemar L. Characterization of Y3 receptor-mediated synaptic inhibition by chimeric neuropeptide Y-peptide YY peptides in the rat brainstem. *Br. J. Pharmacol.* 1997; **120**: 481-487.

105. Hirabayashi A, Nishiwaki K, Shimada Y, Ishikawa N. Role of neuropeptide Y and its receptor subtypes in neurogenic pulmonary edema. *Eur. J. Pharmacol.* 1996; **296**: 297-305.

106. Criscione L, *et al.* Food intake in free-feeding and energy-deprived lean rats is mediated by the neuropeptide Y5 receptor. *J. Clin. Invest.* 1998; **102**: 2136-2145.

107. Kushi A, Sasai H, Koizumi H, Takeda N, Yokoyama M, Nakamura M. Obesity and mild hyperinsulinemia found in neuropeptide Y-Y1 receptor-deficient mice. *Proc. Natl. Acad. Sci. USA* 1998; **95**: 15659-15664.

108. Tang-Christensen M, Kristensen P, Stidsen CE, Brand CL, Larsen PJ. Central administration of Y5 receptor antisense decreases spontaneous food intake and attenuates feeding in response to exogenous neuropeptide Y. *J. Endocrinol.* 1998; **159**: 307-312.

109. Haynes AC, Arch JR, Wilson S, McClue S, Buckingham RE. Characterisation of the neuropeptide Y receptor that mediates feeding in the rat: a role for the Y5 receptor? *Regul. Pept.* 1998; **75-76**: 355-361.

110. Kask A, Rago L, Harro J. Evidence for involvement of neuropeptide Y receptors in the regulation of food intake: studies with Y1-selective antagonist BIBP3226. *Br. J. Pharmacol.* 1998; **124**: 1507-1515.

111. Marsh DJ, Hollopeter G, Kafer KE, Palmiter RD. Role of the Y5 neuropeptide Y receptor in feeding and obesity. *Nat. Med.* 1998; **4**: 718-721.

112. Cabrele C, Wieland HA, Stidsen C, Beck-Sickinger AG. Manuscript submitted.

113. Cabrele C, Beck-Sickinger AG. Manuscript in preparation.

114. Bischoff A, Michel MC. Emerging functions for neuropeptide Y5 receptors. *Trends Pharmacol. Sci.* 1999; **20**: 104-106.

CHAPTER 2

Characterization of Neuropeptide Y Receptor Subtypes by Synthetic NPY Analogues and by Anti-Receptor Antibodies

Christophe P. Eckard[1], Chiara Cabrele[1], Heike A. Wieland[2], and Annette G. Beck-Sickinger[1]

[1]Department of Pharmacy, Federal Institute of Technology, Zurich, Switzerland
[2]Division of Preclinical Research, Boehringer Ingelheim Pharma KG, Biberach, Germany

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2.1 Abstract

Neuropeptide Y (NPY), a 36-mer neuromodulator, binds to the receptors Y_1, Y_2, Y_4 and Y_5 with nanomolar affinity. They all belong to the rhodopsin-like G-protein coupled, seven transmembrane helix spanning receptors. In this study, Ala-substituted and centrally truncated NPY analogues were compared with respect to affinity to the Y-receptors. Furthermore, antibodies against the second (E2) and the third (E3) extracellular loop of NPY Y_1-, Y_2- and Y_5-receptor subtypes were raised, and affinity to intact cells was tested by immunofluorescence assays. Both methods were applied in order to obtain subtype selective tools and to characterize ligand binding. The analogues [A^{13}]-pNPY and [A^{27}]-pNPY showed subtype selectivity for the Y_2-receptor. Sera against the E2 loop of the Y_1-receptor and against the E2 loop of the Y_2-receptor were subtype selective. Two antibodies against the Y_5 E2 and E3 loop recognized the Y_5- and Y_2-receptor subtypes. In combination, these sera are able to distinguish between the Y_1-, Y_2-, and Y_5-receptor subtypes. The analogues and antibodies represent valuable tools to distinguish NPY receptors on membranes and intact cells.

Keywords: neuropeptide Y; NPY analogues; anti-receptor antibodies; NPY receptor; subtype selectivity; ligand-binding site.

2.2 Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide hormone that belongs to the pancreatic polypeptide hormone family [1]. It is widely distributed both peripherally and centrally. Similar to many other neurotransmitters, NPY elicits diverse physiological effects. One of the most striking actions of NPY is the stimulation of food intake in a variety of vertebrate species [2]. But also various other biological effects have been attributed to NPY, e.g., profound effects on secretion of luteinizing hormone as well as on growth hormone and insulin release [3-5]. These observations suggest the important role of NPY in the pathophysiology of obesity and diabetes. Furthermore, it has been reported that NPY elicits potent vasoconstriction [6], facilitates learning and memory retention processes [7], modulates locomotor behaviors [8], produces hypothermia [9, 10], inhibits sexual behavior [11], shifts the circadian rhythms [12], modulates cardiorespiratory parameters [13], generates anxiolytic effects [14], and inhibits alcohol consumption and resistance [15]. Accordingly, NPY is a neuronal and endocrine messenger that exerts its effects via several receptor subtypes. Five distinct NPY receptor subtypes have been cloned and pharmacologically characterized [16]. They are named Y_1- [17-19], Y_2- [20-22], Y_4/PP_1- [23, 24], Y_5- [25] and y_6- [26] receptor subtypes. All subtypes belong to the large superfamily of the G-protein-coupled, heptahelical receptors [27]. The different receptor subtypes are localized in various tissues, in the central nervous system as well as in the periphery. Tissues with high density of NPY receptors are blood vessels, kidney, adrenal glands, colon, heart, pancreas, intestine, nerve endings and brain [2]. The broad physiological relevance of NPY gives reason for an increasing interest in NPY as a new target in drug discovery.

It is not yet fully understood which NPY receptor subtype is related to a certain physiological effect. Available evidence suggests that the Y_1- or Y_5-receptor or even both receptors together regulate food intake [2]. Furthermore, the receptor subtypes are distributed heterogeneously in various tissues. Therefore, receptor subtype selective tools are very important for understanding the biological role and distribution of the receptors and can provide important knowledge for drug design.

Each receptor subtype recognizes specific parts of the hormone folded in an unique conformation. Thus, different receptor subtypes can be characterized by selectively increasing the affinity/activity of peptide analogues in cells or tissue [28]. Generally, full

length analogues are obtained by D-amino acid- or L-alanine-scan or by further amino acid replacements [29]. Frequently, the native ligands are shortened by deletion of the C- or N-terminal parts, or by central truncation [30]. Moreover, analogues with agonistic or antagonistic activity can be a powerful tool to investigate the biological function of a receptor subtype.

Antibodies produced against hormone receptors are valuable tools to investigate the receptor localization on cells or tissue, regulation and desensitization [31-37]. Anti-receptor antisera can be raised by immunization with purified, enriched receptors [33] or by immunization with receptor fragments [32-35]. Antibodies specifically recognizing the NPY receptor subtypes can be used to determine either the localization pattern and the quality of the receptor protein expression or the topology and function of a receptor subtype [38].

In this study we describe synthetic NPY analogues tested in binding assays against the Y-receptors and we compare the binding of antibodies raised against the second and the third extracellular loop of NPY Y_1-, Y_2- and Y_5-receptor subtypes to intact cells in immunofluorescence assays. Both methods are applied in order to get subtype selectivity and to obtain tools to localize the different receptor subtypes.

2.3 Materials and Methods

2.3.1 Synthesis of the NPY Analogues and of the Y-Receptor Segments

The NPY analogues and the peptides for immunization were synthesized by automated multiple solid phase peptide synthesis using a robot system (Syro, MultiSynTech, Bochum, Germany). In order to obtain the C-terminally amidated peptides, the chain assembly was performed on a 0.015 mmol scale using 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Novabiochem, Läufelfingen, Switzerland) and Fmoc/*tert*-butyl chemistry. The side chain protection of the Fmoc-amino acids (Alexis, Läufelfingen, Switzerland) was chosen as follows: Trt for Asn, Gln and His, *tert*-butyl for Asp, Glu, Ser, Thr and Tyr, Boc for Lys and Trp, and 2,2,5,7,8-pentamethylchroman-6-sulfonyl for Arg. A double coupling procedure was performed with diisopropylcarbodiimide/1-hydroxy-benzotriazole (Fluka,

Buchs, Switzerland) activation, seven-fold excess and a coupling time of 40 min. Fmoc removal was accomplished by 30% piperidine (Fluka) in DMF for 15 min. The peptide amides were simultaneously cleaved from the resin and deprotected with TFA/thioanisole/thiocresol (Fluka) for 2.5 hours, and recovered by precipitation from diethyl ether.

The peptides were purified by preparative HPLC and characterized by analytical HPLC on nucleosil C-18 column, 5 µm, 3 x 125 mm (Merck-Hitachi, Darmstadt, Germany). The elution system was 0.1% TFA in acetonitrile (Scharlau, Barcelona, Spain) (A) and 0.1% TFA in water (B); a gradient of 15-60 % A or 25-75 % A over 30 min was used. Correct mass was identified by electrospray ionization mass spectrometry (SSQ710, Finnigan, San Jose, CA). The sequences and the chemical characterization of the peptides are summarized in Tables 1 and 2.

2.3.2 Preparation of the Conjugates and Immunization

For the immunization the peptides were coupled to keyhole limpet hemocyanin (KLH) (Biotrend, Cologne, Germany). Polyclonal antibodies against the KLH-coupled receptor fragments were obtained by immunization of rabbits (Biotrend, Cologne, Germany).

2.3.3 Titer Determination of the Receptor Segment Peptides

In order to determine the titer of the antibodies raised against the peptide-KLH conjugates, the peptides were covalently coupled to preactivated ELISA plates (Life Technologies, Basel, Switzerland). The antibody solutions were diluted and incubated at 37 °C for 90 min. The plates were washed four times and the secondary antibody was incubated for 1 hour. After washing the plates four times, staining was carried out with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and the absorption was subsequently measured at 405 nm in an ELISA-reader.

2.3.4 Cell Culture

SK-N-MC cells (neuroblastoma, hY_1) were cultivated in MEM Earl's salt (Life Technologies, Basel, Switzerland) supplemented with 10% (v/v) fetal calf serum (AMIMED, Allschwil, Switzerland), 4 mM L-glutamine, 1% (v/v) non-essential amino

acids (Life Technologies) and 1 mM sodium pyruvate (Life Technologies). SMS-KAN cells (neuroblastoma, hY_2) were cultivated in Nut-mix HAM's F-12/DMEM 1:1 (Life Technologies) supplemented with 15% fetal calf serum, 1% (v/v) L-glutamine and 1% (v/v) non-essential amino acids. BHK cells (baby hamster kidney cells, transfected with hY_4, rY_5 and hY_5) were cultivated in DMEM (Life Technologies) supplemented with 10% (v/v) fetal calf serum, 1% (v/v) antibiotic-fungicide solution (penicillin 10000 U/ml, streptomycin 10 mg/ml and fungizone 25 µg/ml; AMIMED) and 1 mg/ml geneticin (G418; SIGMA, Buchs, Switzerland). Additionally, 1 mg/ml Hygromicin and 24 hours before harvest 1 mM IPTG were added to the rY_4 expressing BHK cells. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air in 75 cm^2 flasks (Techno Plastic-Products AG, Trasadingen, Switzerland). They were harvested at 100% confluency in phosphate buffered saline (PBS; Life Technologies) containing 0.02% EDTA.

2.3.5 Binding Potency of NPY Analogues

Membrane suspensions obtained from SK-N-MC (hY_1), SMS-KAN (hY_2) and BHK (r/hY_4, r/hY_5) cells (100 µl) were prepared as previously described [28, 30], and incubated with 30 pM ^{125}I-Bolton-Hunter-NPY (^{125}I-BH-NPY) or 1.2 nM [^{3}H]-propionyl-NPY ([^{3}H]-NPY) and different concentrations of the peptide in a total volume of 250 µl for 2 hours at room temperature as recently described [28, 39, 40]. The protein-bound radioactivity was determined in a γ-counter. The nonspecific binding is defined as the radioactivity bound in the presence of 1 µM NPY during the incubation period. Half-maximal inhibition of the specific binding of ^{125}I-BH-NPY or [^{3}H]-NPY of two to three separate experiments, each performed in triplicate, is given as the IC_{50} value.

2.3.6 Immunofluorescence

Affinity of the anti-receptor antibodies was investigated at Y_1-receptor expressing SK-N-MC cells (neuroblastoma, hY_1), SMS-KAN cells (neuroblastoma, hY_2) and transfected BHK cells (baby hamster kidney cells, rY_5). About 500000 cells per well were added to 12 well tissue culture test plates (Techno Plastic-Products) and grown to confluency. Antisera were diluted 1:50 in the corresponding cell culture media, 1.5 ml antisera solution was

added per well. Tissue culture test plates were incubated for 90 min at 37 °C and washed twice with media. Goat anti-rabbit 4(5)-fluoresceinisothiocyanate (FITC) conjugated IgG (SIGMA, Buchs, Switzerland) was diluted 1:80 in media, 1 ml antisera solution was added per well. Tissue culture test plates were incubated for 1 hour at 37 °C and washed twice with media. The cells were viewed in a fluorescence microscope (Zeiss, D-Oberkochen; Axiovert 35, Filters: excitation: BP 450–490 nm, beamsplitter FT 510, emission LP 520). As positive control, one lane of each plate was directly incubated with goat anti-rabbit FITC coupled IgG. For the negative control, anti-receptor antisera were preincubated for 1 hour with the corresponding antigen peptide at the concentrations of 10^{-5} M or 10^{-4} M. Incubation of the antibody solutions as well as detection was performed as described above.

2.4 Results

2.4.1 Synthesis of the Analogues and Receptor Segments

All peptides were designed as amino acid mono-substituted full length or centrally truncated analogues of pNPY and hPP and were obtained by multiple automatic solid phase peptide synthesis using the Fmoc strategy. The size of the receptor segment peptides ranged between 19 and 20 amino acids, in order to increase the probability that the peptides fold into an ordered structure. The peptides were characterized by analytical HPLC and electrospray ionization mass spectrometry.

2.4.2 Characterization of the Sera

The total absorption (405 nm) of the antibodies tested against the peptides ranged from 0.15 to 0.45. The values of preimmune sera ranged from 0.10 to 0.36. They were subtracted from the total absorption to obtain specific binding. The blank values were under 0.05. All antisera showed specific binding.

Table 1. Sequence and analytical data of the peptides that were used to generate anti-receptor antibodies. The peptides are named according to the receptor and the loop from which they were derived.

Name	Sequence	M.W.$_{calc}$ [Da]	M.W.$_{exp}$ [Da]	Receptor segment
Y_1 E2/2	QILTDEPFQNVSLAAFKDK	2163.5	2165	76-94 (rat)
Y_2 E2/1	IFREYSLIEIIPDFEIVAF	2313.7	2314	75-93[a] (human)
Y_5 E2/2	LLSSRYLCVESWPSDSYRIAF	2491.9	2491	186-206 (human)
Y_5 E3	HVVTDFNDNLISNRHFKLV	2267.6	2268	278-296 (human)

[a] At position 93 the natural amino acid Cys was substituted by Phe.

2.4.3 Binding Potency of Full Length NPY Analogues

The affinity to the receptors Y_1 and Y_2 of all 36 Ala-substituted analogues of NPY has previously been reported [40]. In the present work, we have considered sixteen selected Ala-substituted NPY analogues and investigated their binding affinity at the human receptors Y_4 and Y_5. Y_1- and Y_2-receptor binding data are listed for comparison (Table 2). The substitution of Pro at positions 2, 5, 8 and 13 led in general to a decreased affinity. In particular, the substitution of the residues 2 and 5 reduced the hY_1-receptor affinity about 500- and 1000-fold, respectively; the substitution of Pro^5 decreased the hY_2-receptor affinity 600-fold, and the replacement of Pro^8 led to a 11- and 69-fold lower affinity at the hY_4- and hY_5-receptor subtypes, respectively. By single exchange of the aromatic side chains of the Tyr residues, positions 27 and 36 turned out to be the most important for the binding to the hY_1-receptor, with a loss of affinity of 1000- and 4200-fold after substitution. The presence of Ala^{20} reduced the affinity of 300-fold at this receptor. The hY_2-receptor binding was drastically decreased (1200-fold) especially by the substitution of Tyr^{36}. The hY_4-receptor binding was affected in a minor extent by the Tyr/Ala replacement (up to 62-fold loss of affinity), while the hY_5-receptor binding was reduced > 400-fold by replacing Tyr^{27}. By single removal of Arg^{33} or Arg^{35}, we observed a complete loss of affinity for the hY_1-receptor (> 4000-fold); by exchange of Arg^{19} or Arg^{25} the binding was 1200- and 48-fold less potent, respectively. The hY_2-receptor binding was totally lost by substitution of Arg^{35} and was reduced > 1300-fold by Arg^{33} exchange. These two C-

terminal Arg residues proved to be very important also for the binding of the hY_4- and hY_5-receptor subtypes: especially Arg^{35} is required for affinity at the hY_5-receptor (> 1200-fold less affinity after replacement with Ala). Interestingly, [Ala^{19}]-pNPY was almost as potent as the native ligand at the hY_5-receptor and even slightly better at the hY_4-receptor. By removing the negative charge of Asp^{11}, we could obtain a slightly more potent ligand at the receptors hY_4 and hY_5, whereas the potency at the other two subtypes slightly decreased (35- and five-fold at the hY_1- and hY_2-receptor subtypes, respectively). The decrease in hydrophilicity at position 32 after substitution of Thr with Ala corresponded to a decrease in affinity, especially at the hY_1-receptor (> 3000-fold) and hY_2-receptor (> 1000-fold). The substitution of Gln^{34} was poorly tolerated at the hY_1- and hY_2-receptors, while it did not reduced the binding potency for the receptors hY_4 and hY_5. In particular, the analogue [Leu^{34}]-pNPY was 18-fold more potent than pNPY itself at the hY_4-receptor. Instead, the presence of D-Pro^{34} considerably reduced the affinity at all receptor subtypes, while it is known that L-Pro^{34} is perfectly tolerated at the receptors hY_1, hY_4 and hY_5, but not at the hY_2-receptor [25]. IC_{50} values of the full length NPY analogues are summarized in Table 2.

2.4.4 Binding Potency of Centrally Truncated Analogues of NPY and PP

The central segment of NPY was suggested to be important for the peptide conformation and to adopt a turn-like structure that induces the molecule to fold like a hairpin [41]. This was investigated by the preparation of NPY analogues, where the central region was removed and substituded with a spacer like 6-amino hexanoic acid (Ahx) [42]. By varying both the N- and C-terminal cutting positions, it was found that the analogue [Ahx^{5-24}]-pNPY selectively bound to the hY_2-receptor with an affinity of 1.4 nM (Table 2). Instead, analogues with longer N- and C-terminal fragments showed again higher affinities at the hY_1- (IC_{50} 13-74 nM), hY_4- (IC_{50} 45-108 nM) and hY_5- (IC_{50} 11-31 nM) receptors. Among these analogues, the one with the highest number of residues at the N- and C-termini, [Ahx^{9-17}]-pNPY, was the most potent ligand (13 nM at the hY_1, 45 nM at the hY_4 and 11 nM at the hY_5). Accordingly, the region 9-17 is not essential for receptor binding.

Table 2. Biological and analytical data of amino acid mono-substituted and centrally truncated NPY analogues. In brackets the affinity of the peptide divided by the affinity of pNPY is given.

Peptide	M.W.$_{calc}$ [Da]	M.W.$_{exp}$ [Da]	hY$_1$ IC_{50} [nM]	IC_{50} (pep)/IC_{50}(NPY)	hY$_2$ IC_{50} [nM]	IC_{50} (pep)/IC_{50}(NPY)	hY$_4$ IC_{50} [nM]	IC_{50} (pep)/IC_{50}(NPY)	hY$_5$ IC_{50} [nM]	IC_{50} (pep)/IC_{50}(NPY)
pNPY	4253.7	4253	0.23	(1)	0.04	(1)	5.5	(1)	0.8	(1)
[A^{1}]-pNPY	4161.7	4162	21	(91)	0.2	(5)	5.8	(1.1)	2.2	(2.8)
[A^{2}]-pNPY	4227.7	4224	114	(496)	0.3	(8)	7.8	(1.4)	5.5	(7)
[A^{5}]-pNPY	4227.7	4228	228	(991)	24	(600)	25	(4.5)	32	(40)
[A^{8}]-pNPY	4227.7	4225	32	(139)	0.7	(18)	60	(11)	55	(69)
[A^{11}]-pNPY	4209.7	4210	8.0	(35)	0.2	(5)	3.1	(0.6)	0.5	(0.6)
[A^{13}]-pNPY	4227.7	4226	7.5	(33)	0.1	(3)	37	(6.7)	17	(21)
[A^{19}]-pNPY	4168.6	4169	282	(1226)	1.6	(40)	4.1	(0.7)	1.4	(1.8)
[A^{20}]-pNPY	4161.7	4160	71	(309)	1.2	(30)	161	(29)	19	(24)
[A^{21}]-pNPY	4161.7	4160	5.5	(24)	0.2	(5)	66	(12)	32	(40)
[A^{25}]-pNPY	4168.7	4167	11	(48)	0.7	(18)	201	(37)	80	(100)
[A^{27}]-pNPY	4161.7	4159	250	(1087)	1.4	(35)	340	(62)	370	(463)
[A^{32}]-pNPY	4223.8	4221	723	(3143)	45	(1125)	380	(69)	7.7	(9.5)
[A^{33}]-pNPY	4168.7	4167	>1000	(>4348)	54	(1350)	>1000	(>182)	94	(118)
[A^{34}]-pNPY	4196.7	4195	94	(409)	6.0	(150)	7.4	(1.3)	1.3	(1.6)

Table 2. (Continued).

Peptide	M.W.$_{calc}$ [Da]	M.W.$_{exp}$ [Da]	hY$_1$ IC_{50} [nM]	IC_{50} (pep) / IC_{50}(NPY)	hY$_2$ IC_{50} [nM]	IC_{50} (pep) / IC_{50}(NPY)	hY$_4$ IC_{50} [nM]	IC_{50} (pep) / IC_{50}(NPY)	hY$_5$ IC_{50} [nM]	IC_{50} (pep) / IC_{50}(NPY)
[A^{35}]-pNPY	4168.7	4167	>1000	(>4348)	>1000	(>25000)	>1000	(>182)	>1000	(>1250)
[A^{35}]-pNPY	4168.7	4167	>1000	(>4348)	>1000	(>25000)	>1000	(>182)	>1000	(>1250)
[A^{36}]-pNPY	4161.7	4158	970	(4217)	48	(1200)	141	(26)	68	(85)
[L^{34}]-pNPY	4238.8	4238					0.3	(0.05)	1.8	(2.3)
[D-P^{34}]-pNPY	4222.8	4222	266	(1157)			271	(49)	156	(195)
[Ahx$^{5-24}$]-NPY	2220.6	2219	>1000	(>4348)	1.4	(35)	600	(109)	795	(994)
[Ahx$^{5-24,}$ P^{34}]-NPY	2189.6	2190	>1000	(>4348)			514	(93)	>10000	(>12500)
[Ahx$^{8-20}$]-NPY	2981.4	2980	28	(122)			67	(12)	31	(39)
[Ahx$^{8-19}$]-NPY	3144.6	3144	46	(200)			95	(17)	19	(24)
[Ahx$^{9-20}$]-NPY	3078.6	3078	74	(322)			108	(20)	29	(36)
[Ahx$^{9-17}$]-NPY	3469.0	3470	13	(57)			45	(8)	11	(14)
hPP	4181.8	4181	>1000	(>4348)	>1000	(>25000)	0.04	(0.007)	24	(30)
[Ahx$^{5-24}$]-hPP	2161.6	2162	>500	(>2174)	>1000	(>25000)	144	(26)	>1000	(>1250)
[Ahx$^{5-20}$]-hPP	2532.0	2531	>1500	(>6522)	>1000	(>25000)	216	(39)	>7000	(>8750)
[Y$^{5-20}$]-hPP	2582.0	2582	>500	(>2174)	>1000	(>25000)	27	(5)	>5000	(>6250)

Three centrally truncated analogues of hPP were also investigated for binding of the Y-receptors. The ligands containing the Ahx moiety in place of the central segments 5-24 and 5-20 did not show any affinity at the hY_1- and hY_5-receptor subtypes, and their affinity for the hY_4-receptor was considerably reduced. Interestingly, the analogue [Tyr^{5-20}]-hPP was found selectively to bind to the hY_4-receptor, however, with much lesser affinity (670-fold) in comparison with the full length peptide. IC_{50} values of the centrally truncated analogues of NPY and PP are summarized in Table 2.

2.4.5 Immunofluorescence on Cells Expressing Y-Receptor Subtypes

In order to determine the selectivity of the antibodies for the Y-receptors, immunofluorescence experiments were performed on Y-receptor expressing SK-N-MC cells (neuroblastoma, hY_1), SMS-KAN cells (neuroblastoma, hY_2) and BHK cells (baby hamster kidney cells, rY_5). For positive control, cells were directly incubated with goat anti-rabbit FITC coupled IgG. Negative control was made with preimmune sera and by preincubation of the anti-receptor antisera with the corresponding antigen peptide to block specific binding (Figure 1 B).

Antibody Y_1 E2/2 showed selective binding in the fluorescence microscope for the Y_1-receptor (Figure 2 A-C). Selective binding to the Y_2-receptor was detected for the antibody Y_2 E2/1 (Figure 2 D-F). Very strong binding was found for the antibodies Y_5 E2/2 and Y_5 E3 to the receptors Y_2 and Y_5 (Figure 3 A-F). Results of the fluorescence microscope assay are summarized in Table 3.

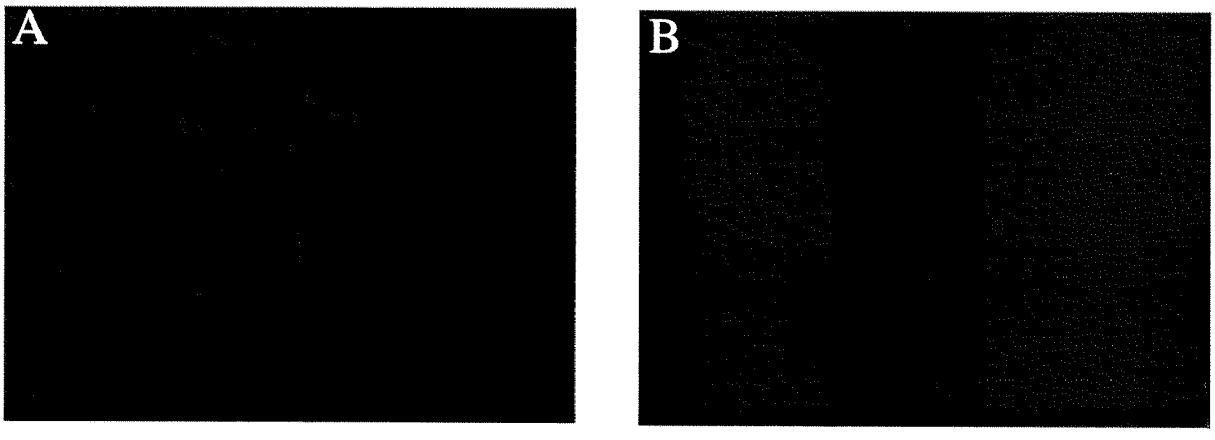

Figure 1. Binding of the antibody Y_5 E2/2 to BHK cells (Y_5). A) Normal conditions, B) blocking of the antibody Y_5 E2/2 with the antigen peptide at the concentration of 10^{-4} M.

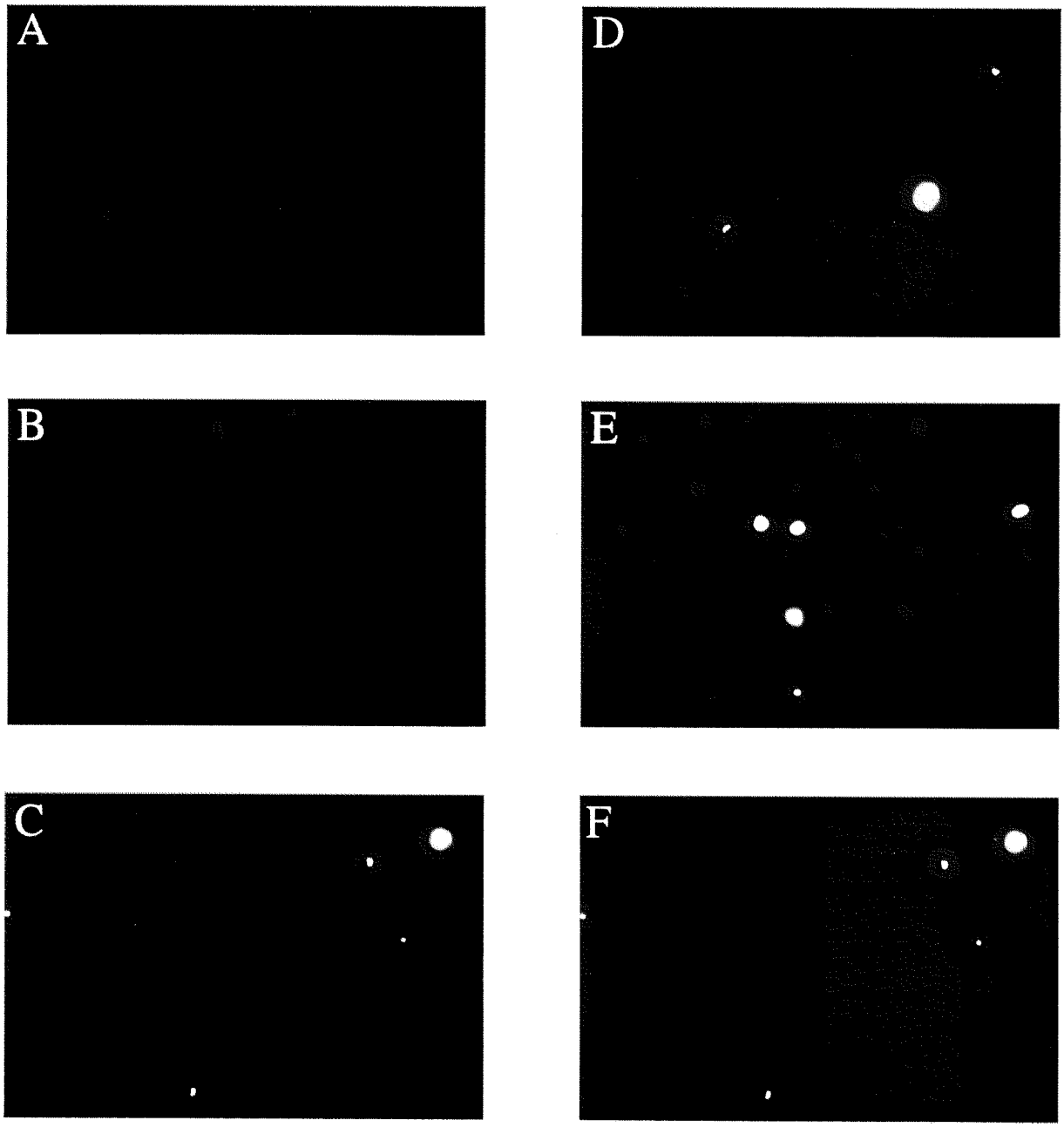

Figure 2. Binding of the antibody Y_1 E2/2 to: A) SK-N-MC cells (Y_1), B) SMS-KAN cells (Y_2), C) BHK cells (Y_5). Binding of the antibody Y_2 E2/1 to: D) SK-N-MC cells (Y_1), E) SMS-KAN cells (Y_2), F) BHK cells (Y_5).

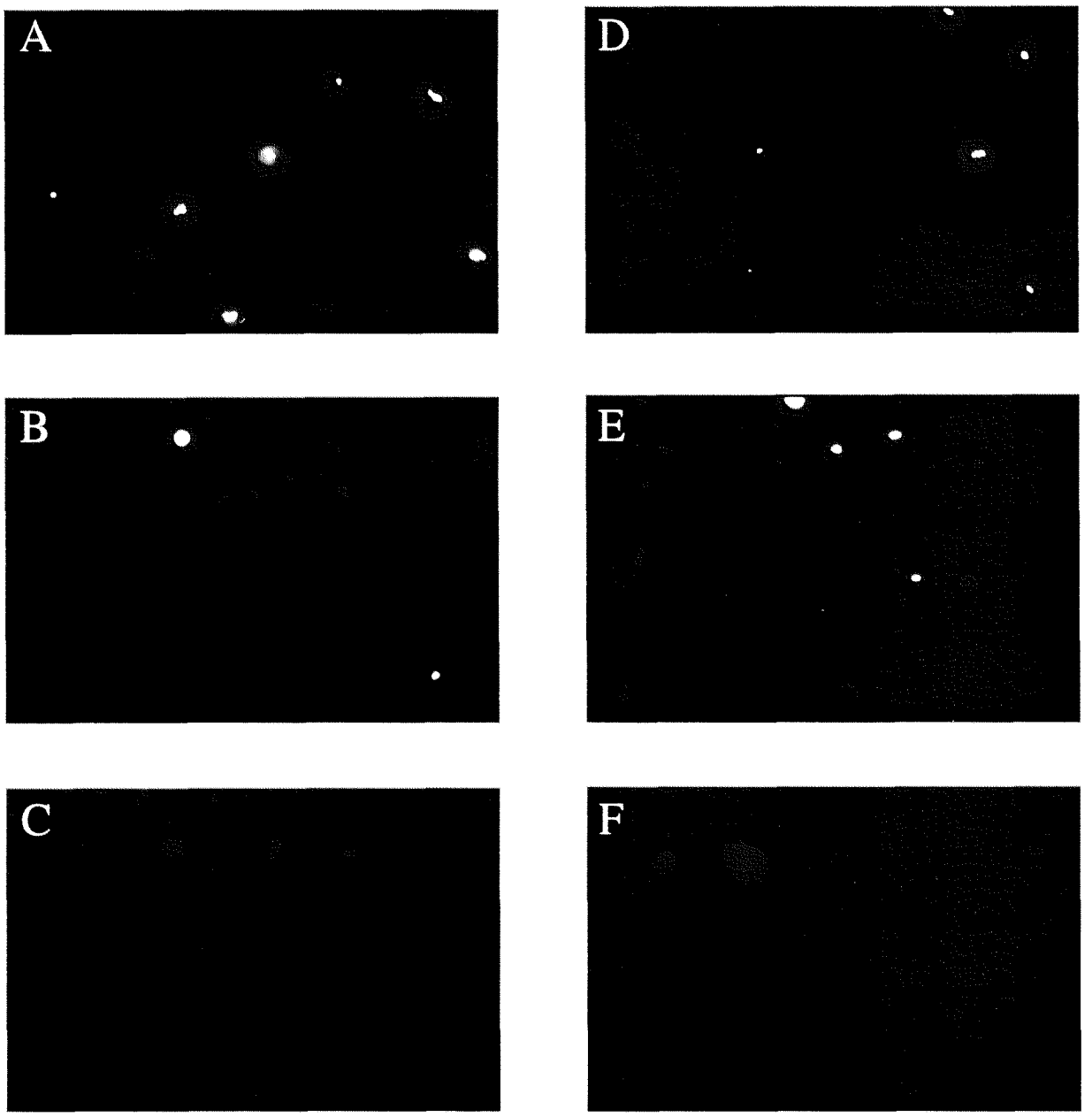

Figure 3. Binding of the antibody Y_5 E2/2 to: A) SK-N-MC cells (Y_1), B) SMS-KAN cells (Y_2), C) BHK cells (Y_5). Binding of the antibody Y_5 E3 to: D) SK-N-MC cells (Y_1), E) SMS-KAN cells (Y_2), F) BHK cells (Y_5).

Table 3. Fluorescence microscope detection of Y-receptors on SK-N-MC cells (neuroblastoma, hY_1), SMS-KAN cells (neuroblastoma, hY_2) and BHK cells (baby hamster kidney cells, rY_5).

