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**Molecular Characterization of the
Neuropeptide Y Y₂-Receptor**

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ABBREVIATIONS

ACSF, artificial cerebrospinal fluid

Ahx, 6-aminohexanoic acid

AMPA, alpha-amino-3-hydroxy-5-methyl-isoxalone-4-propionic acid

aPP, avian (turkey) pancreatic polypeptide

APV, DL-2-amino-5-phosphonovaleric acid

ARC, arcuate nucleus

BH, Bolton-Hunter

BHK, baby hamster kidney

Bpa, *p*-benzoylphenylalanine

BSA, bovine serum albumin

CD, circular dichroism

CF, 4(5)-carboxyfluorescein

CFSE, 4(5)-carboxyfluorescein-*N*-succinimidylester

CNS, central nervous system

DATD, *N,N'*-diallyltartardiamide

DIC, *N,N*-diisopropylcarbodiimide

DMF, *N,N*-dimethylformamide

Dpr, 2,3-diaminopropionic acid

EDTA, ethylenediaminetetraacetic acid

EPSC, excitatory postsynaptic current

FITC, 4(5)-fluoresceinisothiocyanate

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

GABA, γ -amino butyric acid

GPCR, G-protein coupled receptor

HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid

HOBt, 1-hydroxy-benzotriazole

HPLC, high performance liquid chromatography

$^3\text{H-Tmd}27$, [N_α -biotinyl-Ahx $_2$, $^3\text{H-propionyl-Lys}^4$, Ahx $^{5-24}$, (Tmd)Phe 27] NPY

$^3\text{H-Tmd}36$, [N_α -biotinyl-Ahx $_2$, $^3\text{H-propionyl-Lys}^4$, (Tmd)Phe 36] NPY

icv, intracerebroventricular

IPSC, inhibitory postsynaptic current

- KH, Krebs-Henseleit
- LH, lateral hypothalamus
- KLH, keyhole limpet hemocyanin
- LTS, low-threshold spike
- MEM, minimum essential medium
- MS, mass spectrometry
- MW, molecular weight
- NBQX, 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione
- NMDA, N-methyl-D-aspartate
- NMR, nuclear magnetic resonance
- NOE, Nuclear Overhauser Effect
- NPY, neuropeptide Y
- Om, ornithine
- OSu, N-hydroxysuccinimide
- PAGE, polyacrylamide gel electrophoresis
- PBS, phosphate buffered saline
- PFH, perifornical hypothalamus
- PMSF, phenylmethylsulfonylfluoride
- PP, pancreatic polypeptide
- PVN, paraventricular nucleus
- PYY, peptide YY
- s.c.c., short-circuit current
- SDS, sodium dodecyl sulfate
- SON, supraoptic nucleus
- TBS, Tris buffered saline
- TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-tetrafluoroborate
- tBu, *tert*-butyl
- TFA, trifluoroacetic acid
- Tmd27, [N_α-biotinyl-Ahx₂, Ahx⁵⁻²⁴, (Tmd)Phe²⁷] NPY
- Tmd36, [N_α-biotinyl-Ahx₂, (Tmd)Phe³⁶] NPY
- (Tmd)Phe, *p*-(3-trifluoromethyl)diazirinophenylalanine
- Tris, tris(hydroxymethyl)aminomethane
- Tween 20, polyoxyethylene-sorbitan monolaurate

SUMMARY

Neuropeptide Y (NPY) is a 36 amino acid peptide amide and a member of the pancreatic polypeptide hormone family. It is abundant in the mammalian peripheral and central nervous system and acts as a sympathetic co-transmitter that mediates vasoconstriction through direct effects or through potentiation of other vasoconstrictors. Centrally, NPY is involved in regulation of food intake, memory retention and anxiolysis.

The broad physiological relevance of NPY is reflected by the multiplicity of its receptors and gives reason for an increasing interest in NPY as new target in drug discovery. Several subtypes of NPY receptors have been cloned and pharmacologically characterized. The Y_2 -receptor has been characterized as the predominant NPY receptor subtype in the brain and it is particularly abundant in the hippocampus. Various effects are associated with the Y_2 -receptor like modulation of other neurotransmitters like glutamate and GABA, inhibition of lipolysis, antisecretory effects and enhancement of memory retention.