– no fluorescence; + weak fluorescence; ++ moderate fluorescence; +++ strong fluorescence

Antibody	Y_1-Receptor SK-N-MC	Y_2-Receptor SMS-KAN	Y_5-Receptor BHK
Y_1 E2/2	+	–	–
Y_2 E2/1	–	++	–
Y_5 E2/2	–	+++	+++
Y_5 E3	–	+++	+++

2.5 Discussion

The major aim of this work was to compare analogues and anti-receptor antibodies with respect to selectivity and characterization of the ligand binding site. The NPY/PP system consists of two neuropeptides which bind to five distinct receptors: Y_1, Y_2, Y_5 and y_6 are NPY-preferring receptors, while Y_4 is a PP-preferring receptor. Accordingly, selectivity is a major problem. Furthermore, due to the large number of effects and the distribution of the receptors, it is of major importance to develop tools that allow the characterization of the receptors on protein level. Selective ligands, radiolabelled- or fluorescent-labelled-, and anti-receptor-antibodies are useful to address this issue. Furthermore, we were interested in the molecular characterization of the binding site. Again, ligands with specific replacements [43] and anti-receptor antibodies [44] have been used to map ligand binding. Accordingly, we used both approaches and applied them to the NPY/PP system: on the one hand, full length and centrally truncated NPY and PP analogues were synthesized, and their binding potency was tested at the NPY receptor subtypes. On the other hand, polyclonal antibodies raised against synthetic receptor fragments on the second (E2) and third (E3) extracellular loop of the receptor subtypes were tested on intact cells expressing the NPY receptors.

The receptor binding properties of the NPY and PP analogues presented in this work showed how the affinity of the native ligand at its receptor subtypes can be modulated by modifying its primary structure, which is likely to change, consequently, its spatial

conformation. It has been supposed that the members of the PP-family adopt a hairpin-like structure which is stabilized by the interdigitation of the tyrosine side chains on the surface of the C-terminal amphipatic α-helix with the proline residues of the N-terminal part. Accordingly, all NPY receptors have been shown to be sensitive to the positions of Pro and Tyr, however each one in a different way. For example, based on the Ala-scan results, Pro^{2}, Pro^{5}, Tyr^{27} and Tyr^{36} proved to be the most important positions for the hY_1-receptor, Pro^{5} and Tyr^{36} for the hY_2-receptor, Pro^{8} and Tyr^{27} for the hY_4- and hY_5-receptor. These differences might be explained by assuming that the proline rich N-terminal region and the C-terminal helix face each other in a way which depends on the structural features of the specific receptor binding site.

Also the Ala-substitution of the arginine residues led to different receptor affinities: Arg^{19} is important for the receptors hY_1 and hY_2, but not for the receptors hY_4 and hY_5, whereas the replacement of Arg^{25} was well tolerated only at the hY_2-receptor (IC_{50} 0.7 nM). Instead, Arg^{33} and Arg^{35} are essential for the binding of all four subtypes, however, it should be taken into account that the hY_5-receptor was more sentivive to the substitution of Tyr^{27} than to the one of Arg^{33}. Furthermore, Thr^{32} and Gln^{34} replacements did not significantly reduce the affinity at the hY_5-receptor, but led to a very low affinity at the other subtypes. It has well been documented that the C-terminal pentapeptide of NPY represents the binding site for the receptors Y_1 and Y_2 [29]. The results presented in this work have shown that this is also true for the receptors Y_4 and Y_5, however, with some differences. While the Ala-substitution at positions 32-36 led to a dramatic loss of affinity at the receptors hY_1 and hY_2, in the case of hY_5-receptor only the exchange of Arg^{35} led to a significant decrease in the binding potency. At the hY_4-receptor, both arginine residues were found to be crucial for high affinity. These observations suggest that the Y-receptor-ligand interaction is unique for each subtype and it requires a specific spatial conformation of the peptide. Accordingly, the amino acid substitution of natural ligands is extremely useful to have insight into the structural prerequisites that favor the ligand binding. NPY interacts with the receptors Y_1, Y_2 and Y_5 apparently in different ways involving different contact points (Figure 4). To gain more insight into the mechanism of interaction of the ligand with each receptor, selective compounds are required. Among the Ala-substituted full length NPY analogues, $[Ala^{8}]$-pNPY, $[Ala^{13}]$-pNPY and $[Ala^{27}]$-pNPY can be considered hY_2-receptor selective ligands; in particular, $[Ala^{27}]$-pNPY was at least 100-fold more selective for the hY_2-receptor rather than for the other subtypes.

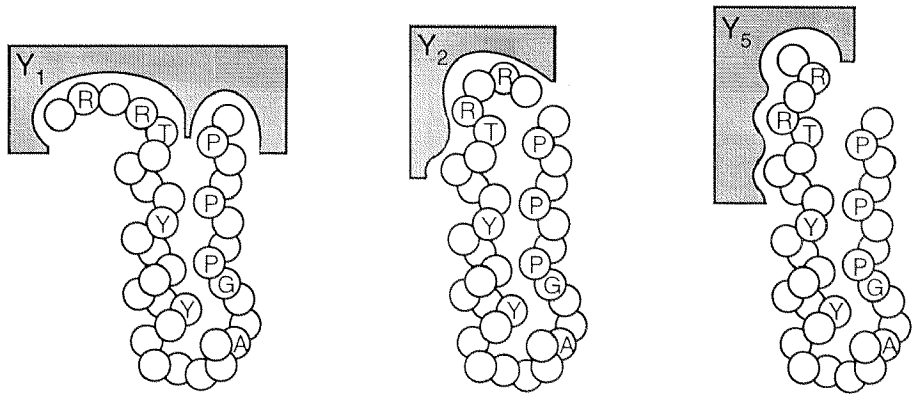

Figure 4. Schematic view of the binding of NPY at the Y_1, Y_2 and Y_5-receptor subtypes.

Among the centrally truncated NPY analogues, only [Ahx^{5-24}]-pNPY has been shown selectively to bind to the hY_2-receptor. Larger analogues did not show any selectivity. Interestingly, in some cases similar affinities were obtained either by replacement of one central position or by removal of a central segment that contained that position. For example, the analogues [Ala^{13}]-pNPY and [Ahx^{8-19}]-pNPY bound to the hY_5-receptor with an affinity of 17 nM and 19 nM, respectively.

To investigate the receptor binding site, also anti-receptor antibodies can be used. If the antibodies are obtained against receptor fragments, then the localization of the functional groups will be possible by competition binding studies with the ligand. For the Y_1-receptor this method has been described by Wieland et al. [44]. In the present work, only few sera were generated for each receptor subtype. To map the binding site with competition experiments, more sera for each receptor subtype are necessary.

To obtain antibodies which can be used to characterize intact cells, we prepared peptides resembling segments of the extracellular loops of the receptors, and they were used for immunization. The extracellular loops of the NPY receptors show some similarity, which makes it difficult to achieve full subtype selectivity. Nevertheless, the generated sera have been shown to be precious tools. For the sera Y_1 E2/2 and Y_2 E2/1, subtype selectivity for the Y_1- and Y_2-receptor subtypes was achieved. The sera Y_5 E2/2 and Y_5 E3 bound to both the Y_2- and Y_5-receptor subtypes. Accordingly, by a combination of these sera it is possible to distinguish between the receptors Y_1, Y_2 and Y_5. Detection of binding is easy and reliable with a second fluorescent-labelled antibody that is directed against the

immunglobulin of the first species. Antibodies also allow receptor localization in tissue, which is important for the understanding of the biological role of a receptor subtype. Moreover, antibodies raised against receptor fragments can be used for many other applications, like molecular mass determination of the receptor protein, affinity purification, receptor regulation, desensitization and localization of functional groups [45].

A rational approach to achieve subtype selectivity is difficult either for NPY analogues or for anti NPY receptor antibodies. Ligand-receptor interaction is usually not-well characterized for each receptor subtype, which makes prediction of selectivity very hard. Because of sequence similarity amongst receptor subtypes, it is difficult to choose receptor fragments for immunization in a way that would avoid any cross-reactivity. Furthermore, peptides for immunization have to be long enough, in order to adopt a conformation that is similar to the one in the receptor.

Production of NPY analogues is fast and cheap in comparison with the preparation of antibodies, but fluorescent labelling of the analogues can influence their binding to the receptor, while anti-receptor antibodies can be detected with a secondary labelled antibody. Furthermore, binding of small peptides on cells or tissue can be difficult to test, because they can be washed out during experimental procedure.

2.6 Conclusions

The goal of this work was the comparison of selective NPY analogues and anti-receptor antibodies with respect to subtype selectivity and mapping of the binding site. For both methods this was partly achieved. The analogues and the antibodies are very good tools to distinguish NPY receptor subtypes on membranes and intact cells. The design of new ligands is usually a successful approach to structurally and biologically characterize different receptor subtypes. In fact, by means of selective analogues it is possible better to understand how each receptor interacts with the ligand and also through which subtype the hormone modulates some physiological functions. Furthermore, these insights are essential for the design of new potent drugs.

In a next step, the analogues and the antibodies have to be tested on tissue, as the knowledge of receptor distribution is important for the understanding of the physiological and biological role of a receptor subtype. Furthermore, localization studies should be also

performed on the protein level, since mRNA localization does not fit necessarily with the localization of the mature protein, as suggested by the observation of the rapid axonal transport for the Y_1-receptor [46].

Acknowledgements

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2.7 References

1. Tatemoto, K., Carlquist, M. and Mutt, V. (1982) Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature **296:** 659-660.

2. Inui, A. (1999) Neuropeptide Y feeding receptors: are multiple subtypes involved? Trends Pharm. Sci. **20:** 43-46.

3. Kalra, S. P. and Crowley, W. R. (1992) Neuropeptide Y: a novel neuroendocrine peptide in the control of pituitary hormone secretion, and its relation to luteinizing hormone. Front. Neuroendocrinol. **13:** 1-46.

4. McDonald, J. K., Lumpkin, M. D., Samson, W. K. and McCann, S. M. (1985) Neuropeptide Y affects secretion of luteinizing hormone and growth hormone in ovariectomized rats. Proc. Natl. Acad. Sci. U.S.A. **82:** 561-564.

5. Moltz, J. H. and McDonald, J. K. (1985) Neuropeptide Y: direct and indirect action on insulin secretion in the rat. Peptides **6:** 1155-1159.

6. Wahlestedt, C., Yanaihara, N. and Håkanson, R. (1986) Evidence for different pre- and postjunctional receptors for neuropeptide Y and related peptides. Regul. Pept. **13:** 307-318.

7. Flood, J. F., Hernandez, E. N. and Morley, J. E. (1987) Modulation of memory processing by neuropeptide Y. Brain Res. **421:** 280-290.

8. Jolicoeur, F. B., Michaud, J. N., Rivest, R., Menard, D., Gaudin, D., Fournier, A. and St-Pierre, S. (1991) Neurobehavioral profile of neuropeptide Y. Brain. Res. Bull. **26:** 265-268.

9. Jolicoeur, F. B., Michaud, J. N., Menard, D. and Fournier, A. (1991) In vivo structure activity study supports the existence of heterogeneous neuropeptide Y receptors. Brain. Res. Bull. **26:** 309-311.

10. Esteban, J., Chover, A. J., Sanchez, P. A., Mico, J. A. and Gibert-Rahola, J. (1989) Central administration of neuropeptide Y induces hypothermia in mice. Possible interaction with central noradrenergic systems. Life Sci. **45:** 2395-2400.

11. Clark, J. T., Kalra, P. S. and Kalra, S. P. (1997) Neuropeptide Y stimulates feeding but inhibits sexual behavior in rats. Obes. Res. **5:** 275-283.

12. Calza, L., Giardino, L., Zanni, M., Velardo, A., Parchi, P. and Marrama, P. (1990) Daily changes of neuropeptide Y-like immunoreactivity in the suprachiasmatic nucleus of the rat. Regul. Pept. **27:** 127-137.

13. McAuley, M. A., Chen, X. and Westfall, T. C. (1993) Central cardiovascular actions of neuropeptide Y. In: The biology of neuropeptide Y and related peptides, pp. 389-418, Colmers, W. F. and Wahlestedt, C. (ed.), Humana Press Inc., Totowa.

14. Heilig, M. (1993) Neuropeptide Y in relation to behavior and psychiatric disorders: some animal and clinical observations. In: The biology of neuropeptide Y and related peptides, pp. 511-555, Colmers, W. F. and Wahlestedt, C. (ed.), Humana Press Inc., Totowa.

15. Thiele, T. E., Marsh, D. J., Marie, L. S., Bernstein, I. L. and Palmiter, R. D. (1998) Ethanol consumption and resistance are inversely related to neuropeptide Y levels. Nature **396:** 366-369.

16. Michel, M. C., Beck-Sickinger, A. G., Cox, H., Doods, H. N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T. and Westfall, T. (1998) XVI. International union of pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. Pharmacol. Rev. **50:** 143-150.

17. Krause, J., Eva, C., Seeburg, P. H. and Sprengel, R. (1992) Neuropeptide Y1 subtype pharmacology of a recombinantly expressed neuropeptide receptor. Mol. Pharmacol. **41:** 817-821.

18. Larhammar, D., Blomqvist, A. G., Yee, F., Jazin, E., Yoo, H. and Wahlestedt, C. (1992) Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y1 type. J. Biol. Chem. **267:** 10935-10938.

19. Herzog, H., Hort, Y. J., Ball, H. J., Hayes, G., Shine, J. and Selbie, L. A. (1992) Cloned human neuropeptide Y receptor couples to two different second messenger systems. Proc. Natl. Acad. Sci. U.S.A. **89:** 5794-5798.

20. Rose, P. M., Fernandes, P., Lynch, J. S., Frazier, S. T., Fisher, S. M., Kodukula, K., Kienzle, B. and Seethala, R. (1995) Cloning and functional expression of a cDNA encoding a human type 2 neuropeptide Y receptor. J. Biol. Chem. **270:** 29038.

21. Gerald, C., Walker, M. W., Vaysse, P. J., He, C., Branchek, T. A. and Weinshank, R. L. (1995) Expression cloning and pharmacological characterization of a human hippocampal neuropeptide Y/peptide YY Y2 receptor subtype. J. Biol. Chem. **270:** 26758-26761.

22. Gehlert, D. R., Beavers, L. S., Johnson, D., Gackenheimer, S. L., Schober, D. A. and Gadski, R. A. (1996) Expression cloning of a human brain neuropeptide Y Y2 receptor. Mol. Pharmacol. **49:** 224-228.

23. Lundell, I., Blomqvist, A. G., Berglund, M. M., Schober, D. A., Johnson, D., Statnick, M. A., Gadski, R. A., Gehlert, D. R. and Larhammar, D. (1995) Cloning of a human receptor of the NPY receptor family with high affinity for pancreatic polypeptide and peptide YY. J. Biol. Chem. **270:** 29123-29128.

24. Bard, J. A., Walker, M. W., Branchek, T. A. and Weinshank, R. L. (1995) Cloning and functional expression of a human Y4 subtype receptor for pancreatic polypeptide, neuropeptide Y, and peptide YY. J. Biol. Chem. **270:** 26762-26765.

25. Gerald, C., Walker, M. W., Criscione, L., Gustafson, E. L., Batzl-Hartmann, C., Smith, K. E., Vaysse, P., Durkin, M. M., Laz, T. M., Linemeyer, D. L., Schaffhauser, A. O., Whitebread, S., Hofbauer, K. G., Taber, R. I., Branchek, T. A. and Weinshank, R. L. (1996) A receptor subtype involved in neuropeptide-Y-induced food intake. Nature **382:** 168-171.

26. Weinberg, D. H., Sirinathsinghji, D. J., Tan, C. P., Shiao, L. L., Morin, N., Rigby, M. R., Heavens, R. H., Rapoport, D. R., Bayne, M. L., Cascieri, M. A., Strader, C. D., Linemeyer, D. L. and MacNeil, D. J. (1996) Cloning and expression of a novel neuropeptide Y receptor. J. Biol. Chem. **271:** 16435-16438.

27. Beck-Sickinger, A. G. (1996) Structural characterization and binding sites of G-protein coupled receptors. Drug Discov. Today **1:** 502-513.

28. Ingenhoven, N. and Beck-Sickinger, A. G. (1997) Fluorescent labelled analogues of neuropeptide Y for the characterization of cells expressing NPY receptor subtypes. J. Recept. & Signal Transduct. Res. **17:** 407-418.

29. Beck-Sickinger, A. G. and Jung, G. (1995) Structure-activity relationships of neuropeptide Y analogues with respect to Y1 and Y2 receptors. Biopolymers **37:** 123-142.

30. Rist, B., Wieland, H. A., Willim, K. D. and Beck-Sickinger, A. G. (1995) A rational approach for the development of reduced-size analogues of neuropeptide Y with high affinity to the Y1 receptor. J. Pept. Sci. **1:** 341-348.

31. Jahns, R., Siegmund, C., Jahns, V., Reilander, H., Maidhof, A., Muller-Esterl, W., Lohse, M. J. and Boege, F. (1996) Probing human beta 1- and beta 2-adrenoceptors with domain-specific fusion protein antibodies. Eur. J. Pharm. **316:** 111-121.

32. Wall, S. J., Yasuda, R. P., Hory, F., Flagg, S., Martin, B. M., Ginns, E. I. and Wolfe, B. B. (1991) Production of antisera selective for m1 muscarinic receptors using fusion proteins: distribution of m1 receptors in rat brain. Mol. Pharmacol. **39:** 643-649.

33. Muller-Newen, G., Kohne, C., Keul, R., Hemmann, U., Muller-Esterl, W., Wijdenes, J., Brakenhoff, J. P., Hart, M. H. and Heinrich, P. C. (1996) Purification and characterization of the soluble interleukin-6 receptor from human plasma and identification of an isoform generated through alternative splicing. Eur. J. Biochem. **236:** 837-842.

34. Abd Alla, S., Godovac-Zimmermann, J., Braun, A., Roscher, A. A., Muller-Esterl, W. and Quitterer, U. (1996) Structure of the bradykinin B2 receptors' amino terminus. Biochemistry **35:** 7514-7519.

35. Van den Pol, A. N., Romano, C. and Ghosh, P. (1995) Metabotropic glutamate receptor mGluR5 subcellular distribution and developmental expression in hypothalamus. J. Comp. Neurol. **362:** 134-150.

36. Westphal, R. S., Backstrom, J. R. and Sanders-Bush, E. (1995) Increased basal phosphorylation of the constitutively active serotonin 2C receptor accompanies agonist-mediated desensitization. Mol. Pharmacol. **48:** 200-205.

37. Palmer, T. M., Gettys, T. W., Jacobson, K. A. and Stiles, G. L. (1994) Desensitization of the canine A2a adenosine receptor: delineation of multiple processes. Mol. Pharmacol. **45:** 1082-1094.

38. Ingenhoven, N., Eckard, C. P., Gehlert, D. R. and Beck-Sickinger, A. G. (1999) Molecular characterization of the human neuropeptide Y Y2-receptor. Biochemistry **38:** 6897-6902.

39. Rist, B., Ingenhoven, N., Scapozza, L., Schnorrenberg, G., Gaida, W., Wieland, H. A. and Beck-Sickinger, A. G. (1997) The bioactive conformation of neuropeptide Y analogues at the human Y2-receptor. Eur. J. Biochem. **247:** 1019-1028.

40. Beck-Sickinger, A. G., Wieland, H. A., Wittneben, H., Willim, K. D., Rudolf, K. and Jung, G. (1994) Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. Eur. J. Biochem. **225:** 947-958.

41. Allen, J., Novotny, J., Martin, J. and Heinrich, G. (1987) Molecular structure of mammalian neuropeptide Y: analysis by molecular cloning and computer-aided comparison with crystal structure of avian homologue. Proc. Nat. Acad. Sci. U.S.A. **84:** 2532-2536.

42. Beck, A. G., Jung, G., Gaida, W., Köppen, H., Lang, R. and Schnorrenberg, G. (1989) Highly potent and small neuropeptide Y agonist obtained by linking NPY 1-4 via spacer to alpha-helical NPY 25-36. FEBS Lett. **244:** 119-122.

43. Rist, B., Entzeroth, M. and Beck-Sickinger, A. G. (1998) From micromolar to nanomolar affinity: a systematic approach to identify the binding site of CGRP at the human calcitonin gene-related peptide 1 receptor. J. Med. Chem. **41:** 117-123.

44. Wieland, H. A., Eckard, C. P., Doods, H. N. and Beck-Sickinger, A. G. (1998) Probing of the neuropeptide Y-Y1-receptors interaction with anti-receptor antibodies. Eur. J. Biochem. **255:** 595-603.

45. Eckard, C. P. and Beck-Sickinger, A. G. (1999) Characterisation of G-protein-coupled receptors by antibodies. Curr. Med. Chem., accepted.

46. Jacques, D., Tong, Y., Dumont, Y., Shen, S. H. and Quirion, R. (1996) Expression of the neuropeptide Y Y1 receptor mRNA in the human brain: an in situ hybridization study. Neuroreport **7:** 1053-1056.

CHAPTER 3

Amino Acid Side Chain Attachment Approach and Its Application to the Synthesis of Tyrosine-Containing Cyclic Peptides

Chiara Cabrele, Michael Langer and Annette G. Beck-Sickinger

Department of Pharmacy, Swiss Federal Institute of Technology, Zurich, Switzerland

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3.1 Abstract

The technique of resin loading by the attachment of the amino acid side chain represents a powerful tool for the synthesis of cyclopeptides by solid phase. We investigated the anchoring of the side chain of *N*-(9-Fluorenylmethoxycarbonyl, Fmoc)-tyrosine methyl ester to benzyl-type resins by the Mitsunobu reaction. Satisfactory loading was obtained for HMPB-MBHA and Wang resins. The suitability of the preloaded resins for solid phase peptide synthesis by using the Fmoc-strategy, combined with the head to tail cyclization on the solid support, was illustrated by the preparation of three cyclic analogs of neuropeptide Y (NPY), a 36-residue peptide hormone and one of the most abundant neuropeptides in the brain. Each peptide contained the N- and C-terminal tetrapeptide segments of NPY, joined by different spacers: 6-aminohexanoic acid, β-alanine, or Ala-Aib. First the synthesis of the peptide methyl esters was performed, followed by saponification and cyclization on the resin. HOBt/DIC or HOBt/TBTU were used for the ring closure. The CD spectra of the three cyclopeptides in 30% trifluoroethanol showed a type I and III β-turns structure, which was already adopted by the (Ala-Aib)-containing cyclopeptide in water. The CD spectra, together with the biological assays, confirmed the suitability of these cyclopeptides as conformationally restricted peptides that may serve as lead structures in drug development.

3.2 Introduction

In the last few years, interest in solid phase synthesis techniques has increased significantly, especially in conjunction with their application to combinatorial chemistry. Libraries of peptides and organic molecules have proven to be powerful tools for facilitating the discovery process of new lead and therapeutic compounds.[1] Linear peptides built by the natural L-amino acids are generally not suitable for use as drugs, because of their poor oral availability and enzymatic degradation. For this reason, much attention is being paid at present to the construction and screening of combinatorial libraries of modified peptides, peptidomimetics and nonpeptide compounds.[2] In particular, C-terminally modified and cyclic peptides have been shown to be diagnostically and therapeutically useful tools in all areas of biomedical research. In many cases, C-terminal peptide modifications have led to the preparation of potent agonists or antagonists, which are also resistant to proteolytic degradation.[3] Furthermore, the peptide cyclization approach has provided conformationally constrained analogs with suitable properties for investigating ligand-receptor interactions and structure-activity relationships,[4] as well as for developing drugs with increased metabolic stability and receptor selectivity.[5] The importance of the cyclic peptides has necessitated the improvement of the method of their preparation.[6] Classic solid phase peptide synthesis does not allow the introduction of modifications at the C-terminus or the head to tail cyclization of peptides that are still bound to the resin. The protected peptide must first be cleaved from the solid support and then modified or cyclized in solution. This synthetic procedure presents some disadvantages, such as the necessity to isolate the desired peptide from the excess reagents, which leads in some cases to a considerable loss of the product. Furthermore, cyclization in solution requires very diluted concentrations of the linear peptide, in order to minimize the formation of the cyclodimer and oligomers.

In the early 1990s, a new strategy has been introduced for peptide synthesis on the solid phase, based on the attachment of the amino acid side chain to the solid support. So far, this method has been applied to aspartic[7, 8] and glutamic[8] acids, lysine,[8] serine and tyrosine[8, 9] in the Boc-strategy, and to aspartic and glutamic acids[10] and lysine[11] in the Fmoc-strategy with different C-terminal protecting groups. In order to extend this method to a broader context, we have investigated further N^{α}-Fmoc amino acids that could be suitable to be linked to the resin through their side chains. In the present work, we address

the possibility of attaching the phenolic group of the tyrosine side chain to the solid support and of carrying out the synthesis and the head to tail cyclization of tyrosine-containing peptides on the solid phase by Fmoc chemistry. We selected three cyclic peptides, corresponding to centrally truncated analogs of the neuropeptide Y (NPY).

Table 1. Amino Acid Sequences of the Cyclopeptides Synthesized by Combination of the Tyrosine Side Chain Attachment Approach with the Resin Bound Peptide Cyclization.

Peptide No.	Amino Acid Sequence[a]	Backbone Atoms No.
10	cyclo-(β-Ala-Tyr-Pro-Ser-Lys-β-Ala-Arg-Gln-Arg-**Tyr**)	32
11	cyclo-(Ahx-Tyr-Pro-Ser-Lys-Ahx-Arg-Gln-Arg-**Tyr**)	38
12	cyclo-(Ala-Aib-Tyr-Pro-Ser-Lys-Ala-Aib-Arg-Gln-Arg-**Tyr**)	36

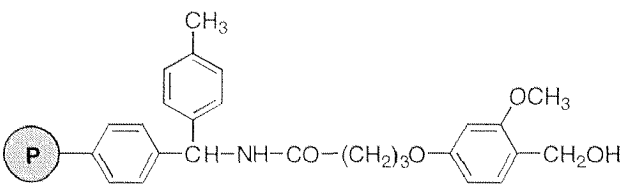
[a] The tyrosine side chain that was attached to the resin is given in **bold**.

NPY, a 36-residue peptide amide of the pancreatic polypeptide family (PP-family), is widely distributed in the peripheral and central nervous systems and is one of the most abundant neuropeptides in the brain.[12] Physiological effects attributed to NPY include vasoconstriction, stimulation of food intake, inhibition of anxiety, increase of memory performance, presynaptic inhibition of neurotransmitter release, and regulation of the sexual hormones. So far, five receptor subtypes of NPY have been identified and characterized: Y_1, Y_2, Y_4, Y_5, and y_6.[13] They belong to the large super-family of the G-protein-coupled receptors and each of them mediates specific biological actions of NPY. It has been found that the N- and C-terminal regions of NPY are involved in the recognition and binding of the ligand to the Y-receptor subtypes.[12] In order to gain insights into the conformational requirements for the receptor affinity and selectivity, we designed three cyclic peptides, **10-12**, containing the two NPY segments Tyr^1-Pro^2-Ser^3-Lys^4 and Arg^{33}-Gln^{34}-Arg^{35}-Tyr^{36}, joined by different types of spacers (Table 1). The synthesis of the cyclopeptides was accomplished totally on the solid phase, starting from the chain assembly of the corresponding linear peptide methyl esters on the resins preloaded by the tyrosine side chain attachment, followed by ester hydrolysis and head to tail cyclization.

3.3 Results and Discussion

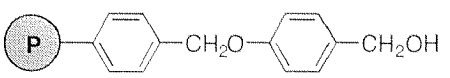
3.3.1 Anchoring of *N*-(9-Fluorenylmethoxycarbonyl)-Tyrosine Methyl Ester to the Resin

N-(9-Fluorenylmethoxycarbonyl, Fmoc)-tyrosine methyl ester was attached to 4-hydroxymethyl-3-methoxyphenoxybutyric acid 4-methyl benzhydrylamine resin (HMPB-MBHA resin, **1a**), 4-benzyloxybenzyl alcohol resin (Wang resin, **1b**) and 4-benzyloxy-2-methoxybenzyl alcohol resin (Sasrin resin, **1c**),



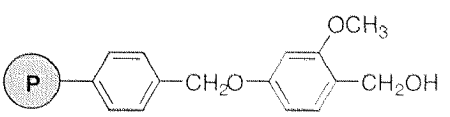
HMPB-MBHA resin

1a



Wang resin

1b



Sasrin resin

1c

by its phenolic group under the conditions of the Mitsunobu reaction,[14] by using triphenylphosphine and diethyl azodicarboxylate (DEAD) (Scheme 1). The loading of the resin was determined by UV quantification of the Fmoc-piperidine adduct,[15] as well as by a quantitative ninhydrin test.[16]

To improve the efficiency of the reaction, extensive variations of the reaction conditions were made. The influence of the solvent was investigated by comparing the results obtained with dichloromethane, *N*-methylmorpholine and tetrahydrofuran. Although *N*-

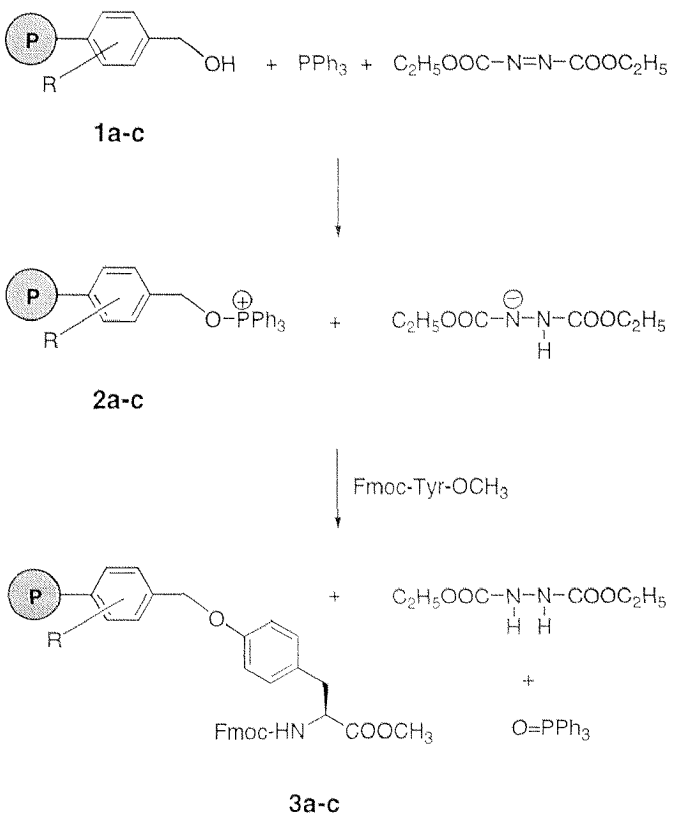
methylmorpholine has been reported to give the best results in coupling of the phenolic group of tyrosine to hydroxymethyl polystyrene resin,[17] the substitution levels of the Sasrin and Wang resins obtained in this solvent upon dropwise addition of diluted DEAD at room temperature were not acceptable, as they were below 0.02 mmol/g. When the etherification was repeated in dichloromethane, the yields for the Sasrin and Wang resins were increased to 0.1 mmol/g. The loading of the HMPB-MBHA resin in dichloromethane gave a substitution level of 0.17 mmol/g. It has been reported that the attachment of Boc-tyrosine *para*-nitrobenzyl ester to hydroxymethyl resin in tetrahydrofuran was successful.[8] However, the loading of the Wang resin in this solvent gave no satisfactory results in our case.

It is known that one side reaction of the Mitsunobu etherification is the thermal decomposition of DEAD with the resulting production of ethyl alcohol; the ethyl alcohol is activated by the remaining DEAD and reacts with the phenolic group of the tyrosine, yielding the corresponding ethyl ether.[18] To avoid the formation of this side product, the reaction mixture was cooled at 0 °C during the dropwise addition of DEAD. The loading obtained for the Wang resin in dichloromethane resulted in 0.23 mmol/g. Some attempts were carried out by using the more stable diisopropyl azodicarboxylate (DIAD) instead of DEAD. However, the loading of the resin could not be increased.

By varying the n-fold excess of the reagents amino acid/triphenylphosphine/DEAD (two- to five-fold), as well as the order of the reagent addition, no increase of the substitution level of the resins was obtained.

Taken together, these results led to the following conclusions: the efficiency of the Mitsunobu etherification of benzyl-type resins with *N*-(9-fluorenylmethoxycarbonyl)-tyrosine methyl ester depends on the ability of the linker to form the oxyphosphonium salt **2** (Scheme 1). This seemed to be favored for the HMPB-MBHA linker (**1a**), where the methoxy *ortho*-position substituent in the phenoxy ring could stabilize the positive charge on the phosphor atom. An *ortho*-methoxy group is present also in the Sasrin linker (**1c**), however, the loading of this resin proved to be more difficult, maybe because of steric hindrance. In fact, the flexibility of a linker influences its solvation and therefore its propensity to undergo the reaction. The Sasrin linker is less flexible than the HMPB-MBHA linker, the latter one containing the butyric acid moiety that acts as a flexible spacer between the polymer and the reactive part of the linker.

Scheme 1. Loading of a Benzyl-Type Resin by Side Chain Attachment of Fmoc-Tyrosine Methyl Ester Using the Mitsunobu Reaction.



Furthermore, the initial substitution level of the Sasrin resin was higher than that of the HMPB-MBHA resin (1.06 mmol/g versus 0.44 mmol/g), and this could also contribute to reduce the mobility of the linker within the polymer.

The attempt to enhance the nucleophilicity of the phenolic group of the tyrosine side chain by choosing *N*-methylmorpholine as the reaction solvent failed, as shown by the unsatisfactory yields of the loading of the Sasrin and Wang resins in this solvent. In contrast, better results were obtained in dichloromethane. In the case of the Wang resin, loading was increased by carrying out the reaction at low temperature, which might prevent the partial thermal decomposition of DEAD. However, the efficiency of the reaction was not dependent on other parameters, such as the alkyl group of the oxidant (DIAD gave no better results than DEAD), the use of the reagents in a large n-fold excess, or the sequence of the reagent addition.

3.3.2 Solid Phase Synthesis of the Peptide Methyl Esters

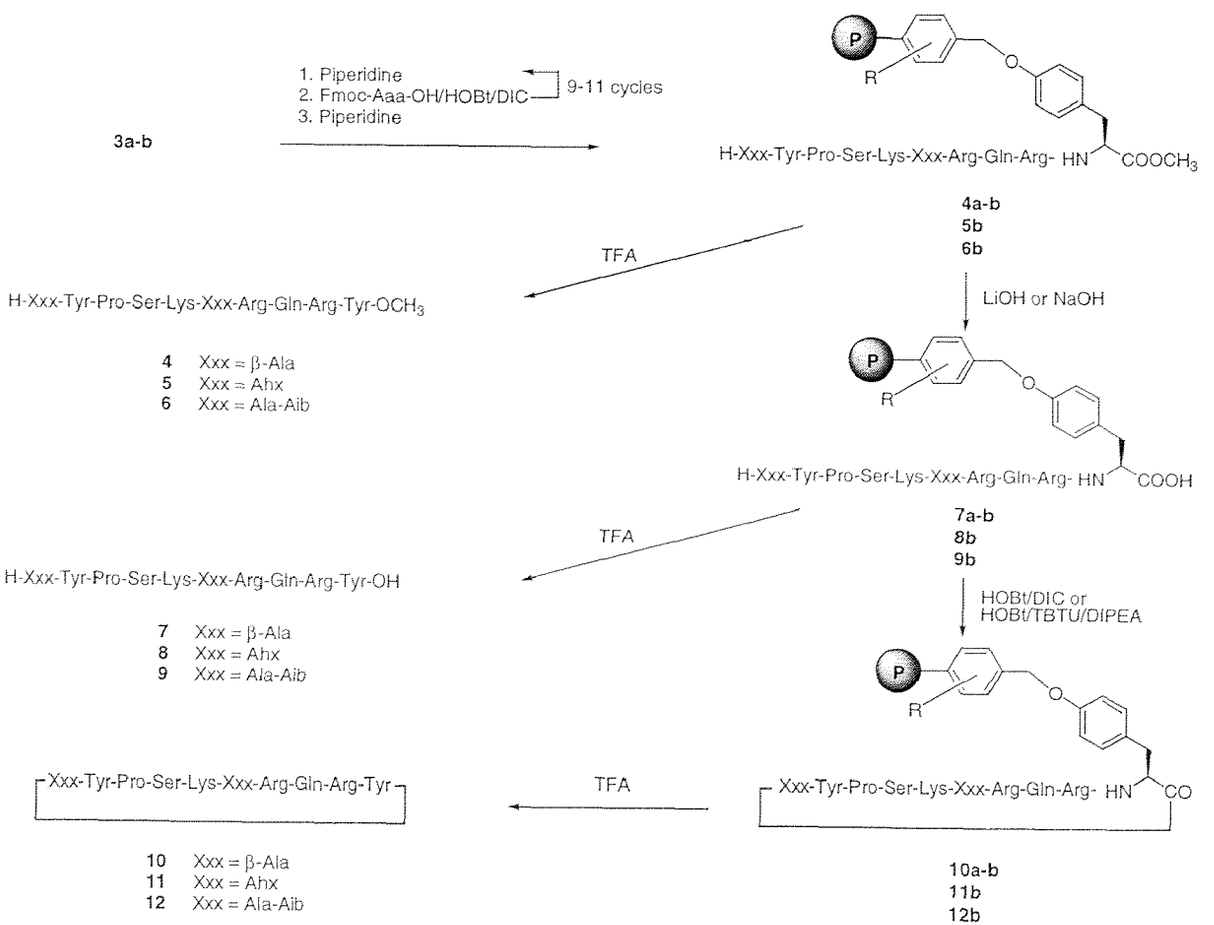
Three peptides with the amino acid sequence Xxx-Tyr-Pro-Ser-Lys-Xxx-Arg-Gln-Arg-Tyr were chosen for study (Table 1). Tyr-Pro-Ser-Lys and Arg-Gln-Arg-Tyr correspond to the N- and C-terminal tetrapeptide fragments of NPY, respectively. Xxx was incorporated into the sequence as a structural element and differed in each of the peptides. 6-Aminohexanoic acid (Ahx) was chosen to play the role of a flexible long spacer, β-alanine was chosen as a shorter spacer able to induce β-turns,[19] and finally the dipeptide segment alanine-α-aminoisobutyric acid (Ala-Aib) was chosen for its high propensity to build turn-like structures.[20]

The synthesis of the linear peptide methyl esters **4-6** was carried out on resins preloaded with the tyrosine side chain attachment, by using the Fmoc-strategy and a double coupling protocol with *N*-hydroxybenzotriazole (HOBt) and *N,N'*-diisopropylcarbodiimide (DIC) in DMF (Scheme 2). Peptide ester **4** was synthesized on both the HMPB-MBHA and Wang resins, whereas the peptide esters **5** and **6** were prepared only on the Wang resin.

3.3.3 Hydrolysis of the C-Terminal Methyl Ester on the Resin

When the stepwise solid phase peptide synthesis was completed, the cleavage of the C-terminal methyl ester was performed on the resin by using methanolic solutions of lithium or sodium hydroxide. Only a partial conversion of the resin bound esters **4a-b**, **5b** and **6b** to the corresponding acids **7a-b**, **8b** and **9b** was obtained with 0.2 M sodium hydroxide in methanol after two days or 0.2 M lithium hydroxide in methanol after four days. The esters were totally converted to the acids by employing 0.33 M lithium hydroxide in methanol/water 97/3 (v/v) for six days or 0.4 M sodium hydroxide in methanol for three days. Despite the strong basic conditions and the long reaction times, the hydrolysis of the peptide methyl esters on the resin went smoothly, and no degradation or racemization products were observed (peptide acid **7**, Figure 1A; peptide acids **8** and **9**, data not shown).

Scheme 2. Solid Phase Synthesis of Linear and Cyclic Peptides by Amino Acid Side Chain Attachment Approach.



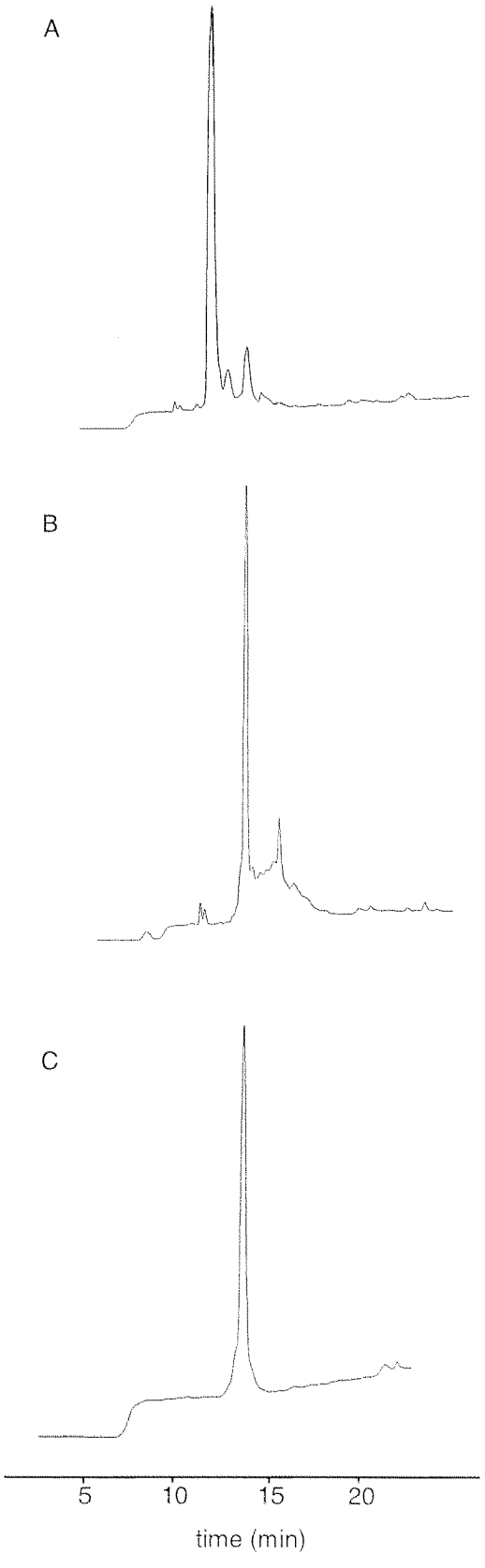


Figure 1. HPLC profiles of the β-alanine-containing peptides synthesized on the Wang resin preloaded by the tyrosine side chain attachment (**3b**), and cleaved by treatment with TFA in the presence of triisopropylsilane and water. Gradient: 0-60% acetonitrile over 30 min; UV detection at 220 nm. A: crude of the peptide acid **7**, obtained by hydrolysis of the ester **4b** with 0.4 M sodium hydroxide in methanol, three days. B: crude of the cyclopeptide **10**, obtained by using a 15-fold excess of HOBt/DIC, 19 hours. C: cyclic monomer **10** after purification by HPLC semi-preparative.

3.3.4 Head to Tail Cyclization on the Resin

In order to establish the optimal reaction conditions for cyclization, the resin bound linear peptide acids **7b**, **8b** and **9b** were cyclized by using different methods for the activation of the carboxy group. The main parameters that affect the conversion rate of the resin bound peptide acid to its reactive form are the efficiency of the activation reagent, the use of the activation reagent in a proper n-fold excess, and the steric hindrance of the solid support. To investigate how the efficiency and the amount of the activation reagent could influence the reaction, different n-fold excesses of the more efficient HOBt/TBTU (1H-benzotriazolium, 1-[bis(dimethylamino)-methylene]-, tetrafluoroborate (1-), 3-oxide, formerly named *O*-(benzotriazol-1-yl) *N,N,N',N'*-tetramethyluronium tetrafluoroborate) activation reagent and of the less effcient HOBt/DIC activation reagent were used. The total conversion of the linear peptides to the corresponding cyclic products was controlled by HPLC and ESI-MS analyses. HOBt/TBTU cyclization was faster than the HOBt/DIC cyclization, however both reagents could drive the reaction to completion. No epimerization was observed to take place during the activation step of the cyclization reaction. The formation of tetramethylguanidinium side products, which has been reported to occur during HOBt/HBTU side chain to side chain cyclization,[21] could not be detected by ESI-MS analysis. The crude cyclopeptides obtained by the two cyclization methods showed similar HPLC profiles: in each case the cyclic monomer (**10-12**) was present as the major product, while the minor products eluting later were attributed to the oligomeric fraction (cyclopeptide **10**, Figure 1B; cyclopeptides **11** and **12**, data not shown). The area percentage corresponding to each cyclopeptide cleaved by TFA in the presence of triisopropylsilane and water (which is shown below to be the most suitable scavengers for minimizing the TFA-promoted side reactions) is reported in the Table 2.

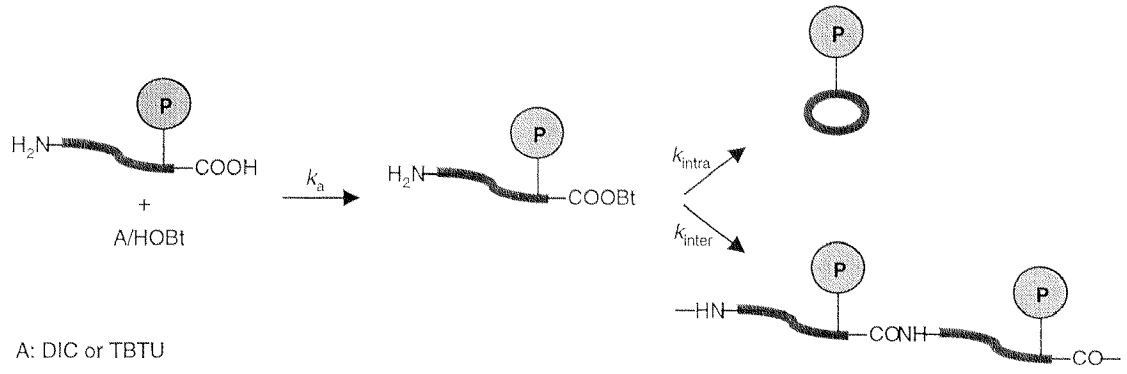
It was observed that the faster HOBt/TBTU cyclization did not prove to be more efficient compared to the slower HOBt/DIC cyclization. Cyclization by a 15-fold excess of HOBt/TBTU (five hours) or by a six-fold excess of HOBt/DIC (22 hours) gave over 70% of the cyclic monomer **12**, while only about 40% of **10** and **11**. In contrast, cyclization by using a 15-fold excess of HOBt/DIC (19 hours) gave over 70% of the cyclopeptides **10** and **11**, but only 54% of the cyclopeptide **12**. These results might be explained by the different effects of the activation conditions on the intra- and intermolecular reaction pathways, in conjunction with the different structural features of the spacer Xxx (Scheme 3). In **8** (the

corresponding linear form of **11**) the two Ahx units, each containing five methylene groups, confer a relatively high flexibility to the peptide chain, which favors the interchain contacts and therefore the strong competition between monocyclization and oligomerization. Similarly, the two units of β-alanine, present in **7** (the corresponding linear form of **10**), introduce more mobility into the backbone, especially into the N-terminal region, while the β-alanine in the middle of the amino acid sequence might weakly force the backbone into a loop structure. Because of the low structural constraint of the peptide chains, the cyclization of **7** and **8** could take place by both the intra- and intermolecular mechanisms with the same probability, and the attempt to favor the former mechanism over the latter one might be very challenging. By carrying out the cyclization of **7** and **8** under different reaction conditions to prepare the cyclic peptides **10** and **11**, respectively, we observed that the intra- and intermolecular reaction pathways were highly competitive when either a large excess of HOBt/TBTU (15-fold) or a moderate excess of HOBt/DIC (six-fold) were applied, whereas the use of a large excess of HOBt/DIC (15-fold) favored the monocyclization over the oligomerization. This seems to suggest that if the peptide chain has a low propensity to undergo an intrachain condensation, such as for **7** and **8**, and the interchain contacts are strongly favored (Scheme 3, $k_{intra} \sim k_{inter}$), the undesired intermolecular cyclization might be minimized by using reaction conditions that determine an intermediate conversion rate of the peptide to its reactive form. Otherwise, when the peptide is converted to the reactive species too quickly or slowly, the intermolecular mechanism will be more likely to occur. On the other hand, if the peptide has a high propensity to undergo an intrachain condensation ($k_{intra} > k_{inter}$), like **9** (the corresponding linear form of **12**) which contains two units of the constrained turn-inducing motif Ala-Aib, the ring closure will be favored, and fast or slow conversion rates of the peptide to the reactive form will both be suitable to maintain the selectivity between the intra- and the intermolecular cyclization, while an intermediate conversion rate might equally favor both the reaction pathways.

Based on these results of the cyclization on the resin, a correlation might be supposed to exist between the activation rate (k_a) and the intra- and intermolecular cyclization rates (k_{intra} and k_{inter}), and the success of the cyclization might depend on a combination of reaction parameters that will drive the cyclization through the intramolecular mechanism rather than through the intermolecular.

By performing the cyclization on the solid phase, the properties of the solid support

Scheme 3. Intra- and Intermolecular Reaction Pathways during Cyclization on the Resin.



certainly play a crucial role.[6] The steric hindrance of the solid support was evaluated by comparing the cyclization of **7** by HOBt/TBTU on the HMPB-MBHA versus the Wang resins. Under conditions of 15-fold excess HOBt/TBTU for five hours, the two resins yielded 65% and 36% of the cyclic monomer **10**, respectively. The higher percentage of the cyclic monomer obtained on the HMPB-MBHA resin might be attributed to the higher flexibility of the HMPB-MBHA linker compared to that of the Wang linker, which increases the solvation of the peptide chain and favors the contact of the reactive sites (the N- and C-termini). Moreover, the lower substitution level of the peptide on the HMPB-MBHA resin compared to that on the Wang resin (ca. 0.13 mmol/g versus 0.17 mmol/g) could provide a large distance between the peptide chains, which favors the intramolecular cyclization over the intermolecular. In fact, it has been reported that the resin loading can be proportionally correlated to the amount of oligomerization in the case of large loop cyclopeptides.[22] Furthermore, it must be stated that, in the case of the peptide bound to the HMPB-MBHA resin, the hydrolysis of the methyl ester did not go to completion, therefore, the ratio between the activated carboxy groups and the free amino groups during the cyclization was lower than 1:1.

3.3.5 Cleavage of the Peptides from the Resin

The peptides **4**, **7** and **10** were cleaved from the HMPB-MBHA resin using 96% TFA in the presence of thioanisole and thiocresol. The analysis of the crude peptides by HPLC and ESI-MS did not reveal the presence of byproducts, confirming that the cleavage of the

tyrosine side chain from the hyperacid sensitive linker went smoothly. When the peptides **4-12** were cleaved from the Wang resin using 90% TFA and the thioanisole and thiocresol scavenger mixture, a major side product was obtained, corresponding to 35% of the yield and having a mass difference of + 106. This additional mass was attributed to the *para*-hydroxybenzyl group, derived from the degradation of the Wang linker, which is likely to occur under strong acid conditions. This observation led us to conclude that the etherification of the Wang linker by the tyrosine side chain strongly destabilized this linker upon TFA treatment, much more than the esterification by the carboxy group of the amino acid. In order to avoid or at least to minimize the amount of the side products derived from the degradation of the Wang linker, we performed the TFA cleavage in the presence of other scavengers. The following combinations were used: triisopropylsilane/water, triisopropylsilane/water/phenol and thioanisole/phenol. The first mixture gave the best results, drastically reducing the formation of byproducts. In contrast, the presence of aromatic compounds as scavengers during the TFA cleavage led to a high amount of side products (Table 2).

3.3.6 Biological Activity of the Cyclopeptides 10-12

To characterize the biological properties of the compounds, cyclopeptides **10-12** were tested for their ability to compete with [^{3}H-propionyl]-NPY at Y_1-, Y_2- or Y_5-receptor subtype expressing cells. No displacement was found at Y_2-receptor expressing cells. This is in agreement with previous papers that showed that modifications at the C-terminal amide are not tolerated.[23] Only moderate displacement was found at Y_5-receptor expressing cells. In contrast, 35-41% of radioactivity was displaced at Y_1-receptor expressing cells in the presence of 1 µM of the cyclopeptides, suggesting micromolar affinity of the compounds. Although the compounds **10-12** reveal only moderate affinity, they show that only eight amino acids, which correspond to the N- and the C-terminal segments of NPY, are required to displace the native ligand. Furthermore, these cyclopeptides confirm the existence of a discontinuous binding site at the Y_1-receptor subtype and – as they selectively bind to the Y_1-receptor versus the Y_2- and Y_5-receptors – could serve as new lead compounds for the development of Y_1-receptor selective ligands.

190 nm. Furthermore, a well defined negative band is now present at 218 nm, which is red shifted and more intense compared to the shoulder observed in water. The trifluoroethanol-induced changes in the CD curves indicate that the cyclopeptides adopt a more ordered structure, which may be interpreted in terms of a mixture of type I and type III β-turns. The spectra of **10** and **11** are very similar, whereas the spectrum of **12** is much more intense in the region 200-225 nm. This observation suggests that the β-turns conformation is more prominent in **12** than in **10** and **11**. Interestingly, **12**, which is the cyclopeptide containing the turn-inducing sequence Ala-Aib, already shows a major propensity to form β-turns in water.

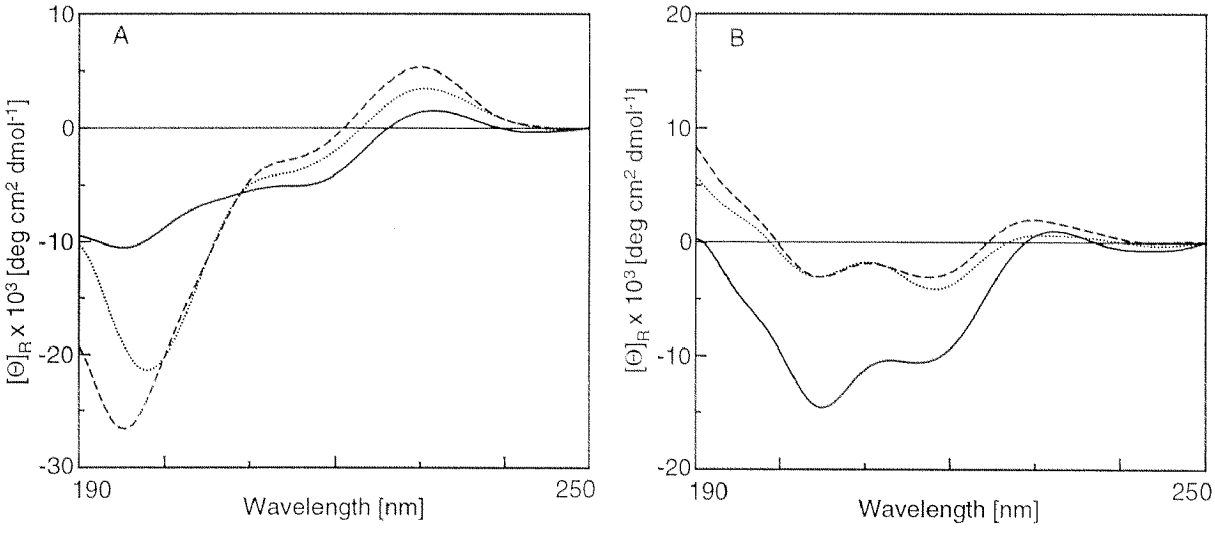


Figure 2. CD spectra of the cyclic peptides **10** (· · ·), **11** (- - - -) and **12** (——) at the concentration of 0.1 mM. A: phosphate buffer 20 mM, pH 7.0. B: 30% trifluoroethanol.

3.4 Conclusions

Our work proves the suitability of the amino acid side chain attachment approach to the synthesis of cyclic peptides on the solid phase also in the Fmoc chemistry. In particular, we have illustrated the anchoring of Fmoc-tyrosine side chain to benzyl-type resin linkers by the Mitsunobu reaction, and the use of the preloaded resins for the preparation of three cyclic analogs of NPY, with a ring size of 32, 36 and 38 atoms. The head to tail cyclization was carried out on the resin by using HOBt/DIC or HOBt/TBTU. No epimerization was

observed to occur during the condensation reaction. Tetramethylguanidinium derivatives that would arise by transfer of the bis(dimethylamino)-methylene moiety from TBTU to the terminal α-amino group of the peptide could not be detected. We observed that the success of the cyclization could depend on the activation conditions, as well as on the structural features of the peptide backbone. Specifically, the optimal reaction conditions for the monocyclization of the two peptides containing β-alanine and Ahx, respectively, were found to be sub-optimal for the peptide containing the segment Ala-Aib. The removal of the cyclic peptides from the solid support could be achieved by TFA treatment. In order to minimize the side products following the degradation of the Wang linker, the triisopropylsilane/water scavengers mixture was shown to be superior to the phenol-, thioanisole- and thiocresol-containing mixtures. The CD spectra in water and in 30% trifluoroethanol confirmed that the more conformationally restricted Aib-containing cyclic peptide has a higher propensity to build β-turns than the other two peptides, which contain more flexible spacers. Furthermore, the cyclopeptides were identified to serve as lead structures for the development of Y_1-receptor selective ligands.

The attachment of the N^{α}-Fmoc protected tyrosine methyl ester to acid-labile resins through its side chain provides the opportunity to perform solid phase reactions that involve selectively the α-amino and the carboxy groups of the aromatic amino acid. For example, after completion of the peptide chain elongation, the C-terminal carboxy group can be selectively deprotected and subjected to the desired chemical modification. Therefore, the Fmoc-tyrosine side chain attachment approach will prove to be valuable for the preparation of C-terminally modified and cyclic peptides that contain this residue at least in one position within their primary sequence, by carrying out each step of the synthesis on the solid support by using the Fmoc-strategy. Moreover, this method should contribute to the broadening of the combinatorial chemistry in the field, which is focussed on the generation of libraries of peptides either chemically modified at the C-terminus or conformationally constrained by backbone cyclization. To date, syntheses of cyclic peptide libraries accomplished totally on the solid support have been reported using the Boc-strategy combined with the attachment of the aspartic acid side chain[1, 7b, 26] or the oxime resin.[27] However, the high interest in modified peptides, cyclopeptides and peptidomimetics as lead compounds for the discovery of more potent, stable and orally available drugs requires future efforts. These include the application of the tyrosine side chain attachment to the combinatorial library approach in the Fmoc chemistry, for the

preparation of head to tail cyclized peptides or peptides modified at the C-terminus upon reaction with amines, alcohols and other organic compounds, as well as for the synthesis of tyrosine-based nonpeptide molecules. Furthermore, we are investigating the side chain attachment of other N^{α}-Fmoc protected trifunctional amino acids, like serine, threonine, histidine and arginine, to the solid support, which would rapidly increase the versatility of this synthetic approach for the solid phase technique.

3.5 Experimental Section

3.5.1 Materials

The *N*-(9-fluorenylmethoxycarbonyl, Fmoc)-protected natural amino acids and 1H-benzotriazolium, 1-[bis(dimethylamino)-methylene]-, tetrafluoroborate (1-), 3-oxide (TBTU) were purchased from Alexis (Läufelfingen, Switzerland). Side chain protecting groups were: *tert*-butyl for Ser and Tyr, Boc for Lys, trityl for Gln and 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg. The Fmoc-protected β-Ala, 6-aminohexanoic acid (Ahx) and α-aminoisobutyric acid (Aib), the 4-benzyloxybenzyl alcohol (Wang) resin and the 4-hydroxymethyl 3-methoxyphenoxybutyric acid 4-methylbenzhydrylamine (HMPB-MBHA) resin were purchased from Novabiochem (Läufelfingen, Switzerland). The 4-benzyloxy-2-methoxybenzyl alcohol (Sasrin) resin was obtained from Bachem (Bubendorf, Switzerland). Tyrosine methyl ester hydrochloride (Novabiochem) was Fmoc-protected in our laboratory by using 9-fluorenylmethyl succinimidyl carbonate[28] (Novabiochem). *N*-hydroxybenzotriazole (HOBt), *N,N*-diisopropylethylamine (DIEA), TFA, thioanisole, triisopropylsilane, piperidine, pyridine, 4-methylmorpholine, 1-methyl-2-pyrrolidinone, *tert*-butanol, 2,2,2-trifluoroethanol, triphenylphosphine, DEAD, DMF (puriss.), phenol (puriss.), sodium hydroxide, sodium hydrogenphosphate, potassium dihydrogenphosphate, ninhydrin, potassium cyanide were obtained from Fluka (Buchs, Switzerland). *N,N'*-diisopropylcarbodiimide (DIC), *p*-thiocresol were purchased from Aldrich (Buchs, Switzerland). DMF (pure), dichloromethane, methanol, diethylether were purchased from Scharlau (La Jota, Barcelona, Spain). Chloroform, ethanol, n-hexane and acetonitrile were obtained from

Romil (Cambridge, England). Lithium hydroxide and acetic anhydride were purchased from Merck (Darmstadt, Germany). All material used for cell culture was purchased from Gibco. [^{3}H-propionyl]-NPY was purchased from Amersham.

3.5.2 General Methods

The assembly of the linear peptides was performed on an automated multiple peptide synthesizer (Syro, MultiSynTech, Bochum). Analytical and semi-preparative reverse phase HPLC was performed on a LiChrospher RP-18 column (5 µm, 3 x 125 mm, Merck, Darmstadt, Germany) and on a Delta-Pak C_{18} column (15 µm, 300 Å, 8 x 100 mm, Waters), respectively, using a Merck-Hitachi L-7100 liquid chromatograph. The following gradients were used: from 0 to 60% A over 30 min, or from 0 to 50% A over 30 min at the flow rate of 0.6 ml/min on the analytical column, from 5 to 36% A over 23 min at the flow rate of 3 ml/min on the semi-preparative column. The binary solvent system (A/B) was as follows: 0.1% TFA in acetonitrile (A), 0.1% TFA in water (B). The absorbance was detected at 220 nm. Electrospray ionization mass spectrometry (ESI-MS) was performed on a Finnigan mass spectrometer. Radioactivity was determined by using a Beckman LS-6500 β^- counter. Circular dichroism was recorded using a JASCO model J720 spectropolarimeter. The CD spectra were measured over 250-190 nm at 20 °C in N_2 atmosphere. The cyclopeptides were dissolved in 20 mM phosphate buffer, pH 7.0, at the concentration of 0.1 mM. Each measurement was performed four times using a thermostatable sample cell with a path of 0.02 cm. Response time was set at 2 s at a scan speed of 20 nm/min, a sensitivity range of 10 mdeg and a step resolution of 0.2 nm. High-frequency noise was reduced by means of a low-path Fourier-transform filter. The CD spectrum of the solvent was subtracted from the CD spectra of the peptide solutions to eliminate the interference from cell, solvent and optical equipment. The values for the mean-residue molar ellipticity $[\Theta]_R$ were expressed in deg cm^2 dmol^{-1}.

3.5.3 Preparation of Cells

SK-N-MC cells were grown in Minimum Essential Medium (MEM) with Earl's salts containing 10% fetal calf serum, 4 mM glutamine, 1 mM sodium pyruvate and 1% non-essential amino acids. SMS-KAN cells were grown under the same conditions using

Dulbecco's MEM/Nutrient mix F12 1:1 with 15% fetal calf serum.[29] BHK cells were cultured in Dulbecco's MEM with 10% fetal calf serum, 5% penstrep and 0.1% geneticin. Cells were grown to confluency at 37 °C and 5% CO_2. The growth medium was removed and the cells were washed with Dulbecco's phosphate buffered saline (PBS). After incubation at room temperature for three minutes with PBS containing 0.02% EDTA, the cells were detached by mechanical agitation and suspended in new medium or incubation buffer.

3.5.4 Binding Assays

Assays were performed using the following incubation buffer: MEM with Earl's salts containing 0.1% bacitracin, 50 µM pefabloc SC and 1% bovine serum albumin (BSA). Growth medium was removed and cells were washed if necessary and resuspended in incubation buffer. 200 µl of the suspension containing ca. 300000 cells were incubated with 25 µl 10 nM [^{3}H-propionyl]-NPY and 25 µl 10 µM NPY or cyclopeptide (**10-12**). Nonspecific binding was defined in the presence of 1 µM cold NPY. After 1.5 hours at room temperature the incubation was terminated by centrifugation at 2000 x g and 4 °C for five minutes. The pellets were then washed once with PBS by centrifugation, resuspended in PBS and mixed with scintillation cocktail. Radioactivity displacements by the cyclopeptides **10-12** were measured at the human Y_1-, Y_2- and Y_5-receptors. Specific binding (i.e., total minus unspecific binding in the presence of 1 µM NPY) was equated to 100%. Each experiment was performed in triplicate and data are given ± S.E.M.. At the Y_1-receptor the following displacements were shown: 37% ± 3 by **10**, 35% ± 2 by **11** and 41% ± 4 by **12**. A displacement < 20% was observed at the Y_2- and Y_5-receptors by all three peptides.

3.5.5 Anchoring of *N*-(9-Fluorenylmethoxycarbonyl)-Tyrosine Methyl Ester to the Resins 1a-c

Anchoring of *N*-(9-Fluorenylmethoxycarbonyl)-Tyrosine Methyl Ester to the Resins **1a-c** was accomplished by using the general procedure described below for the preparation of the resin **3a**.

3.5.5.1 Resin 3a

The HMPB-MBHA resin **1a** (100 mg; loading: 0.44 mmol/g) was swollen in 2 ml dichloromethane for 15 min, then the excess solvent was removed by filtration. *N*-(9-Fluorenylmethoxycarbonyl)-tyrosine methyl ester (96 mg, 0.23 mmol) and triphenylphosphine (72 mg, 0.27 mmol) were dissolved in 2 ml dichloromethane and added to the swollen resin. 42 µl (0.27 mmol) of DEAD were diluted to 200 µl with dichloromethane and added dropwise to the mixture at room temperature. The reaction was run overnight, then the mixture was removed and the resin washed sequentially with dichloromethane (3 x), DMF (3 x), methanol (2 x), DMF (2 x) and finally with diethylether, then dried in vacuo. The substitution level of the loaded resin **3a** was measured spectrophotometrically by Fmoc-cleavage[15] and ninhydrin assay,[16] and was determined to be 0.17 mmol/g. The remaining free hydroxy groups on the resin were acetylated with acetic anhydride (26 µl, 0.28 mmol) in the presence of DIEA (48 µl, 0.28 mmol) in dichloromethane (0.5 ml) for 20 min.

3.5.5.2 Resin 3b

The Wang resin **1b** (237 mg; loading: 0.96 mmol/g) was treated by following the protocol described above for the resin **1a**, with the only variation being that the DEAD solution in dichloromethane was added dropwise at 0 °C. The mixture was kept at 0 °C over one hour after DEAD addition, then it was left to warm to room temperature. The substitution level of the loaded resin **3b** was 0.23 mmol/g.

3.5.5.3 Resin 3c

The Sasrin resin **1c** (100 mg; loading: 1.06 mmol/g) was loaded with *N*-(9-fluorenylmethoxycarbonyl)-tyrosine methyl ester by repeating twice the procedure outlined above for the loading of **1a**. The first time, *N*-methylmorpholine was used as the reaction solvent, while dichloromethane was used the second time. The final amino acid substitution on the loaded resin **3c** was determined to be 0.10 mmol/g.

3.5.6 Synthesis of the Resin Bound Peptide Methyl Esters 4a-b, 5b and 6b

The peptide ester **4** was synthesized on both resins **3a** and **3b**. The peptide esters **5** and **6** were synthesized once starting from the resin **3b**. The chain assembly was performed by the Fmoc-strategy using HOBt/DIC as the activation reagents. The Fmoc-protecting group was removed by treatment with 40% piperidine in DMF for three min, 20% piperidine for seven min and finally 40% piperidine for five min. Each amino acid was introduced by a double coupling (2 x 36 min) using a ten-fold excess of the Fmoc-amino acid and of the activation reagents.

After the peptide chain was completed, a small scale cleavage of the peptide esters **4-6** was performed to characterize them as follows: some peptide resin beads were treated with 0.1 ml of the cleavage mixture TFA/triisopropylsilane/water (93/3.5/3.5 v/v) for three hours, then 1 ml ice cold ether was added to precipitate the peptide. The suspension was centrifuged, the ether decanted, and the product suspended again in ice cold ether. The washing was repeated three times. The peptide was dissolved in 0.1 ml *tert*-butanol/water 4/1 (w/w), while the resin was removed by centrifugation. The peptide esters were characterized by analytical HPLC and ESI-MS. Peptide **4**: t_R 15.26 min; MS calcd for $C_{56}H_{88}N_{18}O_{15}$ $[M+H]^+$ 1254.4, found 1254.3. Peptide **5**: t_R 15.84 min; MS calcd for $C_{62}H_{100}N_{18}O_{15}$ $[M+H]^+$ 1338.6, found 1338.2. Peptide **6**: t_R 16.45 min; MS calcd for $C_{64}H_{102}N_{20}O_{17}$ $[M+H]^+$ 1424.6, found 1424.1.

3.5.7 Hydrolysis of the Resin Bound Peptide Methyl Esters. General Procedure for the Synthesis of 7a-b, 8b and 9b

The peptide resins **4a-b, 5b and 6b** (each 20 mg) were suspended in 0.5 ml methanol for 15 min, then the solvent was removed by filtration. The hydrolysis was performed using 0.6 ml of one of the two following solutions: 0.33 M lithium hydroxide in methanol/water (97/3 v/v) for six days or 0.4 M sodium hydroxide in methanol for three days. After removal of the hydrolysis mixture, the resin was washed with methanol (3 x), DMF (3 x), dichloromethane (2 x), DMF (2 x), methanol (2 x), DMF (2 x) and finally with diethylether, then dried in vacuo. To characterize the peptide acids **7-9**, a small scale cleavage was carried out as described above for the characterization of the peptide esters **4-6**. The peptide acids were characterized by analytical HPLC and ESI-MS. Peptide **7**: t_R

12.10 min; MS calcd for $C_{55}H_{86}N_{18}O_{15}$ $[M+H]^+$ 1240.4, found 1240.4. Peptide **8**: t_R 12.62 min; MS calcd for $C_{61}H_{98}N_{18}O_{15}$ $[M+H]^+$ 1324.6, found 1324.4. Peptide **9**: t_R 12.90 min; MS calcd for $C_{63}H_{100}N_{20}O_{17}$ $[M+H]^+$ 1410.6, found 1410.5.

3.5.8 Cyclization of the Resin Bound Peptide Acids. General Procedure for the Synthesis of 10a-b, 11b and 12b

The peptide resins **7a** (26 mg), **7b**, **8b** and **9b** (each 20 mg) were swollen in 0.6 ml DMF, then the excess solvent was removed by filtration. The cyclization was carried out by using two different coupling reagents in different n-fold excess and over different reaction times. **A:** HOBt/TBTU/DIEA/DMF (0.05 mmol/0.05 mmol/0.09 mmol/0.7 ml), corresponding to 15-fold excess of condensing reagents and 26-fold excess of the base, five hours. **B:** HOBt/TBTU/DIEA/DMF (0.02 mmol/0.02 mmol/0.04 mmol/0.5 ml), corresponding to six-fold excess of condensing reagents and 12-fold excess of the base, four hours. **C:** HOBt/DIC/DIEA/DMF (0.05 mmol/0.05 mmol/0.05 mmol/0.6 ml), corresponding to 15-fold excess of condensing reagents and of the base, 19 hours. **D:** HOBt/DIC/DIEA/DMF (0.02 mmol/0.02 mmol/0.02 mmol/0.7 ml), corresponding to six-fold excess of condensing reagents and of the base, 22 hours. The free amine content on the resin was controlled by a qualitative ninhydrin assay. The coupling mixture was removed and the resin washed with DMF (3 x), dichloromethane (2 x), DMF (2 x), methanol (2 x), DMF (2 x) and finally with diethylether, then dried in vacuo.

3.5.9 Cleavage of the Cyclopeptides 10-12 from the Wang Resin 1b. General Procedure

The dried peptide resins **10b**, **11b** and **12b** (each 20 mg) were treated for three hours with 0.6 ml of one of the following cleavage mixtures: TFA/triisopropylsilane/water (90/7/3 v/v); TFA/triisopropylsilane/water/phenol (93.5/2.6/2.6/1.3 w/w); TFA/thio-anisole/phenol (90.5/4.2/5.3 w/w). Then the resin was filtered off and washed with neat TFA (2 x 0.2 ml). The filtrates were pooled and the peptide was recovered by addition of 4 ml of ice cold ether. The suspension was centrifuged, the ether decanted, and the peptide washed with ice cold ether until removal of the scavengers was complete. After drying under a stream of N_2, the peptide was dissolved in *tert*-butanol/water 4/1 (w/w) and

lyophilized. The crude product was purified by semi-preparative HPLC and characterized by analytical HPLC and ESI-MS. Peptide **10**: 4.0 mg (55% overall yield); t_R 13.48 min (95.1% purity); MS calcd for $C_{55}H_{84}N_{18}O_{14}$ $[M+H]^+$ 1222.4, found 1222.2. Peptide **11**: 3.0 mg (35% overall yield); t_R 14.46 min (95.6% purity); MS calcd for $C_{61}H_{96}N_{18}O_{14}$ $[M+H]^+$ 1306.6, found 1306.2. Peptide **12**: 3.1 mg (36% overall yield); t_R 14.10 min (95.5% purity); MS calcd for $C_{63}H_{98}N_{20}O_{16}$ $[M+H]^+$ 1392.6, found 1392.3.