The major aspect of this work is the molecular characterization of the NPY Y_2 -receptor subtype in order to characterize the molecular mechanisms of its interaction with NPY and the correlated pharmacological responses. Insights in the structural requirements of ligand-receptor interaction provide the knowledge base which facilitates rational drug design and a better comprehension of the complex physiological mechanisms that are associated with NPY. Several aspects of the ligand-receptor interaction of NPY and the Y_2 -receptor subtype have been investigated in this study. NPY analogues have been synthesized in order to provide tools for receptor localization, identification and isolation and for the determination of the structure-affinity and -activity relationship of NPY and its receptors.

In order to distinguish between NPY Y_1 - and Y_2 -receptor subtypes, selective analogues of NPY have been synthesized and modified with a fluorescence label. Using two human neuroblastoma cell lines, SK-N-MC and SMS-KAN, which endogenously express the Y_1 - and Y_2 -receptor subtype, respectively, the fluorescent labeled analogues have been shown to be valuable tools to

characterize cell lines by the identification of receptor subtypes by fluorescence microscopy (Chapter 2). Moreover, irreversible labeling of the receptor subtypes has been achieved using a photoactivatable fluorescent labeled analogue of NPY.

Based on the synthesis and pharmacological investigation of cyclic NPY analogues a model for the bioactive conformation of the cyclic analogues at the Y_2 -receptor has been proposed (Chapter 3). Accordingly, the affinity of a ligand at the Y_2 -receptor is depending on the distance between its N- and C-terminus. The results of this approach suggest that the biologically active conformation of NPY that is required for receptor activation is significantly different for the Y_1 - and the Y_2 -receptor subtype.

Y_1 -, Y_2 -, and Y_5 -receptors appear to be involved in the effect of NPY on the control of the feeding behavior of rats. The receptors appear to coexist on presynaptic terminals of neurons in the paraventricular nucleus (PVN), possibly sharing a mechanism of action. The pharmacology of this response has been shown to resemble that of the induction of food intake, and suggests that a step in eliciting feeding involves the disinhibition of some parvocellular neurons in the paraventricular nucleus of the hypothalamus by a presynaptic action on the GABA release (Chapter 4).

The contribution made by each amino acid sidechain of NPY to receptor binding affinity in centrally located Y_1 - or Y_2 -expressing neuroblastoma cells, and to peripheral Y_2 -like receptor activation in rat jejunal epithelia has been assessed by systematically exchanging single residues of NPY 1 - 36 with L-alanine. Agonist potency showed [Ala³] NPY and [Ala³⁰] NPY to be equipotent with NPY, a 4 - 20-fold loss of activity for [Ala⁵] NPY, [Ala¹³] NPY, [Ala²⁰] NPY, [Ala²¹] NPY and [Ala²²] NPY; a 50 - 100-fold loss of activity for [Ala⁸] NPY, [Ala²⁷] NPY, [Ala²⁸] NPY and [Ala³⁶] NPY, while [Ala³⁴] NPY was inactive. This structure-activity relationship has been shown to be similar to, but not the same as that observed in Y_2 -expressing human neuroblastoma cells (SMS-KAN). [Ala³⁴] NPY for example showed a 75-fold lower affinity at SMS-KAN cells compared to NPY, while it was inactive at rat jejunal epithelia cells (over 270-fold lower activity compared to NPY). This supports the existence of two Y_2 -receptor subtypes, a central and a peripheral one (Chapter 5).

Photoactivatable biotinylated ligand analogues of NPY, which were labeled with ^3H -propionate, have been used for photoaffinity labeling of the Y_2 -receptor subtype and its characterization on the protein level, which was supported by the use of Y_2 -receptor subtype selective antibodies. Photoaffinity labeling of the receptor followed by SDS-PAGE and detection of the bound radioactivity, and SDS-PAGE of solubilized receptors and subsequent Western blotting revealed nearly the same molecular masses. Two proteins with molecular masses of 58 ± 4 kDa and 50 ± 4 kDa correspondingly have been detected for the human neuroblastoma cell line SMS-KAN, which is endogenously expressing the Y_2 -receptor subtype and for CHO-Y2 cells, which have been transfected with Y_2 -receptor cDNA (Chapter 7). Both proteins are suggested to represent the Y_2 -receptor subtype which exhibit different amounts of glycosylation. In combination with a biotin based signal amplification method (Chapter 6) these photoactivatable NPY analogues will be valuable tools for a further detailed identification of the ligand binding sites of the Y_2 -receptor.

In conclusion, a combination of cell biology, synthesis of peptide analogues, pharmacological assays and receptor biochemistry has been used to characterize the human NPY Y_2 -receptor subtype.