3.5.10 Cleavage of the Linear Peptides 4-9 and Cyclopeptides 10-12

Cleavage of the linear peptides **4-9** and cyclopeptides **10-12** in the presence of the scavengers thioanisole and thiocresol was accomplished by following the procedure described below for the cleavage of **10** from the HMPB-MBHA resin **1a**.

3.5.10.1 Cleavage of the Cyclopeptide 10 from the Resin 1a

The dried peptide resin **10a** (10 mg) was treated with 0.3 ml of the cleavage mixture TFA/thioanisole/thiocresol (96/2/2 v/v) for three hours. The resin was removed by filtration and the peptide **10** recovered by addition of ice cold ether and centrifugation. The analytical HPLC and ESI-MS of the purified cyclopeptide were identical to those reported above for the same peptide cleaved from the Wang resin **1b**.

3.6 References

(1) Reviewed in *Combinatorial Peptide and Nonpeptide Libraries: A Handbook*; Jung, G., Ed.; VCH: Weinheim, New York, Basel, Cambridge, Tokyo, 1996.

(2) Reviewed in Gordon, E. M.; Barrett, R. W.; Dower, W. J.; Fodor, S. P.; Gallop, M. A. *J. Med. Chem.* **1994**, *37*, 1385-1401.

(3) (a) Hoffmann, S.; Rist, B.; Videnov, G.; Jung, G.; Beck-Sickinger, A. G. *Regul. Pept.* **1996**, *65*, 61-70. (b) Geraghty, R. F.; Irvine, G. B.; Williams, C. H.; Cottrell, G. A. *Peptides* **1994**, *15*, 73-81. (c) Heimbrook, D. C.; Saari, W. S.; Balishin, N. L.; Fisher, T. W.; Friedman, A.; Kiefer, D. M.; Rotberg, N. S.; Wallen, J. W.; Oliff, A. *J. Med. Chem.* **1991**, *34*, 2102-2107.

(4) (a) Pfaff, M.; Tangemann, K.; Müller, B.; Gurrath, M.; Müller, G.; Kessler, H.; Timpl, R.; Engel, J. *J. Biol. Chem.* **1994**, *269*, 20233-20238. (b) Geyer, A.; Müller, G.; Kessler, H. *J. Am. Chem. Soc.* **1994**, *116*, 7735-7743.

(5) (a) Hruby, V. J.; Bartosz-Bechowski, H.; Davis, P.; Slaninova, J.; Zalewska, T.; Stropova, D.; Porreca, F.; Yamamura, H. I. *J. Med. Chem.* **1997**, *40*, 3957-3962. (b) McMurray, J. S.; Budde, R. J.; Ke, S.; Obeyesekere, N. U.; Wang, W.; Ramdas, L.; Lewis, C. A. *Arch. Biochem. Biophys.* **1998**, *355*, 124-130. (c) Satoh, T.; Li, S.; Friedman, T. M.; Wiaderkiewicz, R.; Korngold, R.; Huang, Z. *Biochem. Biophys. Res. Commun.* **1996**, *224*, 438-443.

(6) Reviewed in Blackburn, C.; Kates, S. A. *Methods Enzymol.* **1997**, *289*, 175-198.

(7) (a) Rovero, P.; Quartara, L.; Fabbri, G. *Tetrahedron Lett.* **1991**, *32*, 2639-2642. (b) Spatola, A. F.; Darlak, K.; Romanovskis, P. *Tetrahedron Lett.* **1996**, *37*, 591-594.

(8) Romanovskis, P.; Spatola, A. F. *J. Peptide Res.* **1998**, *52*, 356-374.

(9) Alsina, J.; Chiva, C.; Ortiz, M.; Rabanal, F.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **1997**, *38*, 883-886.

(10) (a) Breipohl, G.; Knolle, J.; Stüber, W. *Int. J. Pept. Protein Res.* **1990**, *35*, 281-283. (b) Albericio, F.; Van Abel, R.; Barany, G. *Int. J. Pept. Protein Res.* **1990**, *35*, 284-286.

(11) Alsina, J.; Rabanal, F.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **1994**, *35*, 9633-9636.

(12) Beck-Sickinger, A. G.; Jung, G. *Biopolymers* **1995**, *37*, 123-142.

(13) Michel, M. C.; Beck-Sickinger, A. G.; Cox, H.; Doods, H. N.; Herzog, H.; Larhammar, D.; Quirion, R.; Schwartz, T.; Westfall, T. *Pharmacol. Rev.* **1998**, *50*, 143-150.

(14) Reviewed in (a) Mitsunobu, O. *Synthesis* **1981**, 1-28 and (b) Hughes, D. L. *Organic Preparations and Procedures Int.* **1996**, *28*, 127-164.

(15) Meienhofer, J.; Waki, M.; Heimer, E. P.; Lambros, T. J.; Makofske, R. C.; Chang, C. D. *Int. J. Pept. Protein Res.* **1979**, *13*, 35-42.

(16) Sarin, V. K.; Kent, S. B.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147-157.

(17) Richter, L. S.; Gadek, T. R. *Tetrahedron Lett.* **1994**, *35*, 4705-4706.

(18) Krchňák, V.; Flegelová, Z.; Weichsel, A. S.; Lebl, M. *Tetrahedron Lett.* **1995**, *36*, 6193-6196.

(19) Pavone, V.; Lombardi, A.; Saviano, M.; Di Blasio, B.; Nastri, F.; Fattorusso, R.; Maglio, O.; Isernia, C. *Biopolymers* **1994**, *34*, 1505-1515.

(20) (a) Möhle, K.; Gussmann, M.; Hofmann, H. J. *J. Comput. Chem.* **1997**, *18*, 1415-1430. (b) Cann, J. R.; London, R. E.; Unkefer, C. J.; Vavrek, R. J.; Stewart, J. M. *Int. J. Pept. Protein Res.* **1987**, *29*, 486-496. (c) Yasui, S. C.; Keiderling, T. A.; Formaggio, F.; Bonora, G. M.; Toniolo, C. *J. Am. Chem. Soc.* **1986**, *108*, 4988-4993.

(21) Story, S. C.; Aldrich, J. V. *Int. J. Pept. Protein Res.* **1994**, *43*, 292-296.

(22) Plaué, S. *Int. J. Pept. Protein Res.* **1990**, *35*, 510-517.

(23) Beck-Sickinger, A. G., Köppen, H., Hoffmann, E., Gaida, W., Jung, G. *J. Recept. Signal Transduct. Res.* **1993**, *13*, 215-228.

(24) Woody, R. W.; Dunker, A. K. In *Circular Dichroism and the Conformational Analysis of Biomolecules*; Fasman, G. D., Ed.; Plenum Press: New York, 1996, pp 109-157.

(25) Perczel, A.; Hollósi, M. In *Circular Dichroism and the Conformational Analysis of Biomolecules*; Fasman, G. D., Ed.; Plenum Press: New York, 1996, pp 285-380.

(26) Wen, J. J.; Spatola, A. F. *J. Pept. Res.* **1997**, *49*, 3-14.

(27) Mihara, H.; Yamabe, S.; Niidome, T.; Aoyagi, H. *Tetrahedron Lett.* **1995**, *36*, 4837-4840.

(28) Paquet, A. *Can. J. Chem.* **1982**, *60*, 976-980.

(29) Ingenhoven, N.; Beck-Sickinger, A. G. *J. Recept. Signal Transduct. Res.* **1997**, *17*, 407-418.

CHAPTER 4

Y-Receptor Affinity Modulation by the Design of Pancreatic Polypeptide/Neuropeptide Y Chimera Led to Y_5-Receptor Ligands with Picomolar Affinity

Chiara Cabrele[‡], Heike A. Wieland[§], Michael Langer[‡], Carsten Stidsen[†] and Annette G. Beck-Sickinger[‡*]

[‡]Department of Pharmacy, ETH Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
[§]Division of Preclinical Research, Boehringer Ingelheim Pharma KG, Birkendorferstrasse 65, 88397 Biberach, Germany
[†]Molecular Pharmacology, Novo Nordisk Park, 2760 Malov, Denemark

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4.1 Abstract

Neuropeptide Y (NPY) and pancreatic polypeptide (PP) bind to the Y-receptors with very different affinity: NPY has high affinity for the receptors Y_1, Y_2 and Y_5, while PP only binds to Y_4-receptor with picomolar affinity. By exchange of special amino acid positions between the two peptides, we developed 38 full-length PP/NPY chimera with binding properties that are completely different from those of the two native ligands. On the one hand, pig NPY (pNPY) analogs containing the segment 19-23 from human PP (hPP) bound to the Y-receptors with much lesser affinity than NPY. On the other hand, the affinity of the hPP analog containing the pNPY segments 1-7 and 19-23 was comparable to that of pNPY at the Y_1- and Y_5-receptor subtypes, and to that of hPP at the Y_4-receptor. Furthermore, modification of the hPP sequence by the introduction of the segments 1-7 from chicken PP (cPP) and 19-23 from pNPY yielded a ligand with an affinity of 40 pM at the Y_5-receptor. At present, this is the most potent Y_5-receptor known, displaying 15-fold higher affinity than NPY.

4.2 Introduction

Neuropeptide Y (NPY), pancreatic polypeptide (PP) and peptide YY (PYY) are all members of the NPY hormone family.[1, 2] The three peptides consist of 36 residues, are C-terminally amidated and show high sequence identity, which is 69% between NPY and PYY, and 50% between NPY and PP.[3] The tertiary structure of avian PP was determined by X-ray crystallography and shows a characteristic antiparallel fold of a type II polyproline helix (residues 1-8) and of an α-helix (residues 14-31), connected by a loop (residues 9-13).[3, 4] The C-terminal pentapeptide adopts a flexible turn structure in the crystal.

NPY is one of the most abundant neuropeptides in the central and peripheral nervous systems, and is present at high concentrations in most brain structures, especially in the cortical areas, hippocampus and hypothalamus.[5] The most relevant biological actions of NPY are stimulation of food intake, increase in memory retention, inhibition of the sexual behavior, vasoconstriction, anxiolysis, regulation of neurotransmitter release, and modulation of ethanol consumption.[6, 7] These effects are transmitted by at least five different receptor subtypes which belong to the large superfamily of the G-protein coupled receptors and are referred to as the Y_1-, Y_2-, Y_4-, Y_5- and y_6-receptor subtypes.[8] Recently, a strong interest has been focused on the NPY-induced increase in food intake. At present, the Y_1- and Y_5-receptor subtypes are believed to be involved in food intake induced by NPY, however, the individual role of each of these two receptors is still unclear.[9, 10]

NPY and PYY have high affinity for the Y_1-, Y_2-, and Y_5-receptors, while PP is characterized by strong affinity at the Y_4-subtype. NPY is one of the most conserved peptides during evolution, whereas PYY and PP evolved more rapidly.[11] In particular, PP shows only 50% identity between mammals, birds and amphibians. NPY and PYY have been found in all different species investigated so far, while PP is present only in tetrapods. Alignment of all NPY sequences known so far and comparison with the NPY ancestor revealed 22 identical positions among the species, which are positions 2, 4, 5, 8, 9, 12, 13, 18, 20, 23, and 25 to 36 (Table 1). The remaining 14 positions are also highly conserved and the replacement generally involves similar residues: for example, the Glu residue at positions 10 and 15 in the NPY ancestor was replaced by Asp, whereas the Asp residue at position 16 was exchanged by Glu. Further highly conservative substitutions are Leu-Met, Tyr-Phe, Lys-Arg and Ala-Gly. In the case of PYY, 15 positions are perfectly conserved

Table 1. Sequences of porcine NPY, human PYY, and human, rat, chicken and goose PP. Human and porcine NPY simply differ at position 17, where Met is present in hNPY in place of Leu.

Peptide	Amino acid sequence[a]
	1 5 10 15 20 25 30 35
pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY
hPYY	YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY
hPP	APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY
rPP	APLEPMYPGDYATHEQRAQYETQLRRYINTLTRPRY
cPP	GPSQPTYPGDDAPVEDLIRFYNDLQQYLNVVTRHRY
gPP	GPSQPTYPGNDAPVEDLXRFYDNLQQYRLNVFRHRY

[a] The constant positions of NPY, PYY and PP among the different species are underlined.

among all different species, which are positions 2, 4, 5, 8, 12, 14, 20, 25, 27, 29, 30 and 32 to 35 (Table 1), and, with the exception of Ala[14], these positions are also identical in the NPY sequences. In the case of PP, positons 5, 8, 9, 12, 27, 33 and 35 are conserved among the different species. Interestingly, again these positions are not only identical among the PP species but also among the NPY and PYY species, with the only exception of PYY from sea lamprey, where Gly^9 is substituted by Ser. It has to be stressed that the constant residues Pro at positions 5 and 8 and Tyr at position 27 are postulated to stabilize the hairpin-like folding of all PP/NPY peptides by interacting via their side-chains, and, consequently, by keeping the N- and C-terminal segments close to each other.[4] Furthermore, in all three peptides the C-terminal fragment is the most conserved part. Especially, the two Arg residues at positions 33 and 35 are present in all species and, according to previous structure-affinity relationship studies, [12, 13] are responsible for the ligand-receptor interaction. While in most PP species Pro is present at position 34 (only in few species it is exchanged by His), a Gln residue is found in all NPY and PYY peptides. This peculiarity of the PP sequence suggested an important role for this position, which was confirmed by the binding profile of the analogs [Pro^{34}]-NPY and [Leu^{31}, Pro^{34}]-NPY.[14] These compounds bind to the Y_1-, Y_4- and Y_5-receptor subtypes, but not to the Y_2-receptor. Furthermore, the PP analog containing the NPY residues Ile^{31} and Gln^{34} turned out to be a poor Y_4-receptor ligand but fully active at the Y_2-receptor.[2] These three

peptides were the first example of PP/NPY chimera by which a modulatory effect on the binding affinity of the native NPY and PP was obtained.

In this work, we present the results of the design, synthesis and structure-affinity relationship studies of several different sets of PP/NPY chimera. The chimera are based on the sequence of pNPY and hPP and modified by single or multiple exchange position between the two parent peptides. The sequences of the PP/NPY chimera are shown in Table 2. The peptides were synthesized by solid-phase technique; their conformational properties were investigated by circular dichroism (CD) spectroscopy and their receptor binding affinity was determined at the Y_1-, Y_2-, Y_4- and Y_5-receptor subtypes. Our results suggest that the N-terminal segment 1-7 and the central positions 19-23 play a modulatory effect on the binding of the different receptor subtypes: accordingly, the introduction of the hPP sequence 19-23 into pNPY caused a loss of affinity either at the NPY-preferring receptors or at the PP-preferring receptor. On the other hand, hPP was converted to a potent Y_1- and Y_5-receptor ligand by the introduction of the pNPY sequences 1-7 and 19-23. Structurally, the hPP sequence 19-23 caused a decrease in the helical content of pNPY, whereas the pNPY sequence 19-23 induced a conformational change of hPP, without perturbing the stability of the hPP helix.

4.3 Results

4.3.1 Peptide Synthesis

Seven positions are identical for all members of the NPY family. In addition, NPY, PYY and PP exhibit member specific highly conserved positions. We investigated the role of these positions with respect to receptor recognition and binding by the design, synthesis and testing of analogs of each member of the hormone family containing primary structure features of the other parent peptides. Accordingly, characteristic PP and PYY elements were introduced into the NPY sequence, while typical NPY positions were incorporated into the PYY and PP sequences. Furthermore, particular positions present only in one or two species of PP were introduced into the human PP sequence. Accordingly, the peptides **22**, **28** and **24**, **30** contain Glu^{21} and Gln^{23}, respectively, two positions which are present only in rat and mouse PP; the peptides **38**-**40** contain the sequence 1-7 found only in turkey

and chicken PP, and the peptide **40** contains positions 19-23 present only in goose PP (gPP). In total, 38 different PP/NPY chimera, each containing 36 residues, were prepared by multiple solid-phase technique using Fmoc/*tert*-butyl chemistry and HOBt/DIC as the coupling reagents. They were characterized by analytical HPLC and electrospray ionization mass spectrometry. The amino acid sequences are shown in Table 2. Binding affinity of each peptide was determined on Y-receptor selective expressing cell lines, and the IC_{50} values are reported in Table 3.

4.3.2 Receptor Binding Affinity

To characterize the biological properties of the PP/NPY chimera, competition binding assays were performed on cell lines selectively expressing the Y_1-, Y_2-, Y_4- or Y_5- receptor subtypes.[15] Position 7 is highly conserved: Asn^7 is found in most NPY and PYY species, whereas Tyr^7 is present in most PP species. Replacement of Asn^7 with Tyr (peptide **4**) reduced the NPY affinity of three- to seven-fold at the Y_1-, Y_4- and Y_5-receptor subtypes. The double substitution of the NPY residues Lys^4 by Glu and Gln^{34} by Pro (peptide **5**) was characterized by a decrease in the Y_1- (29-fold) and Y_5- (21-fold) receptor affinity, but the Y_4-receptor affinity was increased three-fold. The introduction of the pNPY positions 13-14 into hPYY and vice versa (peptides **3** and **6**) reduced the binding potency, especially at the Y_1-receptor for the PYY analog (20-fold), and at the Y_4-receptor for the NPY analog (six-fold). By the exchange of the whole N-terminal region 1-7 of pNPY with the corresponding hPP segment (peptide **7**), we found a 13-fold decrease at the Y_1-receptor, a four-fold decrease at the Y_5-receptor, but almost two-fold increase at the Y_4-receptor. By extending the N-terminal replacement of hPP up to position 17, the analog **8** turned out to be eight-fold less potent at the Y_1-receptor, but 19-fold more potent at the Y_4-receptor and slightly more potent than NPY at the Y_5-receptor. Moreover, by the additional exchange of Gln^{34} with His, which is present in some PP species like chicken and turkey, the Y_1- and Y_4-receptor subtype affinity was slightly further improved (1.4 nM and 0.15 nM, respectively), while the Y_5-receptor affinity was reduced 2.4-fold (1.1 nM for [hPP^{1-17}, H^{34}]-pNPY, peptide **9**, versus 0.45 nM for [hPP^{1-17}]-pNPY, peptide **8**). A remarkable loss of affinity was obtained after substitution of the pNPY sequence 19-23 (RYYSA) with the corresponding hPP sequence (QYAAD), as the IC_{50} values dropped more than 63-fold at

Table 2. Sequences and analytical data of the PP/NPY chimera. Positions that differ from the parent peptide are underlined.

No.	Peptide	Amino acid sequence	M.W.$_{exp}$ [amu]	M.W.$_{theor}$ [amu]
1	pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY	4253	4253.7
2	hPP	APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY	4181	4181.8
3	[P^{13}, A^{14}]-hPYY	YPIKPEAPGEDAPAEELNRYYASLRHYLNLVTRQRY	4293	4293.8
4	[Y^{7}]-pNPY	YPSKPDYPGEDAPAEDLARYYSALRHYINLITRQRY	4301	4302.8
5	[E^{4}, P^{34}]-pNPY	YPSEPDNPGEDAPAEDLARYYSALRHYINLITRPRY	4222	4223.7
6	[S^{13}, P^{14}]-pNPY	YPSKPDNPGEDASPEDLARYYSALRHYINLITRQRY	4269	4269.7
7	[hPP$^{1-7}$]-pNPY	APLEPVYPGEDAPAEDLARYYSALRHYINLITRQRY	4221	4221.8
8	[hPP$^{1-17}$]-pNPY	APLEPVYPGDNATPEQMARYYSALRHYINLITRQRY	4267	4267.9
9	[hPP$^{1-17}$, H^{34}]-pNPY	APLEPFYPGDNATPEQMARYYSALRHYINLITRHRY	4276	4276.9
10	[hPP$^{19-23}$]-pNPY	YPSKPDNPGEDAPAEDLAQYAADLRHYINLITRQRY	4160	4161.6
11	[hPP$^{19-23}$, P^{34}]-pNPY	YPSKPDNPGEDAPAEDLAQYAADLRHYINLITRPRY	4129	4130.6
12	[hPP$^{19-23}$, H^{34}]-pNPY	YPSKPDNPGEDAPAEDLAQYAADLRHYINLITRHRY	4169	4170.6
13	[rPP$^{19-23}$]-pNPY	YPSKPDNPGEDAPAEDLAQYETQLRHYINLITRQRY	4261	4262.7
14	[rPP$^{19-23}$, P^{34}]-pNPY	YPSKPDNPGEDAPAEDLAQYETQLRHYINLITRPRY	4230	4231.7
15	[rPP$^{19-23}$, H^{34}]-pNPY	YPSKPDNPGEDAPAEDLAQYETQLRHYINLITRHRY	4270	4271.7
16	[Y^{1}]-hPP	YPLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY	4272	4273.9
17	[K^{4}]-hPP	APLKPVYPGDNATPEQMAQYAADLRRYINMLTRPRY	4179	4180.9
18	[N^{7}]-hPP	APLEPVNPGDNATPEQMAQYAADLRRYINMLTRPRY	4131	4132.7
19	[P^{13}, A^{14}]-hPP	APLEPVYPGDNAPAEQMAQYAADLRRYINMLTRPRY	4151	4151.8
20	[pNPY$^{1-7}$]-hPP	YPSKPDNPGDNATPEQMAQYAADLRRYINMLTRPRY	4214	4213.8
21	[Y^{21}]-hPP	APLEPVYPGDNATPEQMAQYYADLRRYINMLTRPRY	4273	4273.9
22	[E^{21}]-hPP	APLEPVYPGDNATPEQMAQYEADLRRYINMLTRPRY	4239	4239.8
23	[A^{23}]-hPP	APLEPVYPGDNATPEQMAQYAAALRRYINMLTRPRY	4137	4137.8

Table 2. (Continued).

No.	Peptide	Amino acid sequence	M.W.$_{exp}$ [amu]	M.W.$_{theor}$ [amu]
24	[Q^{23}]hPP	APLEPVYPGDNATPEQMAQYAAQLRRYINMLTRPRY	4194	4194.8
25	[R^{19}]-hPP	APLEPVYPGDNATPEQMARYAADLRRYINMLTRPRY	4209	4209.9
26	[Q^{34}]-hPP	APLEPVYPGDNATPEQMAQYAADLRRYINMLTRQRY	4211	4212.8
27	[F^{6}, Y^{21}]-hPP	APLEPFYPGDNATPEQMAQYYADLRRYINMLTRPRY	4321	4321.9
28	[F^{6}, E^{21}]-hPP	APLEPFYPGDNATPEQMAQYEADLRRYINMLTRPRY	4287	4287.9
29	[F^{6}, A^{23}]-hPP	APLEPFYPGDNATPEQMAQYAAALRRYINMLTRPRY	4184	4185.8
30	[F^{6}, Q^{23}]-hPP	APLEPFYPGDNATPEQMAQYAAQLRRYINMLTRPRY	4242	4242.9
31	[F^{6}, R^{19}]-hPP	APLEPFYPGDNATPEQMARYAADLRRYINMLTRPRY	4257	4257.9
32	[pNPY$^{19-23}$]-hPP	APLEPVYPGDNATPEQMARYYSALRRYINMLTRPRY	4272	4273.9
33	[pNPY$^{19-23}$, Q^{34}]-hPP	APLEPVYPGDNATPEQMARYYSALRRYINMLTRQRY	4304	4305.0
34	[pNPY$^{19-23}$, H^{34}]-hPP	APLEPVYPGDNATPEQMARYYSALRRYINMLTRHRY	4313	4314.0
35	[F^{6}, pNPY$^{19-23}$, Q^{34}]-hPP	APLEPFYPGDNATPEQMARYYSALRRYINMLTRQRY	4351	4353.0
36	[F^{6}, pNPY$^{19-23}$, H^{34}]-hPP	APLEPFYPGDNATPEQMARYYSALRRYINMLTRHRY	4361	4362.0
37	[pNPY$^{1-7, 19-23}$]-hPP	YPSKPDNPGDNATPEQMARYYSALRRYINMLTRPRY	4305	4305.9
38	[cPP$^{1-7}$, pNPY$^{19-23}$]-hPP	GPSQPTYPGDNATPEQMARYYSALRRYINMLTRPRY	4233	4234.8
39	[cPP$^{1-7}$, pNPY$^{19-23}$, H^{34}]-hPP	GPSQPTYPGDNATPEQMARYYSALRRYINMLTRHRY	4274	4274.8
40	[cPP$^{1-7}$, gPP$^{19-23}$]-hPP	GPSQPTYPGDNATPEQMARFYDNLRRYINMLTRPRY	4289	4289.9

the Y_1-, Y_4- and Y_5-receptor subtypes, and 25-fold at the Y_2-receptor (peptide **10**). Interestingly, the additional substitution of Gln^{34} with Pro yielded the peptide **11** that was significantly more potent than peptide **10** at the Y_1- and Y_4-receptor subtypes (from 14.6 nM to 4.2 nM, and from > 1 000 nM to 14.8 nM, respectively), whereas its affinity at the Y_2-receptor was completely lost (1 000 nM), as expected by the presence of Pro^{34}.[14] However, when His^{34} was introduced in place of Pro, there was again a decrease in affinity. Accordingly, the affinity of the analog [hPP$^{19-23}$, H^{34}]-pNPY (peptide **12**) was found to be 43 nM at the Y_1-receptor, 133 nM at the Y_4-receptor, and 113 nM at the Y_5-receptor.

Interestingly, the presence of His^{34} led to a loss of affinity at the Y_2-receptor, as Pro did. In a similar way, the corresponding three peptides containing the rPP sequence 19-23 (QYETQ) (peptides **13-15**) were found to be poor ligands at all receptor subtypes. Again, the presence of His^{34} further reduced the affinity.

To establish whether the perfectly or highly conserved positions of NPY which are different in PP are major structural elements for high affinity at the NPY-preferring Y-receptor subtypes, we designed a series of hPP analogs, where one or more NPY residues were introduced in place of the corresponding hPP postitions. The analogs [Y^1]-hPP (**16**), [K^4]-hPP (**17**), [N^7]-hPP (**18**) and [P^{13}, A^{14}]-hPP (**19**) were found to have a receptor binding profile that was very similar to that of hPP itself, however they lost affinity at the Y_4-receptor (24- to 48-fold). Only Glu^4/Lys exchange increased the Y_1- and Y_5-receptor subtype affinity ten-fold and three-fold, respectively. Surprisingly, by the introduction of the whole pNPY segment 1-7 that comprises all single replacements described above (peptide **20**), the affinity for the Y_1-, Y_4- and Y_5-receptor subtypes was 3-4 nM, which corresponds to an increase of > 200-fold at the Y_1-receptor, a decrement of more than 100-fold at the Y_4-receptor and a 19-fold increase at the Y_5-receptor.

A different binding potency was obtained by replacement of each single position in the region 19-23 of hPP with the corresponding pNPY or rPP positions (peptides **21-25**). The hPP analogs containing the pNPY residue Tyr^{21} (**21**) in place of Ala^{21}, the pNPY residue Ala^{23} (**23**) or the rPP residue Gln^{23} (**24**) in place of Asp, and the pNPY residue Arg^{19} (**25**) in place of Gln, turned out to have some affinity (14-30 nM) at the Y_1-receptor in comparison with hPP; furthermore, they were as potent as hPP at the Y_4-receptor (0.04 nM to 0.09 nM for the analogs versus 0.04 nM for hPP), and comparable to NPY at the Y_5-receptor (1.1 nM to 2.4 nM for the analogs versus 0.6 nM for pNPY). In contrast, the analog **22**, [E^{21}]-hPP, bound to the Y_4- and Y_5-receptor subtypes with much lesser affinity (0.25 nM and 66 nM, respectively). The latter five hPP analogs were further modified by the replacement of Val^6 with a foreign residue, like Phe (position 6 is relatively variable among the PP species and the replacements are poorly conservative: Glu, Met, Thr, Ala, Lys, His). These peptides (**27-31**) still had good affinity at the Y_4-receptor (0.06-1 nM), as well as at the Y_5-receptor. They did not bind to the Y_2-receptor, but their Y_1-receptor affinity was in the range 40-71 nM, which means an increase of up to 25-fold compared to hPP. The only exception was the analog **28**, [F^6, E^{21}]-pNPY, which bound to the Y_1-receptor with an affinity of 540 nM. A very surprising result was obtained when the whole

pNPY segment 19-23 was introduced into the hPP sequence: this analog (**32**) showed higher affinity than hPP not only at the Y_1- (200-fold) and Y_5- (39-fold) receptor, but also at the Y_2-receptor (> 500-fold, corresponding to an affinity of 23 nM), despite the presence of Pro^{34}. The affinity of the peptide **32** was improved at all receptors by the additional replacements of Pro^{34} with Gln and Val^6 with Phe (peptide **35**), which led to subnanomolar affinities at the receptors Y_2, Y_4, and Y_5 (0.19 nM, 0.04 nM and 0.15 nM), and to an affinity of 14 nM at the Y_1-receptor. The parent analog **36** containing His^{34} was characterized by a loss of affinity at all receptors with the exception of the Y_4-receptor.

It should be underlined that the single substitution of Pro^{34} with Gln (peptide **26**) did not improve the affinity of hPP at the Y_1- and Y_5-receptor subtypes, and the IC_{50} values remained > 1 000 nM and 79 nM, respectively, while the Y_4-receptor affinity decreased (1.6 nM versus 0.04 nM). However, after the introduction of the pNPY segment 19-23 (peptide **33**), not only the Y_1- and Y_5-receptor affinity increased to 10 nM and 0.2 nM, respectively, but also the Y_4-receptor affinity was completely recovered (0.05 nM). Similar behavior was observed for the peptide **34** that differs from the peptide **33** only at position 34, in which His is present in place of Gln.

Based on these results, following observations have been made: (1) the pNPY segment 1-7 was sufficient to increase the affinity of hPP to 3-4 nM at the receptor subtypes Y_1 and Y_5. (2) The central sequence 19-23 of pNPY in hPP led to an affinity of 23 nM at the Y_2-receptor, and of 1.5 nM at the Y_5-receptor. Therefore, we designed a hPP analog containing both the N-terminal and central positions of pNPY, and we obtained a ligand that was as potent as NPY at the Y_1-receptor, comparable to hPP at the Y_4-receptor, and finally five-fold more potent than NPY at the Y_5-receptor (peptide **37**). Furthermore, we substituted the pNPY segment 1-7 with the corresponding segment from chicken (and turkey) PP: this new analog (**38**) had an affinity profile similar to the previous ligand, but it was slightly more potent at the Y_4- and Y_5-receptor subtypes, with an affinity of 0.02 nM and 0.07 nM, respectively. The additional introduction of His^{34} in place of Pro (peptide **39**) further increased the Y_5-receptor affinity to 0.04 nM (15-fold higher than that of NPY itself). In the peptide **40** the combination of the cPP sequence 1-7 with the unique sequence 19-23 from goose PP (RFYDN) was characterized by a decrease in affinity, especially at the Y_1-receptor (10.3 nM) and Y_2-receptor (220 nM) subtypes.

Table 3. Binding affinity of the PP/NPY chimera at the Y-receptors.

No.	Peptide	Y_1			Y_2			Y_4			Y_5		
		IC_{50} [nM]	IC_{50}(analog)/ IC_{50}(NPY)	IC_{50}(analog)/ IC_{50}(hPP)	IC_{50} [nM]	IC_{50}(analog)/ IC_{50}(NPY)	IC_{50}(analog)/ IC_{50}(hPP)	IC_{50} [nM]	IC_{50}(analog)/ IC_{50}(NPY)	IC_{50}(analog)/ IC_{50}(hPP)	IC_{50} [nM]	IC_{50}(analog)/ IC_{50}(NPY)	IC_{50}(analog)/ IC_{50}(hPP)
1	hNPY	0.23	1		0.04	1		5.8	1		0.6	1	
2	hPP	>1 000	>4 000	1	>2 000	>50 000	1	0.04	0.007	1	58	97	1
3	[P^{13}, A^{14}]-hPYY	4.5	19.6					9.8	1.7		11.5	19	
4	[Y^{7}]-pNPY	0.62	2.7					18	3.1	450	3.9	6.5	
5	[E^{4}, P^{34}]-pNPY	6.6	29					2.0	0.34	50	12.5	20.8	
6	[S^{13}, P^{14}]-pNPY	1.4	6					34.1	6	853	3.2	5.3	
7	[hPP^{1-7}]-pNPY	3.0	13					3.7	0.6	93	2.2	3.7	
8	[hPP^{1-17}]-pNPY	1.9	8.3					0.3	0.05	7.5	0.45	0.75	
9	[hPP^{1-17}, H^{34}]-pNPY	1.4	6					0.15	0.02	3.8	1.1	1.8	
10	[hPP^{19-23}]-pNPY	14.6	63		1.0	25		>1 000	>172	25 000	191	318	
11	[hPP^{19-23}, P^{34}]-pNPY	4.2	18		1 000	25 000		14.8	2.6	370	62	103	
12	[hPP^{19-23}, H^{34}]-pNPY	43	187		1 000	25 000		133	23	3 325	113	188	
13	[rPP^{19-23}]-pNPY	70	304		11.5	288		21.2	3.7	530	265	442	
14	[rPP^{19-23}, P^{34}]-pNPY	4.8	21		1 000	25 000		46	8	1 150	32	53	
15	[rPP^{19-23}, H^{34}]-pNPY	112	487		1 000	25 000		460	79	11 500	308	513	
16	[Y^{1}]-hPP	>1 000	>4 000	1				1.0	0.17	25	68	113	1.2
17	[K^{4}]-hPP	>100	>400	0.1				1.3	0.2	33	20	33.3	0.34
18	[N^{7}]-hPP	>1 000	>4 000	1				1.9	0.3	48	146	243	2.5
19	[P^{13}, A^{14}]-hPP	>1 000	>4 000	1				0.97	0.17	24	118	197	2

Table 3. (Continued).

No.	Peptide	Y_1			Y_2			Y_4			Y_5		
		IC_{50} [nM]	IC_{50}(analog)/ IC_{50}(NPY)	IC_{50}(analog)/ IC_{50}(hPP)	IC_{50} [nM]	IC_{50}(analog)/ IC_{50}(NPY)	IC_{50}(analog)/ IC_{50}(hPP)	IC_{50} [nM]	IC_{50}(analog)/ IC_{50}(NPY)	IC_{50}(analog)/ IC_{50}(hPP)	IC_{50} [nM]	IC_{50}(analog)/ IC_{50}(NPY)	IC_{50}(analog)/ IC_{50}(hPP)
20	[pNPY$^{1-7}$]-hPP	4.2	18	< 0.004				4.2	0.7	105	3.0	5	0.05
21	[Y^{21}]-hPP	21	91	< 0.021				0.09	0.02	2.3	1.1	1.8	0.02
22	[E^{21}]-hPP							0.25	0.04	6.3	66	110	1.1
23	[A^{23}]-hPP	19	83	< 0.019				0.11	0.02	2.8	1.8	3	0.03
24	[Q^{23}]hPP	30	130	< 0.03				0.09	0.02	2.3	2.4	4	0.04
25	[R^{19}]-hPP	14	61	< 0.014				0.04	0.007	1	1.2	2	0.02
26	[Q^{34}]-hPP	> 1 000	> 4 000	1				1.6	0.3	40	79	132	1.4
27	[F^{6}, Y^{21}]-hPP	51	222	< 0.05	1 000	25 000	< 0.5	0.16	0.03	4	2.4	4	0.04
28	[F^{6}, E^{21}]-hPP	540	> 2 000	< 0.5	1 000	25 000	< 0.5	0.99	0.17	25	38	63	0.6
29	[F^{6}, A^{23}]-hPP	40	174	< 0.04	1 000	25 000	< 0.5	0.06	0.01	1.5	4	7	0.07
30	[F^{6}, Q^{23}]-hPP	59	256	< 0.06	820	20 500	< 0.4	0.1	0.02	2.5	4.5	8	0.08
31	[F^{6}, R^{19}]-hPP	71	309	< 0.07	740	18 500	< 0.4	0.3	0.05	7.5	5.7	10	0.10
32	[pNPY$^{19-23}$]-hPP	50	217	0.5	23	575	< 0.01	0.5	0.09	12.5	1.5	2.5	0.02
33	[pNPY$^{19-23}$, Q^{34}]-hPP	10	43	< 0.01				0.04	0.007	1	0.2	0.33	0.003
34	[pNPY$^{19-23}$, H^{34}]-hPP	19	83	< 0.019				0.04	0.007	1	0.2	0.33	0.003
35	[F^{6}, pNPY$^{19-23}$, Q^{34}]-hPP	14	61	< 0.014	0.19	4.8	< 0.0001	0.04	0.007	1	0.15	0.25	0.002
36	[F^{6}, pNPY$^{19-23}$, H^{34}]-hPP	72	313	< 0.07	57	1 425	< 0.03	0.02	0.003	0.5	23	38.3	0.4
37	[pNPY$^{1-7}$, $^{19-23}$]-hPP	0.25	1.1	< 0.0003	11.7	292	< 0.006	0.08	0.014	2	0.11	0.18	0.002
38	[cPP$^{1-7}$, pNPY$^{19-23}$]-hPP	0.6	2.6	< 0.0006	28	700	< 0.01	0.02	0.003	0.5	0.07	0.12	0.001
39	[cPP$^{1-7}$,pNPY$^{19-23}$, H^{34}]-hPP	5.7	25	< 0.006	22.2	555	< 0.01	0.06	0.01	1.5	0.04	0.07	0.0007
40	[cPP$^{1-7}$, gPP$^{19-23}$]-hPP	10.3	45	< 0.01	220	5 500	< 0.11	0.06	0.01	1.5	0.2	0.33	0.003

4.3.3 Circular Dichroism

The conformation of the PP/NPY chimera in aqueous solution and at neutral pH was evaluated using circular dichroism (CD) spectroscopy (Figures 1 and 2). The CD spectrum of NPY has the typical profile of the α-helix, which consists of two negative bands at 222 nm and 209 nm, and one positive peak at 189 nm (Fig. 1 A). Analysis of the spectrum according to the method of Yang[16] revealed a helical content of 18% and a β-sheet content of 63% (Table 4, peptide **1**). After the introduction of the hPP segment 19-23, the negative band at 222 nm and the positive peak considerably decreased in intensity (Fig. 1 B), indicating the decrease in helicity of the analog **10** in comparison with NPY (7% versus 18%), in favor of random coil (33% versus 17%). A further decrease in the helical content was caused by the replacement of Gln^{34} with Pro or His (4% for the peptide **11** and 3% for the peptide **12**). Also the introduction of the rPP sequence 19-23 induced a conformational change of NPY (peptide **13**): the helicity increased to 26%, the β-sheet dropped to 22%, and the random coil fraction increased to 47% (Fig. 1 C). Again, the additional substitution of Gln^{34} with Pro or His led to a more drastic loss of helicity (analogs **14** and **15**). On the other hand, the replacement of the NPY segment 1-7 with the corresponding hPP segment induced a partial transition (28%) from α-helix to β-sheet, resulting in 13% of helix and 68% of β-sheet for the analog **7**, [hPP^{1-7}]-pNPY (Fig. 1 D).

In contrast to NPY, hPP was characterized by a much more intensive CD spectrum, and the band at 222 nm was more pronounced than that at 209 nm (Fig. 1 A). The helical content was determined to be 49%, and no β-sheet was detected (Table 4, peptide **2**). The CD curves of the analogs [K^4]-hPP (**17**) and [N^7]-hPP (**18**) turned out to be very similar to that of hPP, in shape as well as in intensity (Fig. 2 A). Instead, the replacement of Ala^1 with Tyr led to a decrease in α-helix of 18% in favor of β-sheet (table 4, peptide **16**). The CD profile of the analog **19** containing the pNPY motif Pro^{13}-Ala^{14} was found to be different from that of the hPP parent peptide. The much more intensive band at 209 nm rather than at 222 nm corresponded to an increase in unordered structure of 28% (51% random coil for the analog **19** versus 40% random coil for hPP). The introduction of the pNPY segment 1-7 produced almost the same CD profile as the replacement Pro^{13}/Ala^{14}, only with a minor intensity at 209 nm, corresponding to a smaller amount of random coil structure: 47% for [$pNPY^{1-7}$]-hPP (peptide **20**) versus 51% for [P^{13}, A^{14}]-hPP (peptide **19**) (Fig. 2 A). The hPP analogs obtained by amino acid replacement in the region 19-23 gave CD curves very

similar to that of hPP, in shape and intensity (Fig. 2 B). There were three exceptions: the CD spectrum of the analog **22**, [E^{21}]-hPP, turned out to be more intensive, with a helical content of 58%. In contrast, the CD spectra of [Y^{21}]-hPP (**21**) and [$pNPY^{19-23}$]-hPP (**32**) were both less intensive and characterized by a helical content equal to or slightly higher than that of hPP, but with a smaller fraction of unordered conformation (21% for the analog **21** and 29% for the analog **32** versus 40% for hPP). Interestingly, the CD curves of the analogs **21** and **32** did not cross through the isodichroic point generated by the other chimera and hPP (Fig. 2 B). By comparison of the CD spectrum of each of the following analogs, [R^{19}]-hPP (**25**), [Y/E ^{21}]-hPP (**21/22**), [A/Q^{23}]-hPP (**23/24**), with its corresponding parent peptide containing Phe^6, it was found that Phe^6 did not induce conformational changes in the case of [R^{19}]-hPP and [Q^{23}]-hPP (Fig. 2 C: CD curves for [R^{19}]-hPP and [F^6, R^{19}]-hPP are shown). In contrast, the aromatic residue caused a partial α-helix to β-sheet transition of the other three peptides, which was 15% for [A^{23}]-hPP, 22% for [Y^{21}]-hPP and 48% for [E^{21}]-hPP (Fig. 2 C: CD curves for [Y^{21}]-hPP and [F^6, Y^{21}]-hPP are shown).

As already mentioned above, [$pNPY^{19-23}$]-hPP showed a CD profile that was completely different from that of hPP. A similar profile was also given by its two parent peptides containing one additional substitution at position 34 with Gln (peptide **33**) or His (peptide **34**). The CD spectra of the latter two analogs were more intensive (61-62% helix content) and gave an isodichroic point with [$pNPY^{19-23}$]-hPP (Fig. 2 D). Again, in the presence of Phe^6, the helix content was reduced and formation of β-sheet occurred (Table 4: 12-17% for the peptides **35** and **36**). The single amino acid exchange of Pro^{34} with Gln (peptide **26**) increased the α-helix to 69%, with a loss of turn and unordered structures (Fig. 2 D). When the peptide chimera [$pNPY^{19-23}$]-hPP was additionally modified by the introduction of the pNPY segment 1-7, there was a 12% increase in the amount of helix (Table 4, peptide **37**), while no significant changes occurred after the introduction of the cPP fragment 1-7 (Fig. 2 E and Table 4, peptide **38**). When in the latter analog, [cPP^{1-7}, NPY^{19-23}]-hPP, Pro^{34} was replaced with His (peptide **39**), or the central NPY pentapeptide was substituted with the goose PP sequence (peptide **40**), both the helix and random coil fractions were reduced and some β-sheet was formed.

Finally, the introduction of the N-terminal hPP sequence 1-17 into NPY (peptide **8**) led to a significant increase in helicity in comparison with NPY (64% versus 18%), which was maintained also in the presence of His^{34} in the analog **9**. The CD curves of these two NPY

analogs were similar in shape and intensity to that of the hPP analog [pNPY$^{1\text{-}7,\ 19\text{-}23}$]-hPP (Fig. 2 F).

Table 4. Calculated secondary structure components (%) for pNPY, hPP and their chimera in aqueous solution, at the concentration of 30 μM and pH 7.[a]

Peptide no.	α-helix	β-sheet	turn	random	Peptide no.	α-helix	β-sheet	turn	random
1	18	63	2	17	**23**	48	0	17	35
2	49	0	11	40	**24**	50	0	14	36
7	13	68	2	17	**25**	51	0	11	38
8	64	0	17	19	**26**	69	0	0	31
9	63	0	20	17	**27**	40	11	26	23
10	7	60	0	33	**28**	31	28	11	30
11	4	61	0	35	**29**	43	6	20	31
12	3	72	0	25	**30**	50	0	15	35
13	26	22	5	47	**31**	48	0	14	38
14	10	39	4	47	**32**	49	0	22	29
15	9	61	0	30	**33**	62	0	17	21
16	40	10	14	36	**34**	61	0	19	20
17	46	0	12	42	**35**	51	12	21	16
18	45	0	11	44	**36**	39	17	24	20
19	45	0	4	51	**37**	55	0	18	27
20	46	0	7	47	**38**	47	0	23	30
21	55	0	24	21	**39**	38	26	15	21
22	58	0	3	39	**40**	38	24	14	24

[a] The CD spectra were analyzed by using the program Secondary Structure Estimation (JASCO) that is based on the reference spectra of Yang et al..[16]

4.4 Discussion

NPY is one of the most conserved peptides during evolution known so far, and it belongs to the peptide hormone family that also includes PP and PYY. As PP was the first one of the three members to be discovered,[17] it gave its name to the family. However, based on the evolutionary studies,[11] NPY is the oldest member of this family that, therefore, is more correctly referred to as the NPY family.[8] It has been suggested that NPY and PYY arose from a common ancestral gene that was duplicated in early ancestral evolution, while PP arose from a more recent duplication of the PYY gene.[11] This can explain the high sequence identity of NPY with PYY but not with PP. Furthermore, NPY and PYY show a similar order of binding affinity at the Y-receptors, which is $Y_2 \geq Y_1 > Y_5 >> Y_4$, while PP has a completely different binding profile ($Y_4 >> Y_5 \geq Y_2 \geq Y_1$). Based on the crystal structure of avian PP,[4] the structural feature of the members of the NPY family was suggested to consist of an antiparallel hairpin-like fold, the so called PP-fold. However, it is not fairly clear yet, whether NPY adopts this three-dimensional structure as well. In many NMR studies, long-range NOEs between the N- and C-terminal part of NPY were not detected.[18-20] Evidence of a PP-fold was reported for NPY by Darbon and co-workers.[21]

The circular dichroism studies presented in this and earlier works[12, 22] show the presence of an α-helix for both NPY and PP, however, there are some differences: the most striking one is the much higher intensity of the CD spectrum of hPP, which corresponds to a helix content of 49% compared to 18% for NPY (Fig. 1 A). Moreover, the ratio $[\Theta]_{222}/[\Theta]_{209}$ is lower for NPY than for hPP. Then, the wavelength corresponding to $[\Theta] = 0$ is red-shifted for the NPY curve compared to that for hPP (203 nm versus 201 nm). These observations suggest that the helical conformation is more stable in hPP than in NPY, which might be due to a different folding of the N- and C-terminal segments in the two peptides. The C-terminal hPP helix might be stabilized by intramolecular hydrophobic interactions with the proline-rich region at the N-terminus, as observed in the X-ray structure of avian PP. The different conformational features of hPP and NPY are probably related to their different biological properties. In order to answer the question whether the primary structure is crucial for the peptide either to fold in a specific way or to be recognized from the various receptor subtypes, we designed chimera based on the combination of NPY, PYY and PP, and investigated their receptor affinity and solution

structure by circular dichroism (Table 2 and Figures 1 and 2). Among the NPY based peptides that contain constant or highly conserved positions of the parent peptides PYY and PP, we observed a reduced affinity at all receptors, with only few exceptions at the Y_4-receptor. The loss of affinity at the Y_1-receptor was three-fold in the best case ([Y^7]-pNPY) and more than 400-fold in the worst case ([rPP^{19-23}, H^{34}]-pNPY). On the one hand, the affinity was partially recovered by the additional introduction of Pro at position 34 in place of the native residue Gln, on the other hand the binding potency was further reduced by the replacement of Gln^{34} with His. The minor affinity of these NPY analogs containing the h/rPP sequence 19-23, in comparison with that of NPY, corresponded to minor helicity and higher content of unordered structure, as suggested by the CD spectra shown in Fig. 1 B-C. The poorest Y_1-receptor analog, [rPP^{19-23}, H^{34}]-pNPY, was characterized by a helical content of 9% only. These results suggest that the central region of NPY plays an important role in inducing and/or stabilizing the helical conformation. Also position 34 influences the helix formation: accordingly, the presence of the helix-breaker Pro led to a decrease in helicity. However, a helix destabilization occurred also after the introduction of His.

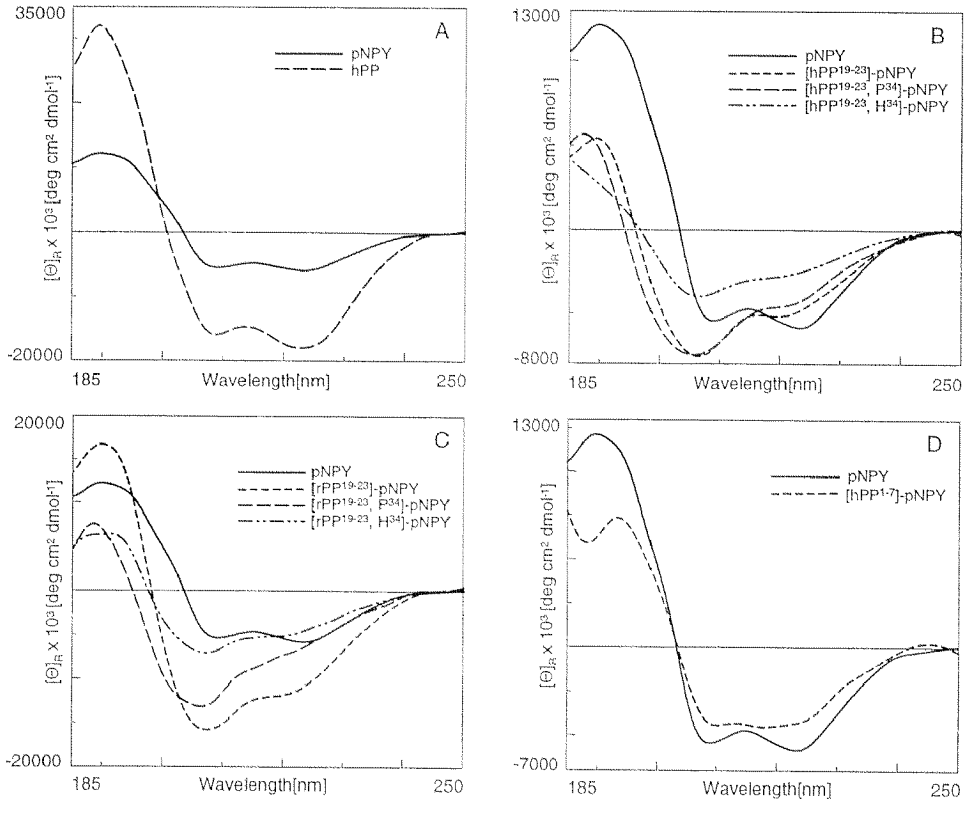

Figure 1. CD spectra of pNPY, hPP and of the PP/NPY chimera **7**, **10-15**.

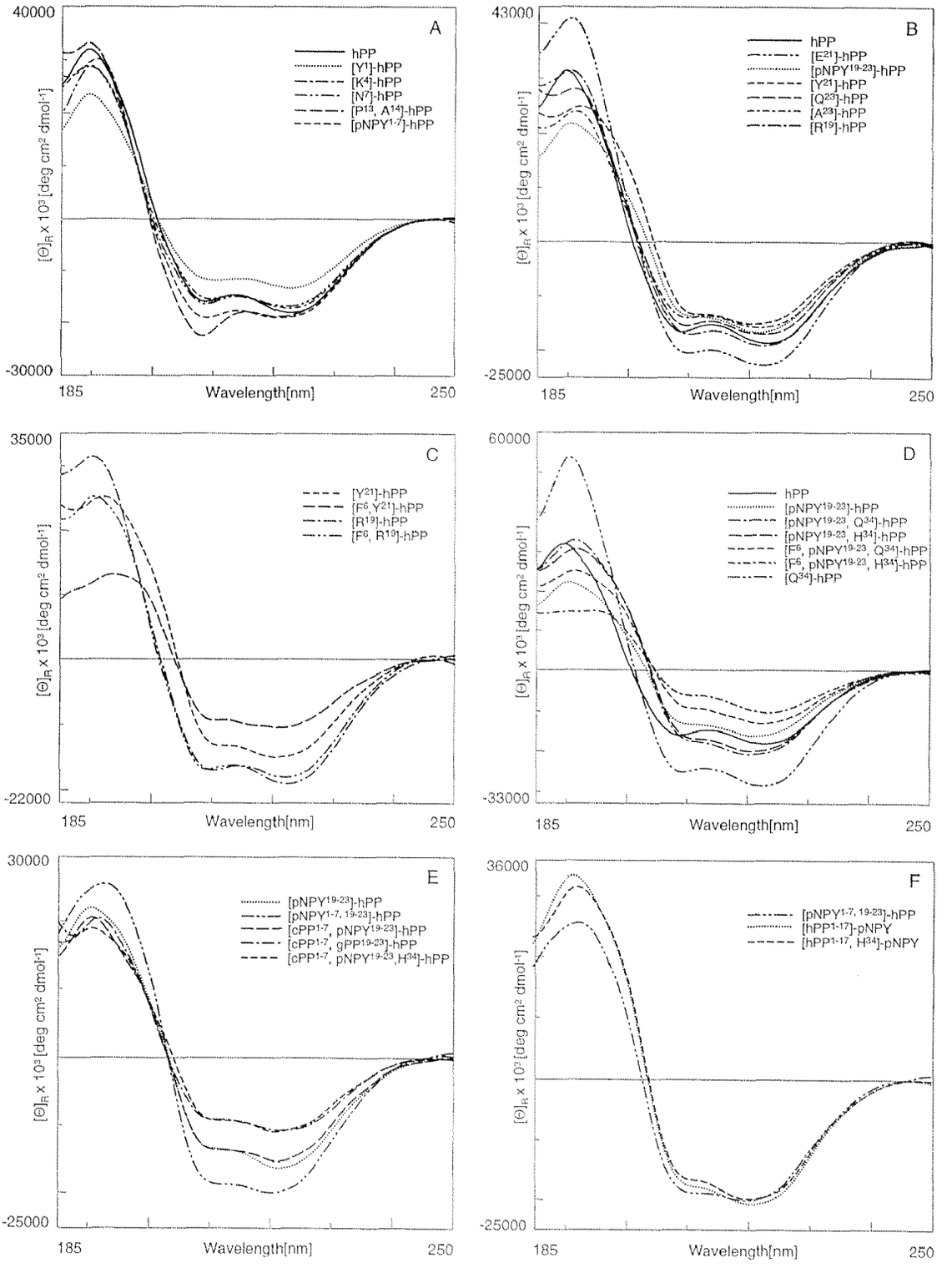

Figure 2. CD spectra of hPP and of the PP/NPY chimera **8**, **9**, **16-27**, **31-40**.

Different results were obtained when the N-terminal part of NPY was substituted with the hPP segments 1-7 or 1-17: the binding affinity at the Y_1-receptor still was in the range 1.4-3 nM (six- to 13-fold lower than that of NPY). Interestingly, the longer the replaced segment the better was the affinity (1.9 nM versus 3 nM). Surprisingly, when the analog [hPP^{1-17}]-pNPY was modified by the introduction of His^{34}, no loss of affinity was observed, as it happened in the case of the centrally substituted NPY analogs, but the peptide bound to the Y_1-receptor even with a slightly increased affinity (1.4 nM versus 1.9 nM). The relative high affinity of the latter three analogs can be explained by the observations of their CD spectra. The CD curves of NPY and [hPP^{1-7}]-pNPY are shown in Fig. 1 D; the hPP segment 1-7 induced only a partial transition from α-helix to β-sheet, without increasing the random coil fraction. The analog containing the longer hPP segment 1-17 was characterized by a very stable helix that was even more pronounced than that of hPP, and was not perturbed by the introduction of His^{34} (Fig. 2 F). Therefore, the N-terminal sequence of hPP seems to be crucial for the stabilization of the C-terminal α-helix. This might explain, for example, why PP is much more α-helical than NPY (Fig. 1 A).

All NPY analogs discussed so far showed a Y_5-receptor binding profile that was very similar to that observed at the Y_1-receptor. Particular attention should be paid to the three analogs containing the hPP segments 1-7 or 1-17 (peptides **7-9**), since they turned out to be as potent as NPY (0.45-2.2 nM). Furthermore, these peptides were found to bind to the Y_4-receptor up to 39-fold better than NPY, correspnding to an affinity of up to 0.15 nM. This observations suggest that the α-helix is an important structural element for the Y_1-, Y_4- and Y_5-receptor subtypes to recognize and bind the ligand. The increase in unordered structure for the analogs [hPP^{19-23}]-pNPY and [rPP^{19-23}]-pNPY corresponded to a loss of affinity at the Y_2-receptor; however, the former analog still bound to the receptor with an affinity of 1.0 nM, and the latter with an affinity of 11.5 nM. On the other hand, micromolar affinities were found for the corresponding analogs containing Pro or His at position 34. While the lack of binding potency was expected in the case of Pro,[14] it was surprising in the case of His. Therefore, it can be concluded, that when the NPY analog adopts a not very stable helix conformation, the presence of His at the C-terminus provides an additional disturbing element which turns out to be a much more efficient helix-breaker than Pro (Fig. 1 B-C). However, if the peptide is highly helical, His does not seem significantly to perturb the secondary structure (Fig. 2 F).

The chimera based on the native sequence of hPP and containing elements of the primary structure of NPY presented all a high helical content (Fig. 2): the lowest helical content was found for the analog [F^{6}, E^{21}]-hPP (31%), while the most pronounced helicity (69%) corresponded to the analog [Q^{34}]-hPP.

The two hPP analogs obtained by Glu^{4}/Lys or Tyr^{7}/Asn substitution gave CD spestra that were very similar to that of hPP (Fig. 2 A). This suggests that these two positions are not determinant for the hPP-like folding. However, these two analogs were differently potent at the Y_1- and Y_5-receptor subtypes. [K^{4}]-hPP bound ten-fold better than [N^{7}]-hPP at the Y_1-receptor, and seven-fold better at the Y_5-receptor. This indicates that position 4 might be involved in the interaction with the two receptor subtypes and that the basic NPY residue Lys is preferred rather than the acid hPP residue Glu. However, the analog [K^{4}]-hPP still remained a very poor ligand at the Y_1- and Y_5-receptor subtypes, with an affinity of > 100 nM and 20 nM, respectively, which indicates that the single exchange at position 4 is not sufficient to increase the hPP affinity at these receptors. This was also observed for the analogs [Y^{1}]-hPP and [P^{13}, A^{14}]-hPP, which showed a micromolar affinity at the Y_1-receptor, and an IC_{50} value of 68 nM and 118 nM at the Y_5-receptor. The introduction of Tyr^{1} in place of Ala induced a partial α-helix to β-sheet transition of hPP, while the double substitution at positions 13-14 decreased the amount of the helix and turn components in favor of the random coil one (Fig. 2 A). Single or double replacements in the N-terminal region did not increase the affinity of hPP at the Y_1- and Y_5-receptor subtypes, and led to a reduced Y_4-receptor binding potency (24- to 48-fold, corresponding to an affinity of 1-1.9 nM). On the other hand, by the substitution of the whole hPP segment 1-7 with the pNPY segment the affinity was increased to 4.2 nM at the Y_1-receptor, and to 3.0 nM at the Y_5-receptor, while the Y_4-receptor affinity further decreased to 4.2 nM. The pNPY segment 1-7 induced a conformational change that was similar to that observed for the double replacement Pro^{13}-Ala^{14}. However, the affinity of the analog [$pNPY^{1-7}$]-hPP at the NPY-preferring receptors was much higher compared to that of [P^{13}, A^{14}]-hPP and hPP itself, which indicates that the N-terminal region of NPY is directly involved in receptor binding or that its more hydrophilic character is favorable to receptor interaction.

Single exchange at positions 19-23 turned out to be more efficient in modulating the binding affinity of hPP, especially at the Y_5-receptor, without loss of affinity at the Y_4-receptor. Accordingly, the hPP analogs [Y^{21}]-hPP, [A/Q^{23}]-hPP and [R^{19}]-hPP bound to the Y_5-receptor with affinities of 1.0-2.4 nM, and their affinities at the Y_4-receptor were

similar to the hPP one. Only the replacement of Ala^{21} with Glu^{21} did not improve the Y_5-receptor affinity (IC_{50} 66 nM). Interestingly, the simultaneous replacement of positions 19-23 with the corresponding pNPY segment led to an affinity of 1.5 nM at the Y_5-receptor, comparable to that obtained by single exchange. Moreover, the affinity at the Y_2-receptor was surprisingly high (23 nM), despite the presence of Pro^{34}. The CD spectra of [R^{19}]-hPP and [A/Q^{23}]-hPP were found to be similar to that of hPP: there was a small increase in helix content for [R^{19}]-hPP, which was probably due to a salt bridge formation between Glu^{15}-Arg^{19} or Arg^{19}-Asp^{23}, and a small increase in turn structure amount for [A/Q^{23}]-hPP (Fig. 2 B). The presence of Glu^{21} in place of Ala was characterized by a transition turn to α-helix, which led to an increase in α-helix of 18% in comparison with hPP. This was probably due to helix stabilization by the formation of a salt bridge between Glu^{21} and Arg^{25}. Also the exchange Ala^{21}/Tyr increased the helix and turn content. The NPY segment 19-23 did not vary the helical content, but induced a random coil to turn transition. In summary, the NPY positions introduced into the central region of hPP by single or multiple exchange did not affect the stability of the helix which was even improved in the presence of Arg^{19}, Tyr^{21} or Glu^{21}. Furthermore, the random coil component was reduced in favor of turn elements, with the only exception of Glu^{21}. The different Y_5-receptor affinity obtained by the substitution of Ala^{21} with Glu (66 nM) or Tyr (1.1 nM) suggests that the hydrophobic character of the side-chain is preferred at this position. At position 19, a basic residue like Arg seems to be more favored than the polar Gln, while at position 23 an aliphatic (Ala) or polar (Gln) side-chain is better tolerated than the negatively charged side-chain of Asp. Interestingly, the additional modification by the introduction of Phe^6 did not induce conformational changes of the two analogs [R^{19}]-hPP and [Q^{23}]-hPP, while it led to partial formation of β-sheet for the other analogs (Fig. 2 C). These peptides were characterized by a micromolar affinity at the Y_2-receptor, however, they bound to the Y_1-receptor with affinities in the range 40-71 nM, with the only exception of the analog [F^6, E^{21}]-hPP (540 nM). Therefore, the presence of Glu^{21} turned out to be poorly tolerated at the Y_5-receptor as well as at the Y_1-receptor.

Replacementof Pro^{34} with Gln in hPP did not improve the affinity at the Y_1- and Y_5-receptor subtypes, and even decreased the Y_4-receptor affinity of 40-fold. However, by the combination of Gln^{34} with the pNPY segment 19-23, the binding potency at the Y_4-receptor was equal to that of hPP, and three-fold better than that of NPY at the Y_5-receptor (0.2 nM versus 0.6 nM). Similar results were obtained in the presence of His^{34}. The two

analogs [pNPY$^{19-23}$, Q^{34}]-hPP and [pNPY$^{19-23}$, H^{34}]-hPP showed very similar CD spectra, characterized by a high helical content (Fig. 2 D). The additional presence of Phe^{6} induced some β-sheet, which, however, did not affect the receptor affinity. The presence of His^{34} in place of Gln was poorly tolerated at the Y_1- and Y_2-receptor subtypes. Finally, also the simultaneous modifications at both the N-terminal and central regions turned out to be suitable for increasing the affinity of hPP at the receptors Y_1, Y_2 and Y_5, without loss of affinity at the Y_4-receptor: the introduction of pNPY 1-7 and 19-23 yielded a ligand that was as potent as NPY at the Y_1-receptor and even five-fold more potent at the Y_5-receptor. Moreover, the affinity at the Y_2-receptor was 11.7 nM, despite the presence of Pro^{34}. This analog contained 55% of helix (Fig. 2 E). The substitution of the pNPY sequence 1-7 by the corresponding cPP segment led to a decrease in helicity, which was accompanied by a decrease in affinity at the Y_1- and Y_2-receptor subtypes, while the Y_5-receptor affinity slightly increased to 0.07 nM. Furthermore, after substitution of Pro^{34} with His, the Y_1-receptor affinity further decreased to 5.7 nM, while the Y_5-receptor affinity increased to 0.04 nM. His^{34} caused a loss of helicity and an increase in β-sheet. Also the substitution of the pNPY sequence 19-23 with the corresponding segment from goose PP led to the formation of β-sheet. In this case, however, there was also loss of affinity: 10.3 nM versus 0.6 nM at the Y_1-receptor, 220 nM versus 28 nM at the Y_2-receptor, and 0.2 nM versus 0.07 nM at the Y_5-receptor. These observations suggest that the decreased Y_1- and Y_2-receptor affinity of the three hPP analogs containing the cPP segment 1-7 compared to the analog containing the corresponding pNPY segment might be related to helix destabilization. This does not seem to be important at the Y_5-receptor, as the affinity increased up to 0.04 nM when the helical content decreased to 38%.

In the case of the analog [cPP$^{1-7}$, gPP$^{19-23}$]-hPP, probably there was a negative contribution of the gPP sequence, due to destabilization of the helix or to an unfavorable interaction with the receptor binding site.

4.5 Conclusions

The structure-affinity relationship study on the chimeric PP/NPY analogs revealed the importance of the central segment of NPY 19-23 (RYYSA) to induce helix conformation as well as high affinity at all four Y-receptors. Accordingly, both PP sequences from rat

(QYETQ) and human (QYAAD) led to a loss of helicity and affinity. Furthermore, position 34 plays a role in inducing the bioactive conformation of the C-terminus of NPY: while the presence of Pro is tolerated at the Y_1- and Y_5-receptor subtypes, and even enhances the affinity for the Y_4-receptor, it leads to complete loss of affinity at the Y_2-receptor. It has been shown that the affinity of NPY at the Y_4-receptor can be increased by coupling of the N-terminal hPP with the C-terminal NPY. Interestingly, the presence of His at position 34 is tolerated only in combination with the N-terminal segment from hPP but not with the central segment from r/hPP, suggesting that the intramolecular interactions between the N- and C-termini may be different in the two analogs. Moreover, the N-terminal hPP sequence has been found strongly to stabilize the helix conformation of NPY in a way which favors the binding of the Y_4- and Y_5-receptor subtypes.

The major role of the central segment of NPY 19-23 in receptor recognition has been shown also in the structure-affinity study on the chimeric hPP analogs, as its presence increased the hPP affinity at the Y_1-, Y_2- and Y_5-receptor subtypes. The simultaneous presence of the NPY sequences 1-7 and 19-23 yielded an analog of hPP that was as potent as NPY at the Y_1-receptor, comparable to hPP at the Y_4-receptor and even better than NPY at the Y_5-receptor. Surprisingly, a subnanomolar affinity at the Y_5-receptor derived from the combination of the cPP sequence 1-7 (GPSQPTY) with the NPY sequence 19-23 (RYYSA) and the cPP residue His^{34}. This chimeric peptide was more helical than NPY but lesser than hPP and other hPP analogs: this observation suggests that a strong helical character of the ligand is not sufficient to have high Y_5-receptor affinity, but also a proper tertiary structure is required to increase the binding potency to the subnanomolar range.

4.6 Experimental Section

4.6.1 Materials

The N^{α}-Fmoc-protected amino acids were purchased from Alexix (Läufelfingen, Switzerland). Side-chain protecting groups were: *tert*-butyl for Asp, Glu, Ser, Thr and Tyr, Boc for Lys, trityl for Asn, Gln and His, 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg. The 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink Amide) and

the 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucylamino methyl (Rink Amide AM) resins were obtained from Novabiochem (Läufelfingen, Switzerland). *N*-hydroxybenzotriazole (HOBt), TFA, thioanisole, *p*-thiocresol, 1,2-ethanedithiol, trimethylbromosilane, piperidine, 1-methyl-2-pyrrolidinone, *tert*-butanol, DMF (puriss.), sodium hydrogenphosphate and potassium dihydrogenphosphate were obtained from Fluka (Buchs, Switzerland). *N,N'*-diisopropylcarbodiimide (DIC) was purchased from Aldrich (Buchs, Switzerland). DMF (pure) and diethylether were purchased from Scharlau (La Jota, Barcelona, Spain). Acetonitrile was obtained from Romil (Cambridge, England). 50% Dulbecco's modified Eagle medium/50% Ham's F12, minimum essential medium with Earl's salts and phosphate buffered saline (PBS) were purchased from Gibco (Life Technologies, Basel, Switzerland). Fetal calf serum, glutamine and non-essential amino acids were obtained from Boehringer Mannheim (Germany). Geneticin, bacitracin and bovine serum albumin were purchased from Sigma (Buchs, Switzerland). ^{3}H-propionyl-NPY was purchased from Amersham.

4.6.2 Peptide Synthesis

The peptides were synthesized by solid-phase technique on an automated multiple peptide synthesizer (Syro, MultiSynTech, Bochum, Germany) starting from the Fmoc-deprotected Rink Amide and Rink Amide AM resins (30 mg, resin loading 0.45-0.51 mmol/g) and using the Fmoc-strategy. The load of the resins with Fmoc-Tyr(*tert*-butyl)-OH and the subsequent chain elongation were performed by double coupling protocol with ten-fold excess Fmoc-amino acid, HOBt, DIC in DMF (2 x 40 min). The Fmoc-deprotection step was accomplished by 40% piperidine in DMF for 3 min, 20% piperidine for 7 min and finally 40% piperidine for 5 min. The removal of the side-chain protecting groups and the cleavage from the resin were accomplished in one step by treatment of the peptide-resin with the cleavage mixture TFA/thioanisole/thiocresol (90/5/5 v/v) for 3 hours. For the cleavage of the Met-containing peptides ethanedithiol was added as scavenger (2-4% v/v). The fully deprotected peptides were precipitated from ice-cold diethylether, the suspensions were centrifuged at 5 °C, the ether was decanted, then the peptides were suspended again in fresh ether and centrifuged. The washings with cold ether were repeated four times. Finally, the peptides were dissolved in *tert*-butanol/water (3/1 w/w) and lyophilized. The peptides containing Met-sulfoxide were reduced after lyophilization by

using the reduction mixture TFA/ethanedithiol/trimethylbromosilane (96/2.4/1.6 v/v) for 30-40 min, under a N_2 atmosphere within the first 5 min, then in tightly closed centrifuge tubes.[23] The reduced peptides were recovered from ice-cold ether, washed as described above, purified to homogenity and finally lyophilized.

The peptides were characterized by electrospray ionization mass spectrometry (SSQ 710, Finnigan MAT, Bremen, Germany) and by analytical reversed-phase HPLC on a LiChrospher RP-18 column (5 µm, 3 x 125 mm, Merck, Darmstadt, Germany) using 0.08% TFA in acetonitrile (A) and 0.1% TFA in water (B) as eluting system (20 to 70% A over 35 min at the flow rate of 0.6 ml/min).

4.6.3 Circular Dichroism

The CD spectra were recorded using a JASCO model J720 spectropolarimeter over 250-180 nm at 20 °C in a N_2 atmosphere. Stock solutions of the peptides were prepared in 20 mM phosphate buffer, pH 7.0, at the concentration range 100-300 µM. The CD spectra were performed on peptide solutions at the concentration range 30-40 µM. Each measurement was repeated four times using a thermostatable sample cell with a path of 0.02 cm and the following parameters: response time of 2 s, scan speed of 20 nm/min, sensitivity of 10 mdeg, step resolution of 0.2 nm and band width of 2 nm. The CD spectrum of the solvent was subtracted from the CD spectra of the peptide solutions to eliminate the interference from cell, solvent and optical equipment. High-frequency noise was reduced by means of a low-path Fourier-transform filter. The ellipticity was expressed as the mean-residue molar ellipticity $[\Theta]_R$ in deg cm^2 $dmol^{-1}$.The secondary structure composition was estimated by using a deconvolution method that employs the reference spectrum of Yang *et al.*.[16]

4.6.4 Cell Culture

BHK cells transfected with hY_1, hY_2, hY_4 or hY_5 receptors were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 5% penstrep and 0.1% geneticin.[15] SMS-KAN cells (hY_2 receptor) were grown in 50% Dulbecco's modified Eagle medium/50% nutrient mix Ham's F12 with 15% fetal calf serum, 4 mM glutamine

and 1% non-essential amino acids.[24] Cells were grown to confluency at 37 °C and 5% CO_2.