The results of this study might be useful in drug targeting and can contribute to a better comprehension of the physiological and potential pathophysiological significance of NPY.

ZUSAMMENFASSUNG

Neuropeptid Y ist eines der am häufigsten vorkommenden Neurohormone des peripheren und zentralen Nervensystems der Säugetiere. Es besteht aus 36 Aminosäuren und ist C-terminal amidiert. Peripher wirkt NPY direkt gefäßverengend, sowie durch die Potenzierung der Aktivität weiterer Neurotransmitter. Zentral beeinflusst NPY die Nahrungsaufnahme, führt zur Steigerung der Gedächtnisleistungen und zur Sedation.

Die große physiologische Relevanz von NPY zeigt sich in der Vielzahl seiner Rezeptoren und ist der Grund für ein steigendes Interesse an NPY in der Arzneimittelforschung. Zahlreiche NPY Rezeptor-Subtypen wurden bereits kloniert und pharmakologisch charakterisiert. Der Y_2 -Rezeptor wurde als der wichtigste Subtyp im Gehirn identifiziert und findet sich im Hippocampus besonders häufig. Er ist mit einer Vielzahl von Effekten, wie der Modulation anderer Neurotransmitter wie Glutamat und GABA (führt z.B. zu einer Verstärkung der Gedächtnisleistung), der Inhibierung der Lipolyse und antisekretorischen Effekten verbunden.

Der Hauptaspekt dieser Arbeit besteht in der Charakterisierung des Y_2 -Rezeptor-Subtyps auf molekularer Ebene, um die molekularen Mechanismen der Interaktion mit NPY und die damit verbundenen pharmakologischen Effekte zu untersuchen.

Einblicke in die strukturellen Verhältnisse von Ligand und Rezeptor ermöglichen ein rationales Design von Arzneimitteln und ein besseres Verständnis der komplexen physiologischen Mechanismen, die mit NPY verbunden sind. In dieser Studie wurden verschiedene Aspekte der Ligand-Rezeptor-Wechselwirkung untersucht. Dazu wurde eine Reihe von NPY Analoga synthetisiert, die zur Lokalisation, Identifizierung und Isolierung und für die Untersuchung der Struktur-Aktivitäts- und -Affinitäts-Beziehungen von NPY und seinen Rezeptoren eingesetzt wurden.

Um eine einfache Unterscheidung zwischen dem NPY Y_1 - und dem Y_2 -Rezeptor-Subtyp zu ermöglichen, wurden subtyp-selektive Analoga synthetisiert und mit einem Fluoreszenz-Label modifiziert. Zwei humane Neuroblastoma Zell-Linien, die den Y_1 - (SK-N-MC) und den Y_2 -Rezeptor-Subtyp (SMS-KAN) exprimieren, wurden als Testsystem eingesetzt. Damit konnte gezeigt werden, daß es mit Hilfe der fluoreszenz-markierten NPY Analoga möglich ist,

Zell-Linien zu charakterisieren, indem die Y_1 - und Y_2 -Rezeptor-Subtypen auf intakten Zellen durch Fluoreszenz-Mikroskopie identifiziert werden (Chapter 2). Darüber hinaus konnten die Rezeptor-Subtypen durch ein photoaktivierbares, fluoreszenz-markiertes NPY Analogon irreversibel markiert werden.

Die Ligand-Rezeptor-Interaktion und die durch NPY induzierten pharmakologischen Effekte wurden durch die Charakterisierung von Affinität, Aktivität und Struktur verschiedener NPY Analoga untersucht.

So wurde auf der Basis der Synthese und der pharmakologischen Untersuchung zyklischer NPY Analoga ein Modell für die bioaktive Konformation dieser Analoga erstellt (Chapter 3). Demnach wird die Affinität eines Liganden an dem Y_2 -Rezeptor-Subtyp durch den Abstand zwischen seinem N- und C-Terminus bestimmt. Diese Resultate deuten darauf hin, daß sich die zur Aktivierung der Rezeptoren notwendige Konformation der Liganden für den Y_1 - und den Y_2 -Rezeptor-Subtyp stark unterscheidet.