4.6.5 Receptor Binding Assays

Cells were resuspended in incubation buffer (minimum essential medium with Earl's salts containing 0.1 % bacitracin, 50 µM pefabloc SC and 1% bovine serum albumin). 200 µl of the suspension containing ca. 440 000 cells were incubated with 25 µl of a 10 nM solution of ^{3}H-propionyl-NPY and 25 µl of a 10 µM solution of NPY or analog. Non specific binding was defined in the presence of 1 µM cold NPY. After 1.5 hours at room temperature, the incubation was terminated by centrifugation at 2 000 x g and 4 °C for 5 min. The pellets were then washed once with PBS by centrifugation, resuspended in PBS and mixed with the scintillation cocktail. Radioactivity was determined by using a β^- counter.

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4.7 References

1. Glover, I. D.; Barlow, D. J.; Pitts, J. E.; Wood, S. P.; Tickle, I. J.; Blundell, T. L.; Tatemoto, K.; Kimmel, J. R.; Wollmer, A.; Strassburger, W.; et al. Conformational studies on the pancreatic polypeptide hormone family. *Eur. J. Biochem.* **1984**, *142*, 379-385.

2. Fuhlendorff, J.; Johansen, N. L.; Melberg, S. G.; Thogersen, H.; Schwartz, T. W. The antiparallel pancreatic polypeptide fold in the binding of neuropeptide Y to Y1 and Y2 receptors. *J. Biol. Chem.* **1990**, *265*, 11706-11712.

3. Allen, J.; Novotny, J.; Martin, J.; Heinrich, G. Molecular structure of mammalian neuropeptide Y: analysis by molecular cloning and computer-aided comparison with crystal structure of avian homologue. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 2532-2536.

4. Blundell, T. L.; Pitts, J. E.; Tickle, I. J.; Wood, S. P.; Wu, C.-W. X-ray analysis (1.4 Å resolution) of avian pancreatic polypeptide: Small globular protein hormone. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 4175-4179.

5. Chronwall, B. M.; DiMaggio, D. A.; Massari, V. J.; Pickel, V. M.; Ruggiero, D. A.; O'Donohue, T. L. The anatomy of neuropeptide Y-containing neurons in rat brain. *Neuroscience* **1985**, *15*, 1159-1181.

6. Turton, M. D.; O'Shea, D.; Bloom, S. R. Central effects of neuropeptide Y with emphasis on its role in obesity and diabetes. In *Neuropeptide Y and drug development*; Grundemar, L., Bloom, S. R., Eds.; Academic Press: 1997; pp 15-39.

7. Thiele, T. E.; Marsh, D. J.; Ste. Marie, L.; Bernstein, I. L.; Palmiter, R. D. Ethanol consumption and resistance are inversely related to neuropeptide Y levels. *Nature* **1998**, *396*, 366-369.

8. Michel, M. C.; Beck-Sickinger, A.; Cox, H.; Doods, H. N.; Herzog, H.; Larhammar, D.; Quirion, R.; Schwartz, T.; Westfall, T. XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol. Rev.* **1998**, *50*, 143-150.

9. Inui, A. Neuropeptide Y feeding receptors: are multiple subtypes involved? *Trends Pharmacol. Sci.* **1999**, *20*, 43-46.

10. Bischoff, A.; Michel, M. C. Emerging functions for neuropeptide Y5 receptors. *Trends Pharmacol. Sci.* **1999**, *20*, 104-106.

11. Larhammar, D. Evolution of neuropeptide Y, peptide YY and pancreatic polypeptide. *Regul. Pept.* **1996**, *62*, 1-11.

12. Beck-Sickinger, A. G.; Wieland, H. A.; Wittneben, H.; Willim, K. D.; Rudolf, K.; Jung, G. Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur. J. Biochem.* **1994**, *225*, 947-958.

13. Eckard, C. P.; Cabrele, C.; Wieland, H. A.; Beck-Sickinger, A. G., manuscript submitted.

14. Fuhlendorff, J.; Gether, U.; Aakerlund, L.; Langeland-Johansen, N.; Thogersen, H.; Melberg, S. G.; Olsen, U. B.; Thastrup, O.; Schwartz, T. W. [Leu31, Pro34]neuropeptide Y: a specific Y1 receptor agonist. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 182-186.

15. Wieland, H. A.; Engel, W.; Eberlein, W.; Rudolf, K.; Doods, H. N. Subtype selectivity of the novel nonpeptide neuropeptide Y Y1 receptor antagonist BIBO 3304 and its effect on feeding in rodents. *Br. J. Pharmacol.* **1998**, *125*, 549-555.

16. Yang, J. T.; Wu, C. S.; Martinez, H. M. Calculation of protein conformation from circular dichroism. *Methods Enzymol.* **1986**, *130*, 208-269.

17. Kimmel, J. R.; Hayden, L. J.; Pollock, H. G. Isolation and characterization of a new pancreatic polypeptide hormone. *J. Biol. Chem.* **1975**, *250*, 9369-9376.

18. Cowley, D. J.; Hoflack, J. M.; Pelton, J. T.; Saudek, V. Structure of neuropeptide Y dimer in solution. *Eur. J. Biochem.* **1992**, *205*, 1099-1106.

19. Mierke, D. F.; Durr, H.; Kessler, H.; Jung, G. Neuropeptide Y. Optimized solid-phase synthesis and conformational analysis in trifluoroethanol. *Eur. J. Biochem.* **1992**, *206*, 39-48.

20. Monks, S. A.; Karagianis, G.; Howlett, G. J.; Norton, R. S. Solution structure of human neuropeptide Y. *J. Biomol. NMR* **1996**, *8*, 379-390.

21. Darbon, H.; Bernassau, J. M.; Deleuze, C.; Chenu, J.; Roussel, A.; Cambillau, C. Solution conformation of human neuropeptide Y by 1H nuclear magnetic resonance and restrained molecular dynamics. *Eur. J. Biochem.* **1992**, *209*, 765-771.

22. Kirby, D. A.; Boublik, J. H.; Rivier, J. E. Neuropeptide Y: Y1 and Y2 affinities of the complete series of analogues with single D-residue substitutions. *J. Med. Chem.* **1993**, *36*, 3802-3808.

23. Beck, W.; Jung, G. Convenient reduction of S-oxides in synthetic peptides, lipopeptides and peptide libraries. *Lett. Pept. Sci.* **1994**, *1*, 31-37.

24. Ingenhoven, N.; Beck-Sickinger, A. G. Fluorescent labelled analogues of neuropeptide Y for the characterization of cells expressing NPY receptor subtypes. *J. Recept. Signal Transduct. Res.* **1997**, *17*, 407-418.

CHAPTER 5

The First Selective Agonist at the Neuropeptide Y Y_5-Receptor Increases Food Intake in Rats

Chiara Cabrele[1], Michael Langer[1], Reto Bader[1], Heike A. Wieland[2], Henry N. Doods[2], Oliver Zerbe[1], and Annette G. Beck-Sickinger[1]

[1]Department of Pharmacy, ETH Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
[2]Division of Preclinical Research, Boehringer Ingelheim Pharma KG, Birkendorferstrasse 65, 88397 Biberach, Germany

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5.1 Abstract

The first Y_5-receptor selective analog of neuropeptide Y (NPY), [Ala^{31}, Aib^{32}]-NPY, has been developed and biologically characterized. By using competition binding assays on Y-receptors selectively expressing cell lines, the affinity was determined to be 5 nM at the Y_5-, > 500 nM at the Y_2-, and > 1 000 nM at the Y_1- and Y_4-receptor. Activity studies were performed *in vitro* by a cAMP enzyme immonoassay, and *in vivo* by food intake studies in rats: the peptide turned out to act as an agonist. Further peptides, obtained by the combination of the motif Ala^{31}-Aib^{32} with some chimera of NPY and pancreatic polypeptide, displayed same selectivity and even higher affinity (up to 0.2 nM) for the Y_5-receptor. *In vivo* administration of the Y_5-receptor selective agonists significantly stimulated feeding in rats. The NMR solution structures of NPY and [Ala^{31}, Aib^{32}]-NPY showed a different conformation in the C-terminal region, where the α-helix of NPY was substituted in the analog by a more flexible 3_{10}-helical turn structure.

5.2 Introduction

Neuropeptide Y (NPY), a 36-residue peptide amide, is a member of the pancreatic polypeptide (PP) hormone family that includes also PP and peptide YY (PYY).[1] NPY is expressed in the central and peripheral nervous systems and is one of the most abundant neuropeptides in the brain. Several important physiological activities such as induction of food intake, inhibition of anxiety, increase in memory retention, presynaptic inhibition of neurotransmitter release, vasoconstriction and regulation of ethanol consumption have been attributed to NPY.[2, 3] Especially, the role of NPY in feeding is of major interest because NPY antagonists would be potential candidates as anti-obesity drugs. Many studies have established the strong influence of NPY in feeding behavior: for example, injection of NPY into the hypothalamus increases food intake[4, 5] and high NPY levels are correlated with leptin deficiency,[6] the hormone secreted by adipocytes that regulates body weight and energy balance.[7, 8] Furthermore, NPY-knockout can reduce obesity in leptin deficient mice (named *ob/ob* mice).[6]

At present, five distinct Y-receptor subtypes that bind NPY, PYY and PP with different affinity have been identified, cloned and characterized. They all belong to the superfamily of the G-protein-coupled receptors and are referred to as Y_1, Y_2, Y_4, Y_5 and y_6.[9] From studies conducted so far by using selective agonists and antagonists, antisense approaches and knockout techniques, the Y_1- and Y_5-receptors have been suggested to be involved in food intake regulation.[10, 11] NPY, PYY, [Leu^{31}, Pro^{34}]-NPY and two N-terminally truncated analogs, NPY (2-36) and (3-36), have been shown to increase food intake. It still remains unclear which receptor (or receptors) madiate feeding, as the peptides described above do not selectively activate a single receptor subtype. NPY and PYY bind to the receptors Y_1, Y_2 and Y_5 with high affinity, the analog [Leu^{31}, Pro^{34}]-NPY has high affinity at the Y_1-, Y_4- and Y_5-receptors, while the N-truncated peptides are potent at the Y_2- as well as at the Y_5-receptor.[9] [D-Trp^{32}]-NPY has been described in literature as a weak Y_5-receptor selective agonist with orexigenic properties,[12] but antagonism against NPY-induced feeding has been observed as well.[13, 14] Furthermore, binding affinity studies on [D-Trp^{32}]-NPY at the Y-receptors have shown a significant Y_2-receptor affinity in addition to the Y_5-receptor one.[15]

As highly specific tools which discriminate the Y_5-receptor activity from that of the other subtypes are missing, we have focused our work on the design of NPY agonists with

high affinity and selectivity, in order to clarify the role of this receptor in the NPY-induced increase in food intake. High affinity of NPY analogs at the Y_5-receptor has been found strongly to depend on the spatial conformation of the C-terminus.[16] Accordingly, we introduced the unnatural amino acid aminoisobutyric acid (Aib) into the C-terminal part of NPY, because this residue can strongly influence the peptide conformation. In particular, Aib-containing peptides have been shown preferentially to adopt turn- or helix-like structures, depending on the main-chain length and on the amino acid that precedes Aib.[17, 18] Since it is well established that the C-terminal part of NPY represents the interaction site with the Y-receptors, and that amino acid exchange is poorly tolerated in the region 33-36,[19, 20] we introduced the dipeptide Ala-Aib into positions 31-32 of porcine NPY (pNPY), in order to induce conformational changes within the peptide region that is determinant for receptor binding. [Ala^{31}, Aib^{32}]-pNPY showed high selectivity at the Y_5-receptor and its activity was tested *in vitro* and *in vivo*, clearly proving its NPY receptor agonism and stimulation of food intake.

The solution structure of [Ala^{31}, Aib^{32}]-pNPY was investigated by circular dichroism (CD) and two-dimensional NMR (2D-NMR). Comparison with the structure of hNPY, determined by Monks and co-workers,[21] revealed a significant conformational change of the C-terminal fragment 28-36: while in the native peptide the α-helix extends to residue 36, the α-helical motif of [Ala^{31}, Aib^{32}]-pNPY ends to residue 31 with a 3_{10}-helical turn between Ile^{28} and Ala^{31}, followed by an apparently not-well defined structure.

In the light of the results obtained with the selective agonist [Ala^{31}, Aib^{32}]-pNPY, we designed a series of peptides based on the combination of some chimera of pNPY and PP with the motif Ala^{31}-Aib^{32}. These peptides maintained high selectivity for the Y_5-receptor, and affinity as well as efficacy were even improved. Accordingly, the Ala^{31}-Aib^{32} motif has been identified as a key structural motif to obtain Y_5-receptor selectivity.

5.3 Results

5.3.1 [Ala^{31}, Aib^{32}]-pNPY: selectivity and agonism at the Y_5-receptor

The peptide was synthesized by solid-phase technique and purified by preparative HPLC (high pressure liquid chromatography). Electrospray ionization mass spectrometry and

Table 1. Amino acid sequences of pNPY, hPP and of the Y_5-receptor selective analogs (Aib is indicated by the letter B).

Peptide	Sequence
pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2
[Ala^{31}, Aib^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLABRQRY-NH_2
[hPP^{1-17}, Ala^{31}, Aib^{32}]-pNPY	APLEPVYPGDNATPEQMARYYSALRHYINLABRQRY-NH_2
[cPP^{1-7}, NPY^{19-23}, Ala^{31}, Aib^{32}, Gln^{34}]-hPP	GPSQPTYPGDNATPEQMARYYSALRRYINMABRQRY-NH_2
hPP	APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-NH_2

analytical HPLC confirmed peptide purity and identity. The amino acid sequence is shown in Table 1.

[Ala^{31}, Aib^{32}]-pNPY has an affinity of 5 nM at the Y_5-receptor, > 500 nM at the Y_2-receptor and >1 000 at the receptors Y_1 and Y_4 (Table 2). Accordingly, [Ala^{31}, Aib^{32}]-pNPY is not only highly selective but additionally retains high affinity for the Y_5-receptor.

To further characterize the newly developed Y_5-receptor selective ligand, we investigated its ability to activate the receptor. Signal transduction of the NPY-activated Y-receptors is mediated by G_i-protein coupling, which leads to the inhibition of the enzyme adenylyl cyclase.[9] As shown in Table 2 and Figure 1, [Ala^{31}, Aib^{32}]-pNPY inhibits the forskolin-stimulated accumulation of intracellular cAMP in a dose-dependent manner. A good correlation between affinity and activity was found for NPY and [Ala^{31}, Aib^{32}]-pNPY, as the analog is nine-fold less potent in binding and five-fold less potent in activating the Y_5-receptor than NPY.

The pharmacological profile of [Ala^{31}, Aib^{32}]-pNPY has all features that are required for a peptide to be referred to as a high selective, full NPY agonist at the Y_5-receptor, and therefore it proves to be a very important tool for the investigation of the biological functions that have been so far attributed or speculated to be related to the Y_5-receptor.

5.3.2 Ala-Aib is the key motif for the Y_5-receptor selectivity

In order to further improve the receptor affinity, we designed several new peptides based on the sequences of PP/NPY chimera which have been found to bind to the Y_5-receptor with very high affinity.[16]

The PP/NPY chimera were modified by the incorporation of the dipeptide Ala-Aib at positions 31-32. These analogs will be referred to as (Ala-Aib)-containing PP/NPY chimera. All peptides were prepared by solid-phase technique and the sequences are shown in Table 1.

As already observed for [Ala^{31}, Aib^{32}]-pNPY, the (Ala-Aib)-containing PP/NPY chimera are selective for the Y_5-receptor as well. The analog [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP is 26-fold more potent than [Ala^{31}, Aib^{32}]-pNPY (0.2 nM against 5.3 nM), and three-fold more potent than the native ligand NPY (0.2 nM against 0.6 nM).

Table 2. Pharmacological properties of hNPY, hPP, Aib-containing peptides and [D-Trp^{32}]-hNPY. In the brackets the ratio $IC_{50}(hY_n)$ / $IC_{50}(hY_5)$ is reported. [a] See Ref. 15.

Peptide	Affinity IC_{50} ± SEM [nM]				Activity EC_{50} ± SEM [nM]
	hY_1	hY_2	hY_4	hY_5	hY_5
hNPY	0.23 ± 0.01	0.04 ± 0.01	5.8 ± 3.5	0.6 ± 0.2	18.5 ± 6.6
[A^{31}, Aib^{32}]-pNPY	> 1 000	> 500	> 1 000	5.3 ± 2.4	98 ± 48
	(> 189)	(> 94)	(> 189)	(1)	
[hPP$^{1-17}$, A^{31}, Aib^{32}]-pNPY	> 1 000	> 500	167 ± 23	0.92 ± 0.06	n. d.
	(> 1 087)	(> 543)	(182)	(1)	
[cPP$^{1-7}$, NPY$^{19-23}$, A^{31}, Aib^{32}, Q^{34}]-hPP	620	> 500	52 ± 5	0.2 ± 0	17.0 ± 6.9
	(3 100)	(> 2 500)	(260)	(1)	
hPP	> 1 000	> 2 000	0.04 ± 0.01	57	n. d.
[D-Trp^{32}]-hNPY	> 1 000	29 ± 11	> 1 000	35[a]	n. d.

Furthermore, in comparison with [Ala^{31}, Aib^{32}]-pNPY, its Y_5-receptor selectivity is slightly increased relative to the Y_4-receptor (1 : 260 versus 1 : > 189), whereas it is significantly increased relative to the receptors Y_1 and Y_2 (1 : 3 100 versus 1 : > 189, and 1 : > 2 500 versus 1 : > 94, respectively). Signal transduction assays confirmed that the ligands behave as agonists of NPY. In particular, [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP is as efficient as NPY itself in receptor activation (Figure 1).

Accordingly, a new class of Y_5-receptor selective ligands has been developed, whose receptor selectivity is provided by the sequence motif Ala^{31}-Aib^{32}.

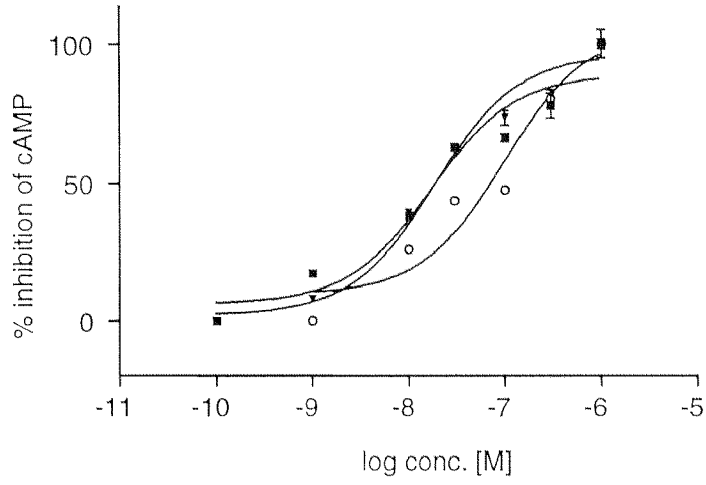

Figure 1. Inhibition of cAMP in the presence of pNPY (▼), [Ala^{31}, Aib^{32}]-pNPY (O) and [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (■) after forskolin-stimulated cAMP production in BHK cells.

5.3.3 Selective Y_5-receptor activation induces food intake in rats

To investigate the *in vivo* potency of [Ala^{31}, Aib^{32}]-pNPY in stimulating feeding by activation of the Y_5-receptor, the selective agonist was administered centrally to rats at three different doses (0.2, 2.0 and 6.0 nmol) and food intake was monitored over 8 hours (firstly after 1 hour and then every 2 hours). The results showed a dose-dependent stimulatory effect on food intake in rats treated with the NPY analog against the control animals (Figure 2a). 1 Hour after administration, the peptide, although being inactive at the low dosis of 0.2 nmol, induced an eight-fold and ten-fold increase in food intake at the higher doses of 2.0 and 6.0 nmol, respectively. After 4 hours, even at the dosis of 0.2 nmol,

a significant increase in food intake was provoked. The stimulation of feeding remained significant even 8 hours after administration: accordingly, the increase in food intake was three-fold at the dosis of 0.2 nmol, four-fold at the dosis of 2.0 nmol and five-fold at the dosis of 6.0 nmol, compared with unstimulated food consumption. After 24 hours no more effect was detected any longer.

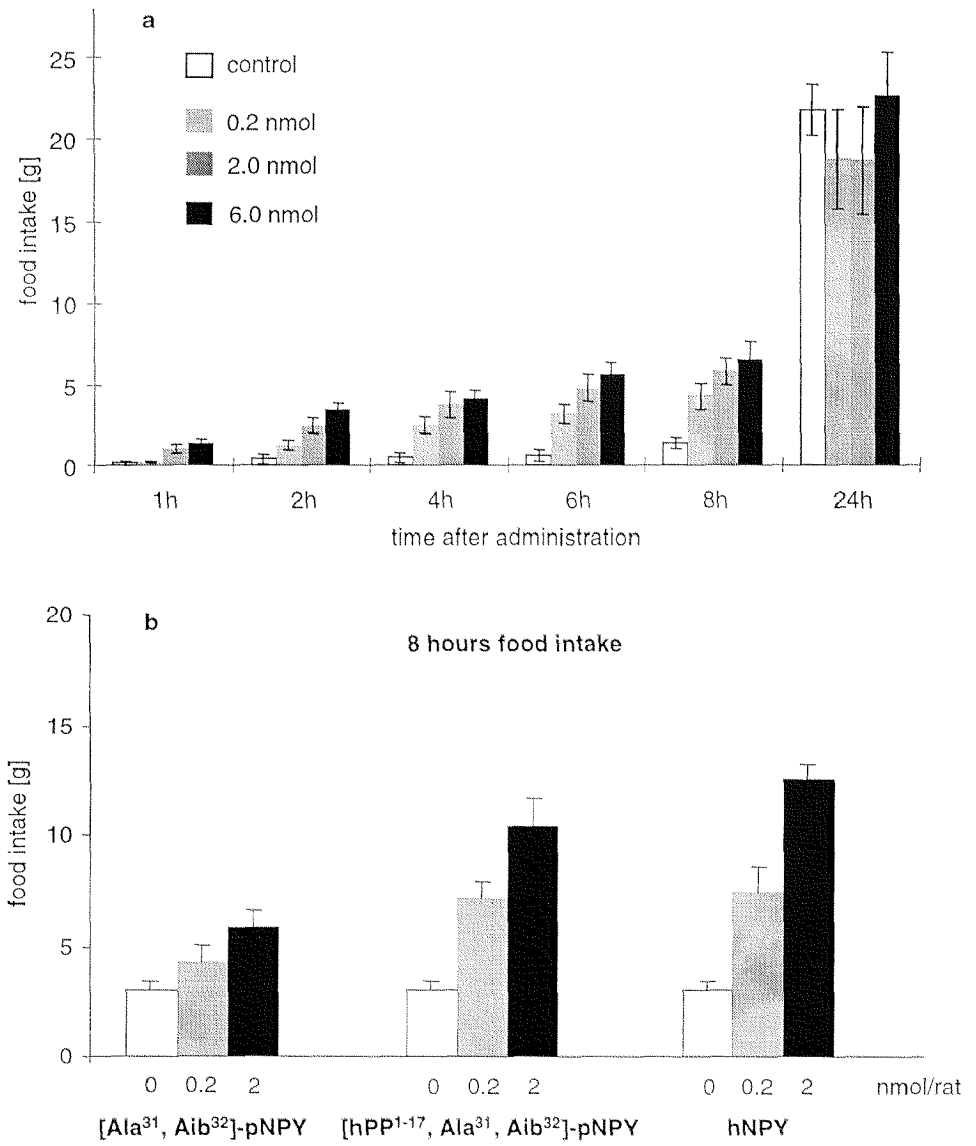

Figure 2. Effect of NPY and of the Y_5-receptor selective analogs on food intake. (**a**) Dose-dependent increase in food intake in rats treated with [Ala^{31}, Aib^{32}]-pNPY. (**b**) Comparison of the dose-dependent stimulation of food intake induced by [Ala^{31}, Aib^{32}]-pNPY, [hPP^{1-17}, Ala^{31}, Aib^{32}]-pNPY and hNPY within 8 hours.

The efficacy of [Ala^{31}, Aib^{32}]-pNPY on stimulation of food intake was compared with that of NPY and of the Y_5-receptor selective analog [hPP$^{1-17}$, Ala^{31}, Aib^{32}]-pNPY. As expected from the binding affinity data, the peptide chimera and NPY were more potent and resulted in a higher amount of food intake in comparison with [Ala^{31}, Aib^{32}]-pNPY, as depicted in Figure 2b.

The results of the *in vivo* feeding experiments clearly show that selective activation of the Y_5-receptor affects food intake in a positive manner. Furthermore, the Y_5-receptor mediated stimulation of feeding depends on the administered dosis of the orexigenic agent.

5.3.4 Ala^{31}-Aib^{32} double substitution induces a different structure of the C-terminal part of NPY

The solution structure of [Ala^{31}, Aib^{32}]-pNPY was investigated by circular dichroism (CD) and 2D-NMR spectroscopy. Figure 3 shows the CD spectra of the pNPY analog compared to the wild-type peptide at pH 3.2. The CD spectrum of pNPY displays the typical features of an α-helix with two negative bands at 220 and 208 nm and a positive one at 186 nm. The CD spectrum of the analog is characterized by a decrease in intensitiy, especially of the positive peak and of the negative band at 220 nm. By measuring the ellipticity at 220 nm and using the equation of Chen & Yang,[22] the fractional helix content was calculated to be approximately 16% and 20% for the modified and the native pNPY, respectively. Accordingly, the substitutions Ile^{31} to Ala and Thr^{32} to Aib led to a reduction of helicity of about 20%.

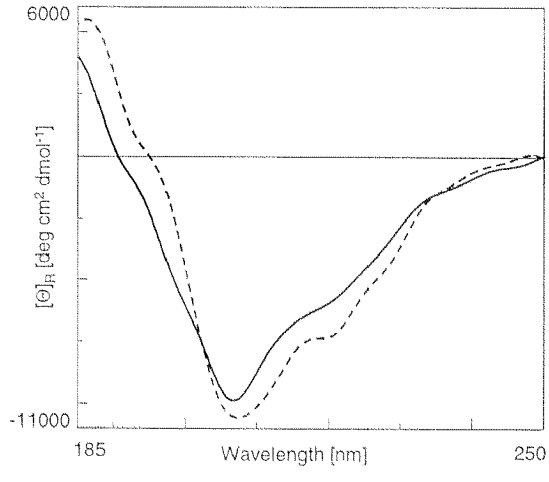

Figure 3. CD spectra of pNPY (---) and [Ala^{31}, Aib^{32}]-pNPY (—) in water at the concentration of 30 μM, pH 3.2 and 20 °C.

Furthermore, the change in the shape of the CD curve is indicative of a partially different conformation of [Ala^{31}, Aib^{32}]-pNPY. Comparison with the CD spectra of known structures suggests an increase in β-turn or 3_{10}-helix in the analog.[23, 24]

In order to gain a more detailed understanding of the structural differences between [Ala^{31}, Aib^{32}]-pNPY and NPY, we used 2D-NMR technique. Distance constraints used for the structure calculation were generated from 200 ms NOESY spectra of a 2 mM sample, at pH 3.2 and 37 °C. Based on the hydrogen bonding pattern, it was concluded that the peptide is α-helical in the region 15-31 with a mean pairwise rms difference of 0.95 Å for the backbone heavy atoms. The N-terminus shows no preferred conformation in solution (Figure 4). In addition, the following observations support the view that the helix is significantly destabilized towards the C-terminus: (1) the lack of a significant number of medium-range NOEs within the C-terminal pentapeptide indicates this segment to be flexible. (2) An *i*, *i*+3 hydrogen bond between positions 28 and 31 (Figure 5) in the absence of the corresponding *i*, *i*+4 reveals the presence of a 3_{10}- instead of an α-helical turn. (3) The chemical shift deviations of the $C^{\alpha}H$ resonances[25] from the corresponding random coil values are smaller in the region 25-30 when compared to hNPY,[21] with the $C^{\alpha}H$ resonance of His^{26} being very close to its random coil value. (4) All $^{3}J_{HN\alpha}$ coupling constants of residues 24-31 and 33-36 are around 7 Hz, which is the value found for rotationally averaged ϕ-backbone angles. In contrast, five $^{3}J_{HN\alpha}$ coupling constants in the region 14-23 were found to be smaller than 6 Hz, clearly showing that the N-terminal part of the helix is more stable. Interestingly, the intermolecular NOEs, as proposed by Monks and co-workers[21] for the dimer structure of wild-type NPY, were not detected in this work.

5.4 Discussion

Obesity has become one of the most common health disorders over the past two decades.[26-28] Several medical problems have been correlated to increased body weight, such as hypertension, cardiovascular diseases, type II diabetes and some forms of cancer. In the past few years, there have been important advances in the understanding of the mechanisms involved in food intake and energy homeostasis. A key role in feeding regulation is played by the adipocyte-derived peptide hormone leptin and its receptor.[29] Leptin levels are correlated with the fat mass: high levels of this hormone reduce food

intake and body weight, while stimulate metabolism. Lack of leptin or its receptor in mutant mice (*ob/ob* mice) induces hyperphagia, obesity, decreased energy expenditure, diabetes and infertility.[30-32] Leptin regulates the hypothalamic expression of several neuropeptides,[27] among them NPY. It has been shown that *ob/ob* mice are characterized by an overproduction of NPY in the hypothalamus,[6] while administration of leptin inhibits NPY secretion. Due to its role in food intake, NPY is a main target for drugs against obesity. Therefore, it is important to understand the mechanisms that underlie the NPY-stimulated feeding. Amongst the NPY receptor subtypes known so far, the Y_1- and Y_5-receptors are the most probable candidates to mediate the orexigenic action of NPY,[10, 11] but many doubts still remain, as several results obtained with different methods are controversial. Wahlestedt and co-workers[33] observed no effect on food intake after administration of Y_1-receptor antisense oligonucleotides, in contrast with the data of Lopez-Valpuesta and co-workers[34] who showed inhibition of NPY-induced feeding. On the other hand, administration of antisense oligonucleotides directed against Y_5-receptors reduced NPY-induced food intake.[35] The knockout studies conducted so far have given unexpected results: both mice with Y_1- and Y_5-receptor knockout exihibited mild obesity, although some differences were observed: the Y_1-receptor deficient animal showed increased body weight without change in feeding behavior, mild-hyperinsulemia, an elevated basal level of plasma insulin and absence of NPY-mediated vasoconstriction,[36, 37] whereas the Y_5-receptor deficient mice presented hyperphagia.[38] Inhibition of NPY-induced feeding was produced by both Y_1- and Y_5-receptor selective nonpeptide antagonists.[39-44] On the other hand, NPY agonists like [Leu^{31}, Pro^{34}]-NPY, NPY (2-36) and (3-36) stimulated food intake.[12] However, these peptides do not selectively bind to one receptor, with [Leu^{31}, Pro^{34}]-NPY displaying equal affinity for the Y_1- and Y_5-receptors. In addition, the N-terminally truncated analogs are ligands for both Y_2- and Y_5-receptors. Although the Y_2-receptor does not seem to mediate the stimulatory effect of NPY on food intake, other effects that are regulated by this receptor can be elicited, which might interfere with the activation of the Y_5-receptor, providing a complex and unclear physiological response. Unfortunately, the use of [D-Trp^{32}]-NPY did not give unambigous results: Gerald and co-workers[12] observed cAMP inhibition and stimulation of food intake mediated by the Y_5-receptor and concluded that this peptide is a weak Y_5-receptor selective agonist *in vitro* and *in vivo*. In contrast, antagonism of NPY-induced feeding by [D-Trp^{32}]-NPY was found by Balasubramaniam and co-workers[13] and Small and co-workers.[14] In the

light of these observations, it must be stated that the *in vivo* profile of this analog of NPY is not-well defined. Furthermore, binding potency at the Y-receptors was tested and found to be in the order $Y_2 \cong Y_5 >> Y_1, Y_4$ (Table 2).

In this work we have presented the first Y_5-receptor selective ligands: the analog [Ala^{31}, Aib^{32}]-pNPY and the (Ala-Aib)-containing PP/NPY chimera. The common sequence Ala^{31}-Aib^{32} represents the key motif for receptor selectivity. Circular dichroism studies on [Ala^{31}, Aib^{32}]-pNPY revealed a reduced α-helix content compared to NPY. The solution structure obtained by 2D-NMR and molecular dynamics confined the destabilization of the helix at the C-terminal end encompassing residues 32-36, for which the backbone conformation is apparently not-well defined (Figure 4). Interestingly, the presence of an *i*, *i*+3 hydrogen bond between residues 28-31 and the absence of the corresponding *i*, *i*+4 hydrogen bond suggests that the α-helix motif ends with a 3_{10}-helical turn (Figure 5).

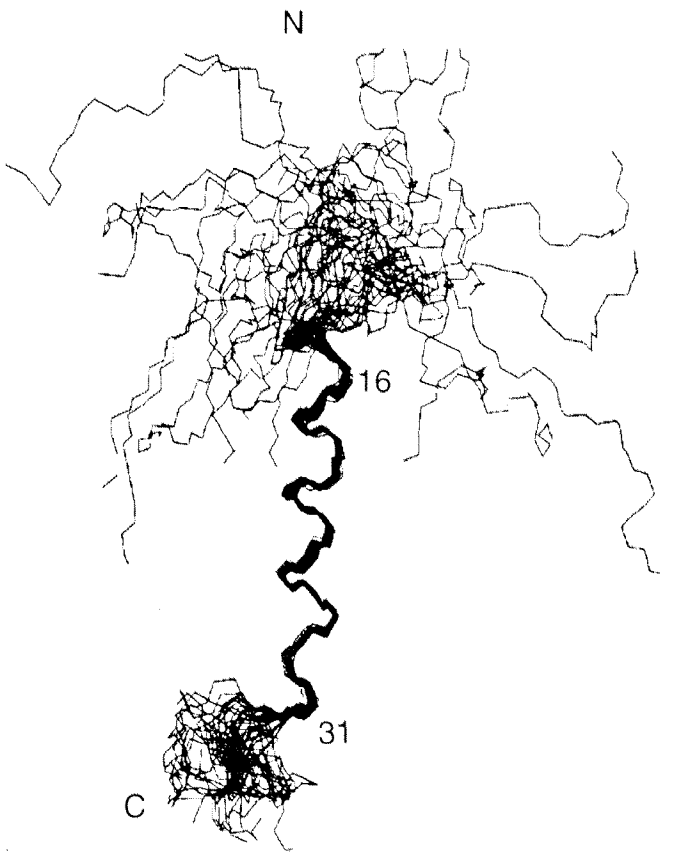

Figure 4. Solution structure of [Ala^{31}, Aib^{32}]-pNPY. The 30 lowest energy structures with DYANA target function values below 0.8 indicating good geometries and compatibility with the NMR constraints are superimposed over the backbone heavy atoms of residues 15-31.

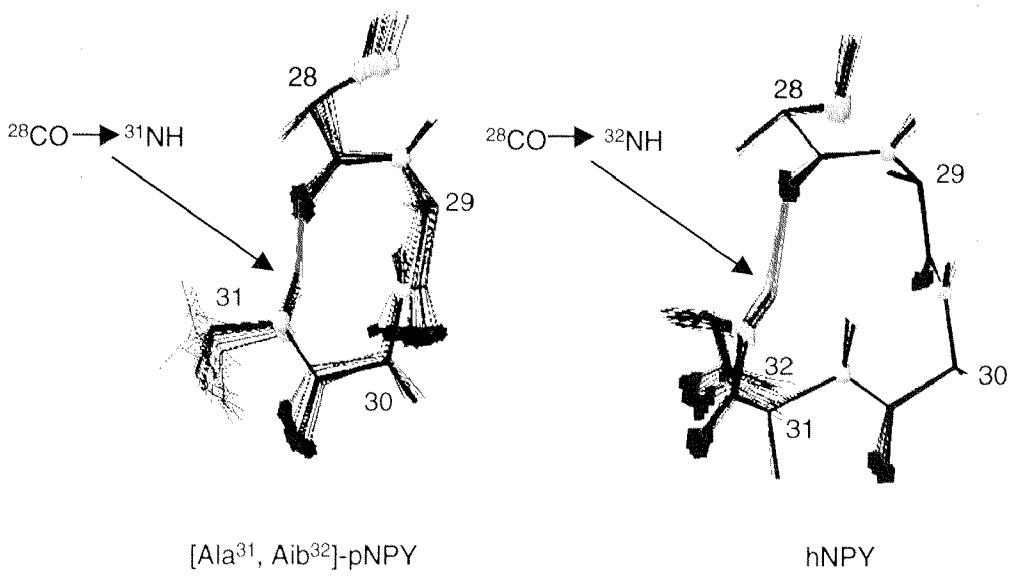

Figure 5. Hydrogen bond pattern over residues 28-32 of [Ala[31], Aib[32]]-pNPY and hNPY. On the left, the 30 structures of the analog with lowest DYANA target function are superimposed over the backbone heavy atoms of residues 28-31. On the right, the structure of hNPY, according to the NMR data of Monks and co-workers, [21] is reported. The arrows indicate the presence of the *i*, *i*+3 hydrogen bond in [Ala[31], Aib[32]]-pNPY and of the *i*, *i*+4 hydrogen bond in hNPY.

The *in vivo* feeding profile of the newly developed Y_5-receptor selective agonists, [Ala[31], Aib[32]]-pNPY and two other (Ala-Aib)-containing PP/NPY chimera, confirms that this receptor subtype is involved in stimulation of food intake. Of course, some aspects still remain to be investigated and more data must be produced. Nonetheless, our results clearly show that the Y_5-receptor certainly plays a decisive role in the complex system that controls hunger. So far, combined Y_1/Y_5 or Y_2/Y_5 ligands have been used to characterize the Y_5-receptor activity. Our new compounds provide highly interesting tools and for the first time allow selectively to target this receptor. Furthermore, radio- or fluorescent-labelled analogs of [Ala[31], Aib[32]]-pNPY might be useful for Y_5-receptor specific assays, for receptor localization and investigation of receptor expression in normal and pathogenic brain structures.

The Y_5-receptor is speculated to be involved in epilepsy, sexual behavior, and circadian rhythm by activation or deactivation of special neurons in the hypothalamus or hippocampus. The (Ala-Aib)-containing NPY agonists can now contribute to elucidate the (patho)-physiological relevance of this receptor.

Accordingly, Ala^{31}-Aib^{32} derived NPY analogs are useful tools to better understand the biological role of NPY. We could already demonstrate that the Y_5-receptor is important for food intake and further controversially discussed activities are currently subjected to further investigation.

5.5 Methods

5.5.1 Peptide synthesis and purification

The peptides were synthesized by Fmoc/*t*-Bu (fluorenylmethoxycarbonyl/*tert*-butyl) solid-phase strategy with an automated multiple peptide synthesizer (Syro MultiSynTech), and 4-(2',4'-dimethoxyphenyl-fluorenylmethoxycarbonyl-aminomethyl)-phenoxyacetamido-norleucylaminomethyl resin (30 mg, 0.45 μmol/mg; Novabiochem, Läufelfingen, Switzerland).[45] Each Fmoc-amino acid (ten-fold excess; Novabiochem) was introduced by double coupling (2 x 36 min) using *in situ* activation with diisopropylcarbodiimide (Aldrich) and hydroxybenzotriazole (Fluka, Buchs, Switzerland). Fmoc removal was carried out with piperidine in dimethylformamide (15 min). Cleavage from the resin and deprotection of the amino acid side-chains were accomplished in one step by trifluoroacetic acid/thioanisole/thiocresol (90/5/5 v/v, 3 hours). For the cleavage of the Met-containing peptides ethanedithiol was added as scavenger (2-4% v/v). The peptides were precipitated from ice-cold diethyl ether and collected by centrifugation, resuspended in ether and centrifuged again. This procedure was repeated four times. The crude peptides were dissolved in *tert*-butanol/water (3/1 w/w), frozen and lyophilized. The Met-containing peptides were subsequently treated with trifluoroacetic acid/trimethylbromosilane/ethanedithiol (96/2.4/1.6 v/v, 30 min) to reduce the Met sulfoxide,[46] then precipitated from ice-cold ether as described before. The lyophilized peptides were purified by preparative HPLC and lyophilized again. The pure products were characterized by analytical HPLC and electrospray ionization mass spectrometry. The peptides were dissolved in diluted HCl (0.5 mM), frozen and lyophilized; this procedure was repeated twice, in order to obtain the corresponding hydrochloride salts.

5.5.2 Cell culture

BHK cells transfected with hY_1, hY_2, hY_4 receptor cDNA and HEK293 cells transfected with the hY_5-receptor were cultured in Dulbecco's modified eagle medium containing 10% fetal calf serum, 5% penstrep and 0.1% geneticin.[39] SMS-KAN cells (hY_2 receptor) were grown in Dulbecco's modified eagle medium/nutrient mix F12 1:1 with 15% fetal calf serum, 4 mM glutamine and 1% non-essential amino acids.[47] Cells were grown to confluency at 37 °C and 5% CO_2.

5.5.3 Binding assays

Cells were resuspended in incubation buffer (Minimum Essential Medium with Earl's salts containing 0.1% bacitracin, 50 µM pefabloc SC and 1% BSA). 200 µl of the suspension containing ca. 440 000 cells were incubated with 25 µl of a 10 nM solution of ^{3}H-propionyl-NPY and 25 µl of a 10 µM solution of NPY or analog. Non specific binding was defined in the presence of 1 µM cold NPY. After 1.5 hours at room temperature, the incubation was terminated by centrifugation at 2 000 x g and 4 °C for 5 min. The pellets were then washed once with PBS by centrifugation, resuspended in PBS and mixed with the scintillation cocktail. Radioactivity was determined by using a β^- counter.

5.5.4 cAMP Enzyme-immunoassay

Cells grown to confluency were resuspended in cAMP-buffer (145 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, 10 mM HEPES, 0.5% BSA, 10 mM glucose, 0.1 mM 3-isobutyl-1-methylxanthine, pH 7.4). 1.5 Mio. cells were incubated with 40 µl of a 1.5 mM solution of forskolin and different concentrations of peptides for 1 hour at 37 °C. Incubation was stopped by addition of 100 µl of a 1M solution of HCl. Cell lysis was done by freezing, followed by centrifugation and the supernatant was diluted 1:30.

The intracellular amount of cAMP was determined by using a cAMP enzyme-immunoassay (Biotrak). Reactions were performed according to the protocol of the manufactor, and optical density was determined at 450 nm.

5.5.5 Food intake studies

Adult male rats weighing between 340 and 400 g were individually housed and maintained on a 12 : 12 hours light-day cycle beginning at 6.00 a. m.. Tap water and standard laboratory chow were available throughout. After 1 week of habituation to their new housing conditions, the animals were anaesthetized with sodium pentobarbital for the placement of stainless steel guide cannulae. Bilateral guide cannulae were placed 1 mm above the paraventricular nucleus according to the stereotaxic coordinates (Paxinos & Watson, 1986): AP:-1.8, L:0.5, V:7.0. Guide cannulae were maintained in place on the skull with small metal screws and dental acrylic cement. Cannulae were closed with a stainless steel stylet when not in use. Rats were allowed to recover for 1 week and were adapted to the injection procedure. On the day of the experiments drugs were injected between 8.00 and 9.00 a. m.. For each experiment eight rats were used and for each dose a different group of rats was used. Food intake was monitored for 24 hours.

5.5.6 CD spectroscopy

The CD spectra were recorded on a JASCO model J720 spectropolarimeter over the range 185-250 nm at 20 °C in a N_2 atmosphere. The peptides were dissolved in water at the concentration of 60 μM. The pH was diminished until 3.2 by addition of a 0.12 M solution of HCl, then water was added to obtain a concentration of 30 μM. Each measurement was performed four times using a thermostatable sample cell with a path of 0.02 cm. Response time was set at 2 s at a scan speed of 20 nm/min, sensitivity of 10 mdeg and step resolution of 0.2 nm. The CD spectrum of the solvent was subtracted from the CD spectra of the peptide solutions to eliminate the interference from cell, solvent and optical equipment. High-frequency noise was reduced by means of a low-path Fourier-transform filter. The values for the mean-residue molar ellipticity $[\Theta]_R$ were expressed in deg cm^2 $dmol^{-1}$.

5.5.7 NMR spectroscopy

NMR samples were prepared by dissolving the peptide in 90% H_2O/10% D_2O (v/v) or 99.9% D_2O. The pH was adjusted to 3.2 by adding small aliquots of 0.1 M solutions of HCl or DCl. All experiments were measured on a 2 mM sample, unless stated otherwise, at 37 °C on a Bruker DRX600 spectrometer. The proton resonances were assigned according

to the standard sequential assignment procedure[48] using data from DQF-COSY, 80 ms TOCSY and 200 ms NOESY recorded on both the 4 mM and 2 mM samples in 90% H_2O/10% D_2O and in 99.9% D_2O. Additional torsion angle constraints were introduced from $^3J_{\alpha\beta}$ coupling constants extracted from an E. COSY experiment recorded in 99.9% D_2O.

5.5.8 Structure calculation

Upper limits for the structure calculation were taken from the integrals of NOESY peaks from all experiments recorded at 2 mM concentration. $^3J_{NH\alpha}$ coupling constants were determined from the splitting of the in-phase dublets of NOESY peaks involving amide protons[49], but only those couplings that were indicative of non rotationally averaged torsion angles were included. From all unambigously assigned NOESY peaks, 14 $^3J_{\alpha\beta}$ coupling constants and five $^3J_{NH\alpha}$ coupling constants < 6 Hz , 274 meaningful upper distance limits as well as 158 ϕ, ψ, χ_1 and χ_2 torsion angle restraints were derived. The calculation was performed by restrained molecular dynamics in torsion angle space using a simulated annealing protocol as implemented in the program DYANA.[50] Figures 4 and 5 were generated using MOLMOL.[51]

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5.6 References

1. Tatemoto, K., Carlquist, M. & Mutt, V. Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* **296**, 659-660 (1982).

2. Reviewed in *Neuropeptide Y and drug development* (eds., Grundemar, L. & Bloom, S. R.) (Academic Press, 1997).

3. Thiele, T. E., Marsh, D. J., Ste. Marie, L., Bernstein, I. L. & Palmiter, R. D. Ethanol consumption and resistance are inversely related to neuropeptide Y levels. *Nature* **396**, 366-369 (1998).

4. Levine, A. S. & Morley, J. E. Neuropeptide Y: a potent inducer of consummatory behavior in rats. *Peptides* **5**, 1025-1029 (1984).

5. Stanley, B. G. & Leibowitz, S. F. Neuropeptide Y injected in the paraventricular hypothalamus: a powerful stimulant of feeding behavior. *Proc. Natl. Acad. Sci. USA* **82**, 3940-3943 (1985).

6. Erickson, J. C., Hollopeter, G. & Palmiter, R. D. Attenuation of the obesity syndrome of ob/ob mice by the loss of neuropeptide Y. *Science* **274**, 1704-1707 (1996).

7. Dallongeville, J., Fruchart, J. C. & Auwerx, J. Leptin, a pleiotropic hormone: physiology, pharmacology, and strategies for discovery of leptin modulators. *J. Med. Chem.* **41**, 5337-5352 (1998).

8. Wang, J., Liu, R., Hawkins, M., Barzilai, N. & Rossetti, L. A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. *Nature* **393**, 684-688 (1998).

9. Michel, M. C., *et al.* XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol. Rev.* **50**, 143-150 (1998).

10. Inui, A. Neuropeptide Y feeding receptors: are multiple subtypes involved? *Trends Pharmacol. Sci.* **20**, 43-46 (1999).

11. Bischoff, A. & Michel, M. C. Emerging functions for neuropeptide Y5 receptors. *Trends Pharmacol. Sci.* **20**, 104-106 (1999).

12. Gerald, C., *et al.* A receptor subtype involved in neuropeptide Y-induced food intake. *Nature* **382**, 168-171 (1996).

13. Balasubramaniam, A., *et al.* [D-Trp32]neuropeptide Y: a competitive antagonist of NPY in rat hypothalamus. *J. Med. Chem.* **37**, 811-815 (1994).

14. Small, C. J., *et al.* Peptide analogue studies of the hypothalamic neuropeptide Y receptor mediating pituitary adrenocorticotrophic hormone release. *Proc. Natl. Acad. Sci. USA* **94**, 11686-11691 (1997).

15. Patent: WO96/16542 PCT/US95/15646.

16. Cabrele, C., Wieland, H. A., Langer, M., Stidsen, C. & Beck-Sickinger, A. G. Manuscript submitted.

17. Karle, I. L. & Balaram, P. Structural characteristics of alpha-helical peptide molecules containing Aib residues. *Biochemistry* **29**, 6747-6756 (1990).

18. Möhle, K., Gussmann, M. & Hofmann, H. J. Structural and energetic relations between beta turns. *J. Comput. Chem.* **18**, 1415-1430 (1997).

19. Beck-Sickinger, A. G., *et al.* Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur. J. Biochem.* **225**, 947-958 (1994).

20. Eckard, C. P., Cabrele, C., Wieland, H. A. & Beck-Sickinger, A. G. Manuscript submitted.

21. Monks, S. A., Karagianis, G., Howlett, G. J. & Norton, R. S. Solution structure of human neuropeptide Y. *J. Biomol. NMR* **8**, 379-390 (1996).

22. Chen, Y.-H., Yang, J. T. & Chau, K. H. Determination of the helix and beta form of proteins in aqueous solution by circular dichroism. *Biochemistry* **13**, 3350-3359 (1974).

23. Woody, R. W. in: *The Peptides*. (eds. Hruby, V. J.) 15-114 (Academic Press, New York, 1985).

24. Toniolo, C., Polese, A., Formaggio, F., Crisma, M. & Kamphuis, J. Circular dichroism spectrum of a peptide 3_{10}-helix. *J. Am. Chem. Soc.* **118**, 2744-2745 (1996).

25. Wishart, D. S., Sykes, B. D. & Richards, F. M. The chemical shift index: a fast and simple method for the assignment of protein secondary structure through NMR spectroscopy. *Biochemistry* **31**, 1647-1651 (1992).

26. Kordik, C. P. & Reitz, A. B. Pharmacological treatment of obesity. Therapeutic strategies. *J. Med. Chem.* **42**, 181-201 (1999).

27. Flier, J. S. & Maratos-Flier, E. Obesity and the hypothalamus: novel peptides for new pathways. *Cell* **92**, 437-440 (1998).

28. Strader, C. D., Hwa, J. J., Van Heek, M. & Parker, E. M. Novel molecular targets for the treatment of obesity. *Drug Discov. Today* **3**, 250-256 (1998).

29. Zhang, Y., *et al.* Positional cloning of the mouse obese gene and its human homologue [published erratum appears in *Nature* **374**, 47 (1995)]. *Nature* **372**, 425-432 (1994).

30. Chen, H., *et al.* Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* **84**, 491-495 (1996).

31. Lee, G. H., *et al.* Abnormal splicing of the leptin receptor in diabetic mice. *Nature* **379**, 632-635 (1996).

32. Chua, S. C. Jr., *et al.* Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Science* **271**, 994-996 (1996).

33. Wahlestedt, C., Pich, E. M., Koob, G. F., Yee, F. & Heilig, M. Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligodeoxynucleotides. *Science* **259**, 528-531 (1993).

34. Lopez-Valpuesta, F. J., Nyce, J. W., Griffin-Biggs, T. A., Ice, J. C. & Myers, R. D. Antisense to NPY-Y1 demonstrates that Y1 receptors in the hypothalamus underlie

NPY hypothermia and feeding in rats. *Proc. R. Soc. Lond. B Biol. Sci.* **263**, 881-886 (1996).

35. Schaffhauser, A. O., *et al.* Inhibition of food intake by neuropeptide Y Y5 receptor antisense oligodeoxynucleotides. *Diabetes* **46**, 1792-1798 (1997).

36. Kushi, A., *et al.* Obesity and mild hyperinsulinemia found in neuropeptide Y-Y1 receptor-deficient mice. *Proc. Natl. Acad. Sci. USA* **95**, 15659-15664 (1998).

37. Pedrazzini, T., *et al.* Cardiovascular response, feeding behavior and locomotor activity in mice lacking the NPY Y1 receptor. *Nat. Med.* **4**, 722-726 (1998).

38. Marsh, D. J., Hollopeter, G., Kafer, K. E. & Palmiter, R. D. Role of the Y5 neuropeptide Y receptor in feeding and obesity. *Nat. Med.* **4**, 718-721 (1998).

39. Wieland, H. A., Engel, W., Eberlein, W., Rudolf, K. & Doods, H. N. Subtype selectivity of the novel nonpeptide neuropeptide Y Y1 receptor antagonist BIBO 3304 and its effect on feeding in rodents. *Br. J. Pharmacol.* **125**, 549-555 (1998).

40. Doods, H. N., *et al.* Effects of the novel Y1 antagonist BIBO3304 on feeding in rodents. *Regul. Pept.* **71**, 212 (1997).

41. Hipskind, P. A., *et al.* Potent and selective 1,2,3-trisubstituted indole NPY Y-1 antagonists. *J. Med. Chem.* **40**, 3712-3714 (1997).

42. Kanatani, A., *et al.* Potent neuropeptide Y Y1 receptor antagonist, 1229U91: blockade of neuropeptide Y-induced and physiological food intake. *Endocrinology* **137**, 3177-3182 (1996).

43. Hofbauer, K. G., *et al.* Antisense oligonucleotides targeted against the NPY Y5 receptor and a selective Y5 receptor antagonist inhibit food intake in rodents. *Regul. Pept.* **71**, 211 (1997).

44. Criscione, L., *et al.* Food intake in free-feeding and energy-deprived lean rats is mediated by the neuropeptide Y5 Receptor. *J. Clin. Invest.* **102**, 2136-2145 (1998).

45. Rist, B., *et al.* The bioactive conformation of neuropeptide Y analogues at the human Y2-receptor. *Eur. J. Biochem.* **247**, 1019-1028 (1997).

46. Beck, W. & Jung, G. Convenient reduction of S-oxides in synthetic peptides, lipopeptides and peptide libraries. *Lett. Pept. Sci.* **1**, 31-37 (1994).

47. Ingenhoven, N. & Beck-Sickinger, A. G. Fluorescent labelled analogues of neuropeptide Y for the characterization of cells expressing NPY receptor subtypes. *J. Recept. Signal Transduct. Res.* **17**, 407-418 (1997).

48. Wüthrich, K. *NMR of proteins and nucleic acids* (eds., John Wiley & Sons) (Wiley Interscience, New York, 1986).

49. Szyperski, T., Güntert, P., Otting, G. & Wüthrich, K. Determination of scalar coupling constants by inverse Fourier transformation of in-phase multiplets. *J. Magn. Resonance* **99**, 552-560 (1992).

50. Güntert, P., Mumenthaler, C. & Wüthrich, K. Torsion angle dynamics for NMR structure calculation with the new program DYANA. *J. Mol. Biol.* **273**, 283-298 (1997).

51. Koradi, R., Billeter, M. & Wuthrich, K. MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* **14**, 51-55, 29-32 (1996).

CHAPTER 6

Ala^{31}-Aib^{32}: Identification of the Key Motif for High Affinity and Selectivity of Neuropeptide Y at the Y_5-Receptor

Chiara Cabrele[‡], Heike A. Wieland[§], Carsten Stidsen[†] and Annette G. Beck-Sickinger[‡*]

[‡]Department of Pharmacy, ETH Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
[§]Division of Preclinical Research, Boehringer Ingelheim Pharma KG, Birkendorferstrasse 65, 88397 Biberach, Germany
[†]Molecular Pharmacology, Novo Nordisk Park, 2760 Malov, Denemark

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6.1 Abstract

The turn-inducing sequence Ala-Aib introduced into positions 31 and 32 of neuropeptide Y (NPY) and its analogs has been identified as the key structure for Y_5-receptor selectivity. Full length, N-terminally and centrally truncated analogs of NPY and PP/NPY chimera containing the motif Ala-Aib were prepared: all peptides turned out to be selective for the Y_5-receptor. The affinity of the NPY based peptides was in the range 4-70 nM, while the affinity of three (Ala-Aib)-contaning PP/NPY chimera was in the range 0.2-0.9 nM. The circular dichroism spectra of the Aib-analogs were all characteristic of an α-helix, however, they had different intensities of the two negative bands at 220 nm and 208 nm. Affinity and selectivity for the Y_5-receptor were correlated with the ratio of the ellipticity at 220 nm versus the one at 208 nm (R). When R was in the range 0.74-0.96, the affinity at the Y_5-receptor was in the range 4-70 nM, while there was complete loss of affinity at the Y_4-receptor. $R > 1.15$ was associated with very high affinity at the Y_5-receptor and weak affinity at the Y_4-receptor. These results suggest that the selectivity of the Ala^{31}-Aib^{32} motif for the Y_5-receptor derives from a specific conformation that must be correlated with the bioactive conformation of NPY at this subtype.

6.2 Introduction

Neuropeptide Y (NPY) (1) is a 36 amino acid peptide amide and is the most conserved member of a hormone family, the so called NPY family, which further contains the pancreatic polypeptide (PP) and the peptide YY (PYY). NPY is widely distributed within the central and peripheral nervous systems and exerts several physiological functions, such as vasoconstriction, anxyolysis, regulation of neurotransmitter release, increase in memory retention and stimulation of food intake. NPY and related peptides activate at least four different receptor subtypes that belong to the large family of the G-protein coupled receptors and are designated by a capital Y: Y_1, Y_2, Y_4 and Y_5 (2). A fifth receptor has been found in mice, but only an inactivated protein is expressed in human (3). There is strong evidence that the Y_1- and Y_5-receptor subtypes mediate the NPY-induced increase in food intake (4). So far, feeding studies have been carried out *in vivo* by administration of NPY analogs which are Y_1- or Y_5-receptor preferring ligands, but they are not highly specific relative to the other subtypes (5,6): for example, NPY (2-36) has been shown to be equally potent at the receptors Y_2 and Y_5 and still activates the Y_1-receptor. The shorter analogs of NPY, (3-36) and (13-36), prefer the Y_2- and Y_5-receptor subtypes, but some Y_1-receptor activity is also maintained. [Leu^{31}, Pro^{34}]-NPY and [Pro^{34}]-PYY are equipotent at the receptors Y_1, Y_4 and Y_5. Only [D-Trp^{32}]-NPY has been shown selectively to activate the Y_5-receptor, however, it is a weak agonist (5) and, additionally, we found that it also binds to the Y_2-receptor with nanomolar affinity (7). All these analogs have shown orexigenic properties (5,6). Because of the lack of specificity, these peptides are not very suitable tools to distinguish the individual role of the Y_1- and Y_5-receptor subtypes in the stimulation of food intake induced by NPY agonists.

A further approach to the *in vivo* characterization of the feeding receptors has been based on the application of antagonists. BIBP 3226 (8) and BIBO 3304 (9) are potent and highly selective ligands at the Y_1-receptor, and their administration inhibited the NPY-stimulated increase in food intake. However, it has been observed that the injection of BIBP 3226 is associated with abnormal behavior and this has suggested that the compound might inhibit NPY-induced feeding via a non-specific mechanism (10). Additionally, Haynes and co-workers have reported that BIBP 3226 failed to inhibit the response to rat NPY in rats (11). 1229U91, an antagonist at the Y_1-receptor (12), was successful in inhibiting the NPY-induced increase in food intake (11,13,14), however, it should be taken into account that

1229U91 acts as an agonist at the Y_4-receptor (15). Recently, inhibition of food intake has been observed after administration of the Y_5-receptor selective antagonist CGP 71683A (16). Again, the results obtained by using NPY antagonists at the receptors Y_1 and Y_5 can not clarify the role of NPY and its receptors in feeding behavior.

In this work we present a new class of NPY analogs and PP/NPY chimera which selectively bind to the Y_5-receptor. The high specificity for the Y_5-receptor subtype relative to the receptors Y_1, Y_2 and Y_4 has been obtained by the incorporation of the dipeptide Ala-Xxx at positions 31-32, where Xxx is Aib, Pro or hydroxyproline (Hyp). These selective analogs have been shown to act as agonists of NPY at the Y_5-receptor and to be very useful tools to characterize this receptor *in vivo*.

6.3 Experimental Procedures

6.3.1 Materials

The N^{α}-Fmoc-protected amino acids were purchased from Alexix (Läufelfingen, Switzerland). Side-chain protecting groups were: *tert*-butyl for Asp, Glu, Ser, Thr and Tyr, Boc for Lys, trityl for Asn, Gln and His, 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg. The 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink Amide) and the 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucylamino-methyl (Rink Amide AM) resins were obtained from Novabiochem (Läufelfingen, Switzerland). *N*-hydroxybenzotriazole (HOBt), TFA, thioanisole, *p*-thiocresol, 1,2-ethanedithiol, trimethylbromosilane, piperidine, 1-methyl-2-pyrrolidinone, *tert*-butanol, DMF (puriss.), sodium hydrogenphosphate and potassium dihydrogenphosphate were obtained from Fluka (Buchs, Switzerland). *N,N'*-diisopropylcarbodiimide (DIC) was purchased from Aldrich (Buchs, Switzerland). DMF (pure) and diethylether were purchased from Scharlau (La Jota, Barcelona, Spain). Acetonitrile was obtained from Romil (Cambridge, England). 50% Dulbecco's modified eagle medium/50% Ham's F12, minimum essential medium with Earl's salts and phosphate buffered saline (PBS) were purchased from Gibco (Life Technologies, Basel, Switzerland). Fetal calf serum, glutamine and non-essential amino acids were obtained from Boehringer Mannheim (Germany).

Geneticin, bacitracin and bovine serum albumin were purchased from Sigma (Buchs, Switzerland). ^{3}H-propionyl-NPY was purchased from Amersham.

6.3.2 Peptide Synthesis

The peptides were synthesized by solid-phase technique on an automated multiple peptide synthesizer (Syro, MultiSynTech, Bochum, Germany) using Rink Amide and Rink Amide AM resins (30 mg, resin loading 0.45-0.51 mmol/g). Peptide chain assembly was performed by using Fmoc-strategy and a double coupling procedure with ten-fold excess Fmoc-amino acid, HOBt, DIC in DMF (2 x 40 min). The Fmoc-deprotection step was accomplished by 40% piperidine in DMF for 3 min, 20% piperidine for 7 min and finally 40% piperidine for 5 min. The removal of the amino acid side-chain protecting groups and the peptide cleavage from the resin were accomplished in one step by using the cleavage mixture TFA/thioanisole/thiocresol (90/5/5 v/v) for 3 hours. For the cleavage of the Met-containing peptides ethanedithiol was added as scavenger (2-4% v/v). The fully deprotected peptides were precipitated from ice-cold diethylether, the suspensions were centrifuged at 5 °C, the ether was decanted, then the peptides were suspended again in fresh ether and centrifuged. The washings with cold ether were repeated four times. Finally, the peptides were dissolved in *tert*-butanol/water (3/1 w/w) and lyophilized. The peptides containing Met-sulfoxide were reduced after lyophilization by using the reduction mixture TFA/ethanedithiol/trimethylbromosilane (96/2.4/1.6 v/v) for 30-40 min, under a N_2 atmosphere within the first 5 min, then in tightly closed centrifuge tubes (17). The reduced peptides were recovered from ice-cold ether, washed as described above, purified to homogenity and finally lyophilized.

The peptides were characterized by electrospray ionization mass spectrometry (SSQ 710, Finnigan MAT, Bremen, Germany) and by analytical reversed-phase HPLC on a LiChrospher RP-18 column (5 µm, 3 x 125 mm, Merck, Darmstadt, Germany) using 0.08% TFA in acetonitrile (A) and 0.1% TFA in water (B) as eluting system (20 to 70% A over 35 min at the flow rate of 0.6 ml/min).

6.3.3 Circular Dichroism

The CD spectra were recorded using a JASCO model J720 spectropolarimeter over 250-180 nm at 20 °C in a N_2 atmosphere. The CD spectra were performed on peptide solutions at the concentration range 30-40 µM. Each measurement was repeated four times using a thermostatable sample cell with a path of 0.02 cm and the following parameters: response time of 2 s, scan speed of 20 nm/min, sensitivity of 10 mdeg, step resolution of 0.2 nm and band width of 2 nm. The CD spectrum of the solvent was subtracted from the CD spectra of the peptide solutions to eliminate the interference from cell, solvent and optical equipment. High-frequency noise was reduced by means of a low-path Fourier-transform filter. The ellipticity was expressed as the mean-residue molar ellipticity $[\Theta]_R$ in deg cm^2 $dmol^{-1}$.

6.3.4 Cell Culture

BHK cells transfected with hY_1, hY_2, hY_4 or hY_5 receptors were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 5% penstrep and 0.1% geneticin (9). SMS-KAN cells (hY_2 receptor) were grown in 50% Dulbecco's modified Eagle medium/50% nutrient mix Ham's F12 with 15% fetal calf serum, 4 mM glutamine and 1% non-essential amino acids (18). Cells were grown to confluency at 37 °C and 5% CO_2.

6.3.5 Receptor Binding Assays

Cells were resuspended in incubation buffer (minimum essential medium with Earl's salts containing 0.1 % bacitracin, 50 µM pefabloc SC and 1% bovine serum albumin). 200 µl of the suspension containing ca. 440 000 cells were incubated with 25 µl of a 10 nM solution of ^{3}H-propionyl-NPY and 25 µl of a 10 µM solution of NPY or analog. Non specific binding was defined in the presence of 1 µM cold NPY. After 1.5 hours at room temperature, the incubation was terminated by centrifugation at 2 000 x g and 4 °C for 5 min. The pellets were then washed once with PBS by centrifugation, resuspended in PBS and mixed with the scintillation cocktail. Radioactivity was determined by using a β^- counter.

6.4 Results

6.4.1 Peptide Synthesis

The amino acid sequence of the NPY analogs and of the PP/NPY chimera are shown in Table 1. The synthesis was accomplished by multiple solid-phase technique using the Fmoc/*tert*-butyl chemistry and HOBt/DIC as coupling reagents. The acylation of Aib by Ala, Leu or Tyr went smoothly and run to completion without the need of special and strong activating reagents. Only the sequence Ala-Aib-Ala-Aib at positions 29-32 of peptide **3** turned out to be more difficult, as shown by the presence of a minor peak (15%) in the analytical HPLC chromatogram of the crude product (data not shown). The inpurity was found to have a mass difference of –156 that was attributed to Ala-Aib.

Methionine was introduced into the peptide chain without side-chain protection. The analogs were characterized by analytical HPLC and ESI-MS. The HPLC chromatograms and the ESI-MS spectra of the crude methionine-containing peptides (**7-11**) revealed the presence of side-products that corresponded to the methionine sulfoxide which was formed during the peptide chain assembly and/or the TFA cleavage. The methionine sulfoxide was completely reduced by using trimethylbromosilane in the presence of ethanedithiol under acidic conditions (TFA) (17).

6.4.2 Receptor Binding Affinity

Binding of the peptides to the Y-receptors was tested by using a competition assay on cell lines selectively expressing the Y_1-, Y_2-, Y_4- or Y_5-receptor subtypes (9). The binding affinity is reported as the IC_{50} value in Table 1. The full-length NPY analogs that contain the motif Ala^{31}-Aib^{32} (**1-5**) were found to be selective at the Y_5-receptor, with an affinity that was in the range 4-70.5 nM. The IC_{50} values at the Y_2-receptor were > 480 nM, and the binding potency at the receptors Y_1 and Y_4 was in the micromolar range. The only exception was peptide **2**, [Ala^{31}, Aib^{32}, Pro^{34}]-pNPY, which had a reduced Y_5-receptor selectivity relative to the Y_4-receptor. Accordingly, it bound to the Y_4- and Y_5-receptors with an affinity of 260 nM and 49.7 nM, respectively.

Table 1. Amino acid sequence, analytical and biological data of the NPY analogs and of the PP/NPY chimera. Ala^{31} is shown in **bold**; Aib is indicated as **B**, D-Pro as **p**, Hyp as **U**, Ahx as **X**. The hPP positions introduced into NPY are underlined with a solid line; the pNPY positions introduced into hPP are underlined with a dotted line, and the cPP positions introduced into hPP are underlined with a broken line.

No.	Peptide	Amino acid sequence	M.W.$_{exp}$ [amu]	M.W.$_{calc}$ [amu]	Binding affinity (IC_{50} [nM])			
					Y_1	Y_2	Y_4	Y_5
1	[Ala^{31}, Aib^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINL**AB**RQRY	4195.0	4195.7	1 000	760	1 000	5.86±2.73
2	[Ala^{31}, Aib^{32}, Pro^{34}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINL**AB**RPRY	4162.8	4164.6	> 400		260	49.7±33.3
3	[Ala^{29}, Aib^{30}, Ala^{31}, Aib^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYI**ABAB**RQRY	4125.5	4124.6	> 1 000	480	4 000	4
4	[Aib^{24}, Ala^{31}, Aib^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSA**B**RHYINL**AB**RQRY	4166.9	4167.6	> 1 000		>1 000	14.4±4.7
5	[Aib^{24}, Aib^{28}, Ala^{31}, Aib^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSA**B**RHY**B**NL**AB**RQRY	4139.1	4139.6	> 1 000		>1 000	70.5±0.5
6	[hPP^{1-7}, Ala^{31}, Aib^{32}]-pNPY	APLEPVYPGEDAPAEDLARYYSALRHYINL**AB**RQRY	4163.2	4163.7	3 000		1 000	6.05±1.35
7	[hPP^{1-17}, Ala^{31}, Aib^{32}]-pNPY	APLEPVYPGDNATPEQMARYYSALRHYINL**AB**RQRY	4207.7	4209.8	2 000		192	0.92±0.06
8	[$pNPY^{1-7}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP	YPSKPDNPGDNATPEQMAQYAADLRRYINM**AB**RQRY	4185.8	4186.7	2 000		920	12.1±3.9
9	[$pNPY^{1-7, 19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP	YPSKPDNPGDNATPEQMARYYSALRRYINM**AB**RQRY	4278.3	4278.8	500		81	0.82±0.32
10	[cPP^{1-7}, $pNPY^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP	GPSQPTYPGDNATPEQMARYYSALRRYINM**AB**RQRY	4208.3	4207.8	620		50	0.20
11	[cPP^{1-7}, $pNPY^{19-23}$, Ala^{31}, Aib^{32}, His^{34}]-hPP	GPSQPTYPGDNATPEQMARYYSALRRYINM**AB**RHRY	4216.1	4216.8	560		27.3	3.56±3.04
12	[Ala^{31}, Pro^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINL**AP**RQRY	4206.8	4207.7	> 1 000	666	3 000	12

Table 1. (Continued)

No.	Peptide	Amino acid sequence	M.W.$_{exp}$ [amu]	M.W.$_{calc}$ [amu]	Binding affinity (IC_{50} [nM]) Y_1	Y_2	Y_4	Y_5
13	[Ala^{31}, D-Pro^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINL**Ap**RQRY	4207.4	4207.7	> 1 000	>1 000	2 000	689
14	[Ala^{31}, Hyp^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINL**AU**RQRY	4223.3	4222.7	> 1 000	1 000	4 000	40
15	[Ala^{31}, Aib^{34}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINL**A**TR**B**RY	4167.9	4168.6	1 000	1 000	99	20.0±2.6
16	[Ala^{31}, Aib^{32}]-pNPY (2-36)	PSKPDNPGEDAPAEDLARYYSALRHYINL**AB**RQRY	4031.7	4032.5	> 1 000		> 1 000	7
17	[Ala^{31}, Aib^{32}]-pNPY (18-36)	ARYYSALRHYINL**AB**RQRY	2398.1	2398.8	325	39	307	16
18	[Ahx$^{5-24}$, Ala^{31}, Aib^{32}]-pNPY	YPSK**X**RHYINL**AB**RQRY	2162.4	2162.6	951	65	901	19
19	[Ahx$^{8-20}$, Ala^{31}, Aib^{32}]-pNPY	YPSKPDN**X**YSALRHYINL**AB**RQRY	2923.1	2923.4	> 1 000		> 1 000	31

The NPY analog **3**, in which the motif Ala-Aib was repeated twice consecutively at positions 29-32, had the best affinity at the Y_5-receptor (4 nM). In contrast, the presence of one or two Aib residues in the region 24-28 led to a minor affinity (14.4 nM for peptide **4** and 70.5 nM for peptide **5**).

The NPY chimera **6** and **7** contain the N-terminal part of hPP, the segments 1-7 and 1-17, respectively. While the binding profile of peptide **6** was very similar to that of the analog **1**, peptide **7** turned out to be five- to six-fold more potent at the receptors Y_4 and Y_5, thus displaying very high Y_5-receptor affinity (IC_{50} 0.92 nM) as well as selectivity.

Among the PP chimera **8-11**, peptides **9** and **10** turned out to bind to the Y_5-receptor with a subnanomolar affinity (0.82 nM and 0.20 nM, respectively), while only with moderate affinity to the Y_4- (> 50 nM) and Y_1- (> 500 nM) receptor. The modification of peptide **10** by the replacement of Gln^{34} with His (analog **11**) led to a partial loss of affinity at the Y_5-receptor (18-fold), but to a slight increase at the Y_4-receptor (two-fold), with a subsequent loss of specificity for the Y_5-receptor. The analog [$pNPY^{1-7}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (**8**) was less potent at the Y-receptors compared to the other PP chimera. By comparison of the amino acid sequences of peptides **8** and **9**, the only difference is the absence of the pNPY segment 19-23 in **8**. The lack of this primary structure element in the analog **8** led to a reduction of affinity of 15-fold at the Y_5-receptor, 11-fold at the Y_4-receptor and four-fold at the Y_1-receptor.

The two NPY analogs containing Ala at position 31 and Pro or Hyp at position 32 (peptides **12** and **14**) turned out to be Y_5-receptor selective as the corresponding NPY analogs containing Aib at position 32. Pro was better tolerated than Hyp, as suggested by the reduced affinity (three-fold) of the analog [Ala^{31}, Hyp^{32}]-pNPY (**14**) compared to [Ala^{31}, Pro^{32}]-pNPY (**12**). The introduction of D-Pro caused a dramatic loss of affinity at the Y_5-receptor (689 nM for [Ala^{31}, D-Pro^{32}]-pNPY versus 12 nM for [Ala^{31}, Pro^{32}]-pNPY).

The shift of the Aib-substitution from position 32 to position 34 (peptide **15**) reduced the Y_5-receptor affinity but enhanced the Y_4-receptor one: this led to a lack of Y_5-receptor specifcity relative to the Y_4-receptor but not to the Y_1- and Y_2-receptor subtypes.

The N-terminally truncated NPY analogs [Ala^{31}, Aib^{32}]-pNPY (2-36), (**16**) and (18-36) (**17**) had low affinity at the receptors Y_1 and Y_4 and turned out to be Y_5-receptor preferring ligands (IC_{50} 7 nM and 16 nM, respectively). Peptide **17** had some affinity also for the Y_2-receptor, with an IC_{50} value of 39 nM. A similar binding profile was found for the centrally

truncated analog [Ahx$^{5-24}$, Ala^{31}, Aib^{32}]-pNPY (**18**) which bound to the Y_1- and Y_4-receptor subtypes with more than 900 nM affinity, whereas the Y_2-receptor affinity was 65 nM and the Y_5-receptor one was 19 nM.

6.4.3 Circular Dichroism

The conformation of peptides **1-16** was investigated in aqueous solution and at neutral pH by using circular dichroism (CD) spectroscopy. As shown in Figure 1, all peptides adopted a helical structure; however, the CD profiles were different in the relative intensity of the two negative bands at 220 nm and 208 nm. The analogs [Aib^{24}, Ala^{31}, Aib^{32}]-pNPY (**4**) and [Ala^{31}, Pro^{32}]-pNPY (**12**) showed a minimum at 208 nm and a shoulder at 220 nm, and the ratio of the ellipticity at 220 nm versus the ellipticity at 208 nm (R) was 0.74 for the first peptide and 0.76 for the second one (Figure 1, panel A). Surprisingly, the CD spectrum of [Ala^{31}, D-Pro^{32}]-pNPY (**13**) turned out to be superimposable to that of the corresponding analog containing the L-isomer of Pro (data not shown). The CD curves of the peptides [Aib^{24}, Aib^{28}, Ala^{31}, Aib^{32}]-pNPY (**5**) and [Ala^{31}, Hyp^{32}]-pNPY (**14**) were characterized by a R value of 0.85 and 0.82, respectively (Figure 1, panel B). The CD spectra of [Ala^{31}, Aib^{32}]-pNPY (**1**), [Ala^{29}, Aib^{30}, Ala^{31}, Aib^{32}]-pNPY (**3**) and of the NPY chimera containing the hPP segment 1-7, [hPP$^{1-7}$, Ala^{31}, Aib^{32}]-pNPY (**6**), had a higher R value, which was 0.96 for peptide **1** and 0.94 for peptides **3** and **6** (Figure 1, panel C). The shortened NPY analog [Ala^{31}, Aib^{32}]-pNPY (2-36) (**16**) gave a CD profile similar to that of the corresponding full-length analog, with the same R value. $R > 1$ was found for the hPP analogs **9-11** and for the NPY chimera [hPP$^{1-17}$, Ala^{31}, Aib^{32}]-pNPY (**7**) ($1.15 \leq R \leq 1.32$) (Figure 1, panel D).

The two NPY analogs containing Pro or Aib at position 34, [Ala^{31}, Aib^{32}, Pro^{34}]-pNPY (**2**) and [Ala^{31}, Aib^{34}]-pNPY (**15**) and the PP chimera **8** were characterized by CD spectra displaying some differences compared to the CD profiles described above. In Figure 2, panel A, the CD curves of peptides **2** and **15** are reported and compared to that of the analog [Ala^{31}, Aib^{32}]-pNPY (**1**): the additional presence of Pro^{34} in **2** caused a blue-shift of the negative band at 220 nm as well as an increase in its intensity, and the R value rose from 0.96 to 1.02. In contrast, the movement of Aib from position 32 to position 34 resulted in a red-shift of the CD spectrum and also increased the R value from 0.96 to 1.06. In Figure 2, panel B, the CD spectrum of the hPP analog [pNPY$^{1-7}$, Ala^{31}, Aib^{32}, Gln^{34}]-

hPP (**8**) is compared to that of the corresponding analog, where the hPP fragmet 19-23 was replaced by that of pNPY (peptide **9**). The most striking difference between the two curves was the more pronounced negative band at 208 nm for the peptide lacking the central pNPY segment (**8**); accordingly, the R value decreased from 1.15 to 1.06.

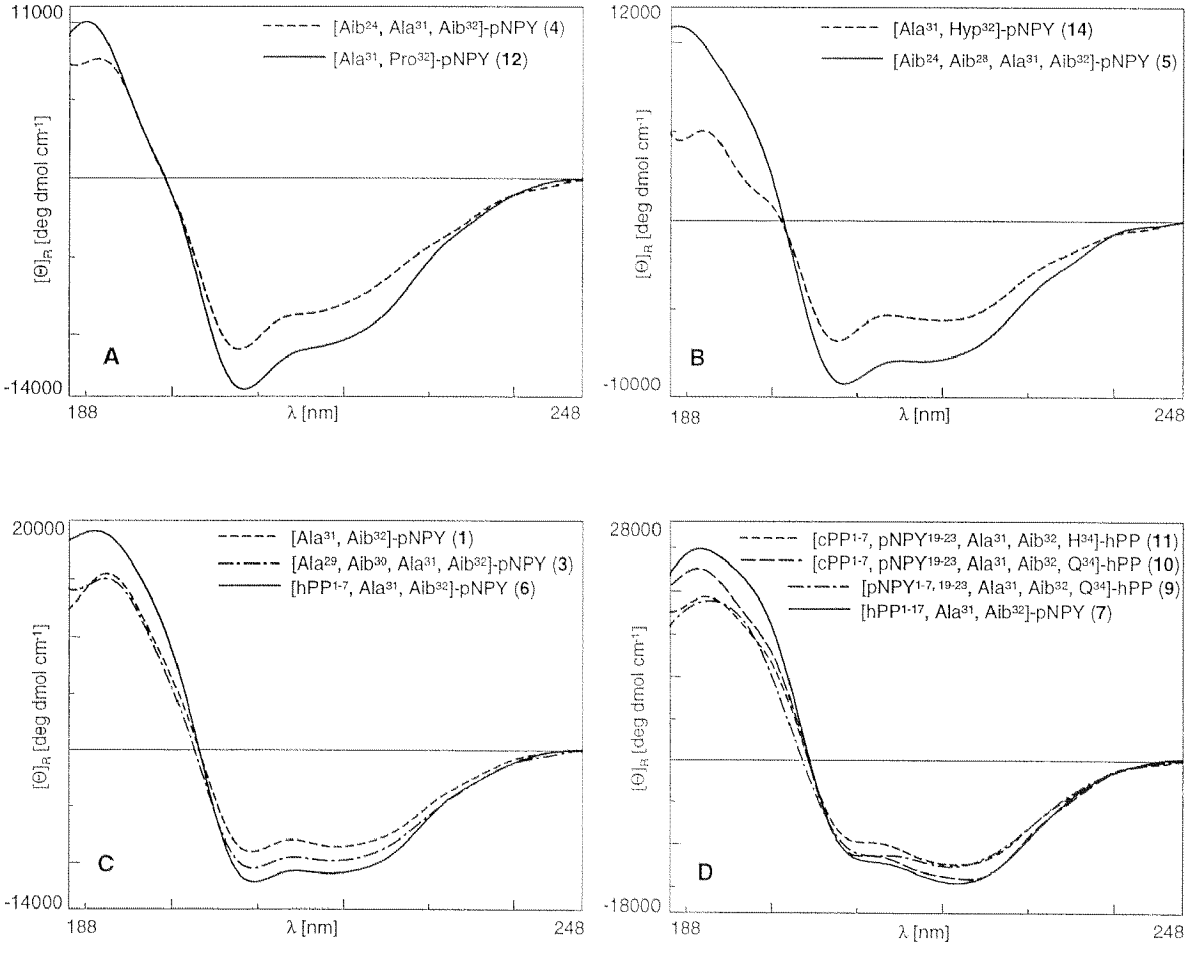

Figure 1. CD spectra of the NPY analogs and PP/NPY chimera in aqueous solution, at pH 7 and 20 °C. Panel A) The ratio between the ellipticity at 220 nm and 208 nm (R) is 0.74-0.76 and the Y_5-receptor affinity is 12-14 nM. Panel B) R is 0.82-0.85 and the Y_5-receptor affinity is 40-70 nM. Panel C) R is 0.94-0.96 and the Y_5-receptor affinity is 4-6 nM. Panel D) R is 1.15-1.32 and the Y_5-receptor affinity is 0.2-3.56 nM.

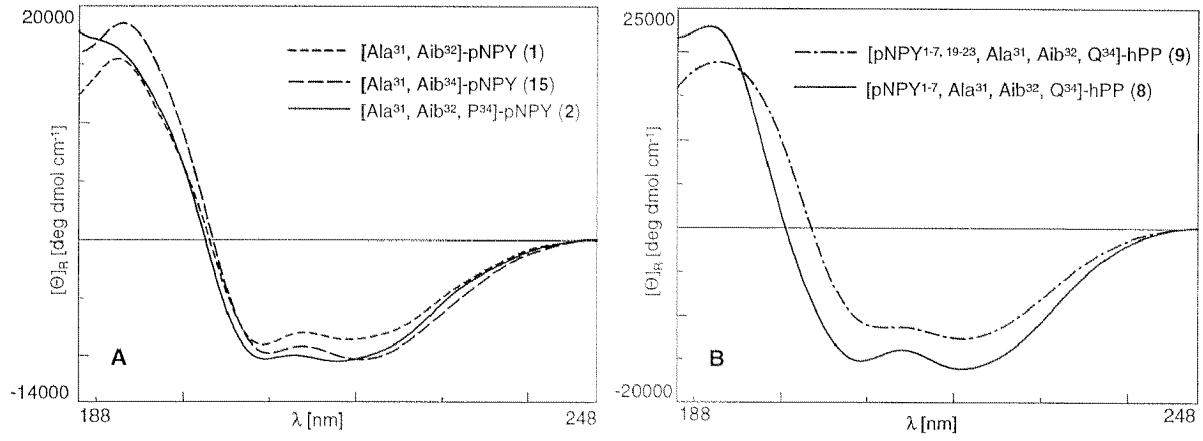

Figure 2. CD spectra of the NPY analogs [Ala^{31}, Aib^{32}, Pro^{34}]-pNPY (**2**) and [Ala^{31}, Aib^{34}]-pNPY (**15**) and comparison with [Ala^{31}, Aib^{32}]-pNPY (**1**) (panel A). CD spectrum of [pNPY$^{1-7}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (**8**) and comparison with [pNPY$^{1-7, 19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (**9**) (panel B).

6.5 Discussion

From structure-affinity and structure-activity relationship studies it is known that the C-terminus of NPY and of the other members of the NPY family represents the functional part of the ligands. Amino acid replacements as well as chemical modifications that involve the C-terminal pentapeptide are poorly tolerated at all NPY receptors. In particular, the two arginine side-chains at positions 33 and 35 play a key role in the binding and activation of the four receptor subtypes Y_1, Y_2, Y_4 and Y_5 (19). In addition, the C-terminal amide is essential, as suggested by the complete loss of affinity after its conversion to a carboxy group or ester (20). The analog [Ala^{34}]-pNPY is as potent as NPY at the Y_4- and Y_5-receptor subtypes, while some affinity is lost at the Y_1- and Y_2-receptors (19). Pro^{34} has been shown to be perfectly tolerated at the receptors Y_1, Y_4 and Y_5, but not at the Y_2-receptor (2,21). The receptors Y_1, Y_2 and Y_4 were sensitive to the Ala-substitution at position 32 (Y_1>Y_2>Y_4), with a loss of affinity in the range 70-3 000-fold, while the affinity at the Y_5-receptor was reduced only ten-fold (19).

So far, there is no information about the bioactive conformation adopted by the C-terminus of NPY in the binding pocket of the different NPY receptors. The solution structure of NPY has been investigated by circular dichroism, 2D-NMR combined with molecular dynamics, and molecular modelling based on the crystal structure of avian PP (aPP). It is well established that there is an amphipatic α-helix in the C-terminal region

(19-34 (22), 15-35 (23), 11-36 (24), 13-36 (25)). In the crystalline form of aPP (26) and in some NMR studies (22) the C-terminal end is structurally not-well defined. The observation that proline is tolerated at postion 34 by all receptors with the exception of the Y_2-receptor suggests that a turn motif might be present in the bioactive conformation of the ligand at the receptors Y_1, Y_4 and Y_5. Therefore, we decided to introduce a turn-inducing motif into positions 31 and 32 which are the residues next to the very sensitive C-terminal tetrapeptide. We chose the dipeptide Ala-Aib because of its property to induce β-turns, especially 3_{10}-helical turns (27,28). [Ala^{31}, Aib^{32}]-pNPY has been recently investigated by 2D-NMR (7): the most striking difference between this analog and native NPY has been identified in the C-terminal nonapeptide that was α-helical in NPY, while in the analog a 3_{10}-turn in the region 28-31 was present, followed by a flexible carboxy end. Interestingly, the presence of the key motif Ala^{31}-Aib^{32} conferred high selectivity of NPY analogs for the Y_5-receptor; moreover, the repeat of the key motif at positions 29-30 modulated the affinity as well as the selectivity at the Y_5-receptor in a positive manner. In fact, [Ala^{29}, Aib^{30}, Ala^{31}, Aib^{32}]-pNPY bound to the Y_5-receptor slightly better than [Ala^{31}, Aib^{32}]-pNPY and showed a higher selectivity with respect to the Y_4-receptor. Instead, the presence of further Aib residues in the region 24-28 reduced the affinity at the Y_5-receptor, which consequently led to loss of selectivity relative to the other receptors. The combination of Ala^{31}-Aib^{32} with Pro^{34} modulated the Y_4- and Y_5-receptor affinity in an opposite way: while the Y_4-receptor affinity was improved, the Y_5-receptor affinity was decreased. The loss of specificity with respect to the Y_4-receptor may be attributed to Pro^{34}, which is present in most species of PP (29). The results obtained with the (Ala-Aib)-containing NPY analogs lead to the conclusion that a C-terminal turn-inducing element provides selectivity for the Y_5-receptor. Thus, the next step is the improvement of affinity at this subtype without loosing specificity.

In a recent work, we developed a series of PP/NPY chimera, some of which turned out to be highly potent at the Y_5-receptor (up to 15-fold more potent than NPY itself), however they had no selectivity (30). Therefore, we decided to combine the primary sequence of the highly potent chimera with the key motif Ala-Aib, in order to obtain peptides with high affinity as well as high selectivity at the Y_5-receptor, which would prove the concept of the turn-inducing motif as the key structure for selectivity. As expected, all (Ala-Aib)-containing chimera bound to the Y_5-receptor with moderate to high affinity, which was comparable to that of NPY for the analogs **7** and **9**, and even three-fold higher for the

analog **10**. His^{34} in the analog **11** turned out to be less favorable at the Y_5-receptor than Gln^{34} (analog **10**), as peptide **11** had 18-fold lower affinity than peptide **10**. Instead, the affinity at the Y_4-receptor increased almost two-fold in the presence of His^{34}, with consequent loss of specificity of the peptide **11** for the Y_5-receptor. Furthermore, the hPP chimera lacking the central pNPY segment 19-23 (peptide **8**) was characterized by a reduced affinity at the receptors Y_4 and Y_5 compared to the corresponding hPP analog that contained pNPY 19-23. This suggests that the sequence 19-23 of pNPY also plays an important role in inducing the bioactive conformation. By comparing the two NPY chimera that contain the hPP sequences 1-7 or 1-17, it was observed that the longer hPP-segment favored the binding at the Y_4- and Y_5-receptor subtypes; however, the peptide [hPP^{1-17}, Ala^{31}, Aib^{32}]-pNPY remained selective for the Y_5-receptor.

To prove the hypothesis that a turn-inducing motif may be used for selectivity at the Y_5-receptor, we investigated the effect of Pro at position 32. Interestingly, also the analog [Ala^{31}, Pro^{32}]-pNPY (**12**) showed a binding profile that was very similar to that of the NPY analogs that contain Aib^{32}, especially to [Aib^{24}, Ala^{31}, Aib^{32}]-pNPY (**4**). In fact, peptides **4** and **12** had similar affinity at the Y_5-receptor (14.4 nM and 12 nM, respectively), and bound to the Y_4-receptor with more than 1 000 nM affinity. When L-Pro^{32} was substituted with the D-isomer (peptide **13**), the Y_5-receptor affinity decreased 57-fold. This suggests that the stereochemistry of the turn-inducing residue 32 is of major importance for the binding conformation. Furthermore, we tested the influence of the trans 4-hydroxy group of hydroxyproline on receptor binding (peptide **14**). The affinity at the Y_5-receptor was three-fold lower than that of the analog [Ala^{31}, Pro^{32}]-pNPY and was more similar to that of the analog [Aib^{24}, Aib^{28}, Ala^{31}, Aib^{32}]-pNPY (**5**) (40 nM for the peptide **14** and 70.5 nM for the peptide **5**).

To investigate the importance of the position of the Aib residue in the C-terminal sequence, we shifted Aib from position 32 to position 34. We found a loss of Y_5-receptor affinity as well as selectivity relative to the Y_4-receptor. This suggests that position 32 may be highly specific for the Y_5-receptor. This seems to be supported by the observation that the Y_2-receptor selective centrally truncated analog [Ahx^{5-24}]-pNPY was converted to a Y_5-receptor preferring ligand by the incorporation of the motif Ala^{31}-Aib^{32} (peptide **18**): accordingly, the affinity at the Y_2-receptor dropped from 1.4 nM to 65 nM (46-fold lower), while the affinity at the Y_5-receptor rose from 795 nM to 19 nM (42-fold higher) (for the affinity values of [Ahx^{5-24}]-pNPY at the Y-receptors see Eckard and co-workers (19)).

Therefore, the first Y_5-receptor preferring ligand with nanomolar affinity and reduced size has been identified.

By the analysis of the solution structure of the Aib-containing analogs by circular dichroism, it was possible to correlate the conformational and biological properties of the peptides and to classify them in four different groups (Figure 1, panels A-D). (A) Peptides **4** and **12** showed a Y_4-receptor affinity of > 1 000 nM and a Y_5-receptor affinity of 12-14.4 nM; they were characterized by a similar helical CD profile, with a ratio (R) of the ellipticity at 220 nm versus the one at 208 nm of 0.74-0.76. (B) The analogs **5** and **14** also bound to the Y_4-receptor in the micromolar range, but their Y_5-receptor affinity was reduced to 40-70.5 nM. Their CD spectra presented a very similar R value that was 0.82-0.85. (C) A micromolar affinity at the Y_4-receptor and an affinity in the range 4-6 nM at the Y_5-receptor were found for the peptides **1**, **3** and **6**. The R value obtained from their CD curves was in the range 0.94-0.96. (D) Finally, the hPP analogs **9-11** and the NPY chimera **7** were the most potent ligands at the Y_5-receptor (0.2-3.6 nM), and bound to the Y_4-receptor with affinities which were in the range 27.3-167 nM. The R value from the CD spectra of this group was determined to be > 1.15.

The analogs [Ala^{31}, Aib^{32}, Pro^{34}]-pNPY (**2**), [Ala^{31}, Aib^{34}]-pNPY (**15**) and [$pNPY^{1-7}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (**8**) could be classified in none of the four groups described above: the peptide **2** was similar to the members of the group B with respect to its affinity at the Y_5-receptor, however, it turned out to be at least four-fold more potent at the Y_4-receptor. The peptide **15** showed a Y_5-receptor affinity comparable to that of the members of the group A, but it was at least ten-fold more potent at the Y_4-receptor. Both the peptides **2** and **15** showed an increase in the intensity of the band at 220 nm (R=1.02-1.06) (Figure 2, panel A). The hPP chimera **8** showed a much lower affinity at the Y_5-receptor than the other chimera (at least three-fold and up to 60-fold lower) and also a more reduced affinity for the Y_4-receptor. To the different biological behavior corresponded a different CD profile, characterized by a more pronounced band at 208 nm (R=1.06), as shown in Figure 2, panel B.

6.6 Conclusions

We developed a new class of Y_5-receptor specific ligands which are characterized by the presence of a turn-inducing element at position 32. We found that Aib as well as Pro induce selectivity at the Y_5-receptor, with Aib being more suitable than Pro, as Aib led to a two-fold higher affinity at the Y_5-receptor than Pro. The NMR structure of [Ala^{31}, Aib^{32}]-pNPY showed a 3_{10}-turn involving residues 28-31. This may suggest that the specific conformation adopted by the ligand in this region is of major importance for receptor recognition and binding. Based on this supposition, we are currently carrying out studies based on peptide and peptidomimetics design, in order to further characterize the structural requirements for high affinity and specificity at the Y_5-receptor. Preliminary results seem to suggest that the affinity at the Y_5-receptor can be increased by the inverse motif Aib-Ala (data not shown).

These selective analogs are very interesting also for their ability to act as agonists of NPY at the Y_5-receptor: they have been recently shown to stimulate food intake in rats (7). Accordingly, they are promising tools to understand the role of NPY as orexigenic neuropeptide.

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6.7 References

1. Tatemoto, K. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5485-5489

2. Michel, M. C., Beck-Sickinger, A., Cox, H., Doods, H. N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., and Westfall, T. (1998) *Pharmacol. Rev.* **50**, 143-150

3. Gregor, P., Feng, Y., DeCarr, L. B., Cornfield, L. J., and McCaleb, M. L. (1996) *J. Biol. Chem.* **271**, 27776-27781

4. Inui, A. (1999) *Trends Pharmacol. Sci.* **20**, 43-46

5. Gerald, C., Walker, M. W., Criscione, L., Gustafson, E. L., Batzl-Hartmann, C., Smith, K. E., Vaysse, P., Durkin, M. M., Laz, T. M., Linemeyer, D. L., Schaffhauser, A. O., Whitebread, S., Hofbauer, K. G., Taber, R. I., Branchek, T. A., and Weinshank, R. L. (1996) *Nature* **382**, 168-171

6. Wyss, P., Stricker-Krongrad, A., Brunner, L., Miller, J., Crossthwaite, A., Whitebread, S., and Criscione, L. (1998) *Regul. Pept.* **75-76**, 363-371

7. Cabrele, C., Langer, M., Bader, R., Wieland, H. A., Doods, H. N., Zerbe, O., and Beck-Sickinger, A. G., manuscript submitted

8. Rudolf, K., Eberlein, W., Engel, W., Wieland, H. A., Willim, K. D., Entzeroth, M., Wienen, W., Beck-Sickinger, A. G., and Doods, H. N. (1994) *Eur. J. Pharmacol.* **271**, R11-13

9. Wieland, H. A., Engel, W., Eberlein, W., Rudolf, K., and Doods, H. N. (1998) *Br. J. Pharmacol.* **125**, 549-555

10. Morgan, D. G., Small, C. J., Abusnana, S., Turton, M., Gunn, I., Heath, M., Rossi, M., Goldstone, A. P., O'Shea, D., Meeran, K., Ghatei, M., Smith, D. M., and Bloom, S. (1998) *Regul. Pept.* **75-76**, 377-382

11. Haynes, A. C., Arch, J. R., Wilson, S., McClue, S., and Buckingham, R. E. (1998) *Regul. Pept.* **75-76**, 355-361

12. Daniels, A. J., Matthews, J. E., Slepetis, R. J., Jansen, M., Viveros, O. H., Tadepalli, A., Harrington, W., Heyer, D., Landavazo, A., Leban, J. J., and et al. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 9067-9071

13. Kanatani, A., Ishihara, A., Asahi, S., Tanaka, T., Ozaki, S., and Ihara, M. (1996) *Endocrinology* **137**, 3177-3182

14. Ishihara, A., Tanaka, T., Kanatani, A., Fukami, T., Ihara, M., and Fukuroda, T. (1998) *Am. J. Physiol.* **274**, R1500-1504

15. Kanatani, A., Ito, J., Ishihara, A., Iwaasa, H., Fukuroda, T., Fukami, T., MacNeil, D. J., Van der Ploeg, L. H., and Ihara, M. (1998) *Regul. Pept.* **75-76**, 409-415

16. Criscione, L., Rigollier, P., Batzl-Hartmann, C., Rüeger, H., Stricker-Krongrad, A., Wyss, P., Brunner, L., Whitebread, S., Yamaguchi, Y., Gerald, C., Heurich, R. O., Walker, M. W., Chiesi, M., Schilling, W., Hofbauer, K. G., and Levens, N. (1998) *J. Clin. Invest.* **102**, 2136-2145

17. Beck, W., and Jung, G. (1994) *Lett. Pept. Sci.* **1**, 31-37

18. Ingenhoven, N., and Beck-Sickinger, A. G. (1997) *J. Recept. Signal Transduct. Res.* **17**, 407-418

19. Eckard, C. P., Cabrele, C., Wieland, H. A., and Beck-Sickinger, A. G., manuscript submitted

20. Hoffmann, S., Rist, B., Videnov, G., Jung, G., and Beck-Sickinger, A. G. (1996) *Regul. Pept.* **65**, 61-70

21. Fuhlendorff, J., Gether, U., Aakerlund, L., Langeland-Johansen, N., Thogersen, H., Melberg, S. G., Olsen, U. B., Thastrup, O., and Schwartz, T. W. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 182-186

22. Mierke, D. F., Durr, H., Kessler, H., and Jung, G. (1992) *Eur. J. Biochem.* **206**, 39-48

23. Darbon, H., Bernassau, J. M., Deleuze, C., Chenu, J., Roussel, A., and Cambillau, C. (1992) *Eur. J. Biochem.* **209**, 765-771

24. Cowley, D. J., Hoflack, J. M., Pelton, J. T., and Saudek, V. (1992) *Eur. J. Biochem.* **205**, 1099-1106

25. Monks, S. A., Karagianis, G., Howlett, G. J., and Norton, R. S. (1996) *J. Biomol. NMR* **8**, 379-390

26. Blundell, T. L., Pitts, J. E., Tickle, I. J., Wood, S. P., and Wu, C.-W. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4175-4179

27. Möhle, K., Gussmann, M., and Hofmann, H. J. (1997) *J. Comput. Chem.* **18**, 1415-1430

28. Toniolo, C., Polese, A., Formaggio, F., Crisma, M., and Kamphuis, J. (1996) *J. Am. Chem. Soc.* **118**, 2744-2745

29. Larhammar, D. (1996) *Regul. Pept.* **62**, 1-11

30. Cabrele, C., Wieland, H. A., Langer, M., Stidsen, C. and Beck-Sickinger, A. G., manuscript submitted

PUBLICATIONS

A. Zaramella, O. Curcuruto, M. Hamdan; C. Cabrele, E. Peggion (1995) **Investigation of Crudes of Synthesis of [Leu^{31}, Pro^{34}]-Neuropeptide Y by Capillary Zone Electrophoresis/Mass Spectrometry**. *Rapid. Commun. in Mass Spectrometry* **9**, 1386-1390.

C. Cabrele, S. Furin, M.Gurrath, S. Mammi, E. Schievano, E. Peggion (1997) **Aggregation and Conformational Transition in Aqueous Solution of a Bombolitin III Analogue Containing a Photoreactive Side-Chain Group**. *Biopolymers* **42**, 147-156.

C. Bubert, C. Cabrele, O. Reiser (1997) **Novel Strategies for the Synthesis of Peptides Containing Cis- or Trans-β-Aminocyclopropane-Carboxylic Acids**. *Synlett*, 827-829.

C. Cabrele, M. Langer, A. G. Beck-Sickinger (1999) **The Amino Acid Side Chain Attachment Approach and Its Application to The Synthesis of Tyrosine-Containing Cyclic Peptides**. *J. Org. Chem.* **64**, 3453-4361.

C. Cabrele, A. G. Beck-Sickinger (1999) **Amino Acid Side-Chain Attachment: A Convenient Strategy to Obtain C-Terminal Modified or Cyclic Peptides by Solid Phase Synthesis**. In *Peptides 1998*, S. Bajusz and F. Hudecz, Ed.; pp. 202-203, Akadémiai Kiadó, Budapest 1999. 25th European Peptide Symposium, EPS, Budapest, Hungary.

A. G. Beck-Sickinger, C. Cabrele, R. M. Söll (1999) **Neuropeptide Y: A Molecule for Hunger, Stress and Memory**. In *Bioorganic Chemistry: Highlights and New Aspects* (U. Diederichsen, T. K. Lindhorst, B. Westermann, L. A. Wessjohann, eds.), Wiley-VCH, Weinheim, 1999, 291-297.

C. Bubert, C. Cabrele, R. Beumer, O. Vielhauer, M. Pietzsch, O. Reiser (1999) **The Synthesis of Enantiomerically Pure β-Aminocyclopropane Carboxylic Acids**. *J. Org. Chem.*, submitted.

C. P. Eckard, C. Cabrele, H. A. Wieland, A. G. Beck-Sickinger (1999) **Characterization of Neuropeptide Y Receptor Subtypes By Synthetic NPY Analogues and By Anti-Receptor Antibodies**. *Cell. Mol. Life Sci.*, submitted.

C. Cabrele, M. Langer, R. Bader, H. A. Wieland, H. N. Doods, O. Zerbe, A. G. Beck-Sickinger (1999) **The First Selective Agonist at the Neuropeptide Y Y_5-Receptor Increases Food Intake in Rats**. *Nature Neuroscience*, submitted.

C. Cabrele, H. A. Wieland, M. Langer, C. Stidsen, A. G. Beck-Sickinger (1999) **Y-Receptor Affinity Modulation by the Design of Pancreatic Polypeptide/Neuropeptide Y Chimera Led to Y_5-Receptor Ligands with Picomolar Affinity**. *J. Med. Chem.*, submitted.

C. Cabrele, H. A. Wieland, C. Stidsen, A. G. Beck-Sickinger (1999) **Ala^{31}-Aib^{32}: Identification of the Key Motif for High Affinity and Selectivity of Neuropeptide Y at the Y_5-Receptor**, *J. Biol. Chem.*, submitted.

C. Cabrele, A. G. Beck-Sickinger (1999) **Molecular Characterization of the Interaction Ligand-Receptor of the Neuropeptide Y Family (Review)**. *J. Pept. Sci.*, in press.

POSTERS

C. Cabrele, A. G. Beck-Sickinger (4/1999) **At the Search of Highly Potent Ligands of the NPY-Receptor Subtypes: NPY/hPP Chimeras**. Peptido- and Proteino-Mimetics, Houffalize, Belgium.

C. Cabrele, M. Langer, A. G. Beck-Sickinger (11/1998) **The First Y_5-Receptor Selective Agonist of Neuropeptide Y**. Posterday Pharmacology-Pharmacy, ETH Zurich, Switzerland.

R. Bader, C. Cabrele, A. G. Beck-Sickinger, O. Zerbe (11/1998) **Solution Structure of an NPY-Y_5 Agonist from 2D ^{1}H NMR Data**. Posterday Pharmacology-Pharmacy, ETH Zurich, Switzerland.

R. Söll, C. Cabrele, A. G. Beck-Sickinger (10/1998) **Neuropeptide Y: A Molecule for Hunger, Stress and Memory**. Neuroscience Center Zurich: Opening Symposium, Zurich.

C. Cabrele, A. G. Beck-Sickinger (8/1998) **Amino Acid Side-Chain Attachment: A Convenient Strategy to Obtain C-Terminal Modified or Cyclic Peptides by Solid Phase Synthesis**. 25th European Peptide Symposium, EPS, Budapest, Hungary.

C. Cabrele, A. G. Beck-Sickinger (10/1997) **Solid Phase Synthesis of Modified NPY Fragments Using the Side Chain Amino Acid Attachment Approach**. 4th Meeting on Peptide and Protein Drugs, ETH Zurich.

C. Bubert, C. Cabrele, O. Vielhauer, M. Pietzsch, O. Reiser (9/1997) **Conformationally Restricted β-Amino Acids as New Peptide Building Blocks**. Workshop: Peptide Modification by Non-Natural Amino Acids, Leipzig, Germany.

ORAL PRESENTATIONS

The First Agonist of Neuropeptide Y with High Affinity and Selectivity at the Y_5-Receptor (3/1999), 4th Deutsches Peptidsymposium, Leipzig, Germany.

Design and Synthesis of Conformationally Constrained Analogs of Neuropeptide Y with High Selectivity for the Y-Receptor Subtypes (10/1998), Aktuelle Forschung Pharmazie, ETH Zurich, Switzerland.

Conformationally Constrained Peptides (9/1996), Seminar of the Pharmaceutical Biochemistry Group, Department of Pharmacy, ETH Zurich, Vira, Switzerland.

Solid Phase Synthesis of Bioactive Peptides: Bombolitin III, NPY and Analogs. Conformational Studies in Aqueous Solution by Circular Dichroism (6/1995), Max-Planck-Institut für Experimentelle Medizin, Göttingen, Germany.

CURRICULUM VITAE

1969	Born on June 6th in Padova, Italy
7/1988	High school diploma (Abitur), Liceo classico C. Marchesi, Padova, Italy
10/1988 - 7/1994	Chemistry studies at the University of Padova, Italy
10/1992 - 12/1992	Visiting Research Fellow (Erasmus Student Exchange Program) with Prof. Dr. O. Reiser, University of Göttingen, Germany *Model Studies on Dihydropyrans as Building Blocks in Organic Synthesis*
10/1993 - 7/1994	Diploma thesis with Prof. E. Peggion, University of Padova, Italy *Structural Variations of Bombolitin III in Aqueous Solution Induced by Self-Aggregation*
7/1994	Diploma in Chemistry, University of Padova, Italy
11/1994 - 10/1995	Research Fellow with Prof. E. Peggion, University of Padova, Italy. Research position (Glaxo Wellcome) at the University of Padova, Italy *Synthesis and Conformational Studies of Bioactive Peptides*
3/1996 - 10/1996	Research Fellow with Prof. Dr. O. Reiser, University of Göttingen, Germany. Research position (Deutsche Forschungsgemeinschaft) at the University of Göttingen *Synthesis of β-Amino-Cyclopropane-Carboxylic Acid Derivatives* *Lipases and Esterases Induced Enantioselective Hydrolysis of Methyl Carboxylates*
Since 11/1996	Ph. D. student with Prof. Dr. Annette G. Beck-Sickinger at the Swiss Federal Institute of Technology (ETH) Zurich, Switzerland, Department of Pharmacy, section of Pharmaceutical Biochemistry
1/1997 - 2/1999:	Teaching assistant in Pharmaceutical Chemistry, Swiss Federal Institute of Technology, Zurich
8/1999	Final examination to obtain the degree of Doctor of Natural Sciences, Swiss Federal Institute of Technology, Zurich

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