Weiterhin scheinen die NPY Rezeptor-Subtypen Y_1 , Y_2 und Y_5 an dem Mechanismus beteiligt zu sein, über den NPY das Fressverhalten von Ratten beeinflußt. Die Rezeptoren koexistieren auf den präsynaptischen Termini von Neuronen im Paraventriculären Nucleus (PVN) und interagieren möglicherweise. Es konnte gezeigt werden, daß die Pharmakologie dieser Interaktion große Ähnlichkeit mit der der Induktion der Steigerung der Nahrungsaufnahme durch NPY aufweist. Dies deutet darauf hin, daß ein Schritt zur Steigerung der Nahrungsaufnahme die Inhibierung einiger parvozellulärer Neuronen im PVN des Hypothalamus durch eine präsynaptische Beeinflussung der GABA-Ausschüttung einschließt (Chapter 4).

Der Bedeutung jeder einzelnen Aminosäure-Seitenkette von NPY, sowohl für die Affinität von NPY an Y_1 - und Y_2 -Rezeptor-Subtypen in Neuroblastoma-Zellen, als auch für die Aktivierung von Rezeptoren in Epithel-Zellen des Dünndarms, wurde durch einen systematischen Austausch jeder einzelnen Aminosäure der NPY-Sequenz durch L-Alanin untersucht. Hierbei zeigte sich, daß [Ala³] NPY und [Ala³⁰] NPY die gleiche Aktivität wie NPY aufweisen, [Ala⁵] NPY, [Ala¹³] NPY, [Ala²⁰] NPY, [Ala²¹] NPY and [Ala²²] NPY eine 4 - 20-fach geringere Aktivität und [Ala⁸] NPY, [Ala²⁷] NPY, [Ala²⁸] NPY und [Ala³⁶] NPY eine 50 - 100-fach schwächere Aktivität zeigen, während [Ala³⁴] NPY inaktiv ist. Die so ermittelte Struktur-Aktivitäts-Beziehung ist sehr ähnlich wie die für den Y_2 -Rezeptor-Subtyp in humanen Neuroblastoma-Zellen (SMS-KAN) beobachtete, aber nicht exakt die

gleiche. So zeigt z.B. [Ala³⁴] NPY im Vergleich zu NPY eine 75-fach geringere Affinität an SMS-KAN Zellen, während es an Epithel-Zellen des Dünndarms inaktiv ist, d.h. eine über 270-fach geringere Aktivität im Vergleich mit NPY aufweist. Dies unterstützt die These, nach der ein zentraler und ein peripherer Y₂-Rezeptor-Subtyp existiert (Chapter 5).

Die Charakterisierung des Y₂-Rezeptor-Subtyps auf der Protein-Ebene wurde mit Hilfe photoaktivierbarer, biotinylierter Analoga von NPY, die mit ³H-Propionsäure modifiziert wurden, erreicht. Die Liganden wurden für das Photocrosslinking des Y₂-Rezeptor-Subtyps eingesetzt und die Ergebnisse durch den Einsatz subtyp-selektiver Antikörper verifiziert. Hierzu wurden entsprechende Membran-Präparationen durch Crosslinking kovalent mit dem Ligand verbunden, die Proteine mittels SDS-PAGE getrennt und die gebundene Radioaktivität gemessen. Parallel dazu wurden solubilisierete Membrane ebenfalls mittels SDS-PAGE getrennt und die Molekulargewichte der Rezeptoren durch Western-Blotting unter Einsatz der subtyp-selektiven Antikörper ermittelt. Es wurden jeweils zwei Proteine mit molekularen Massen von 58 ± 4 kDa und 50 ± 4 kDa identifiziert, sowohl in humanen Neuroblastoma-Zellen (SMS-KAN), die den Y₂-Rezeptor-Subtyp endogen exprimieren, als auch in Eierstock-Zellen des Chinesischen Hamsters, die mit humaner cDNA transfiziert wurden (Chapter 7). Diese beiden Proteine entsprechen vermutlich zwei unterschiedlich stark glykosylierten Formen des Y₂-Rezeptor-Subtyps. In Kombination mit einer auf dem Biotin/Streptavidin-System basierenden Signalverstärkungs-Technik (Chapter 6) stellen die verwendeten photoaktivierbaren NPY Analoga wertvolle Hilfsmittel für die weitergehende detaillierte Identifizierung der NPY-Bindungsstellen des Y₂-Rezeptor-Subtyps dar.

Durch diese Studie konnte gezeigt werden, daß eine Charakterisierung des humanen NPY Y₂-Rezeptor-Subtyps durch eine Kombination von Zellbiologie, der Synthese von Peptid-Analoga, pharmakologischen Assays und Rezeptor-Biochemie möglich ist.

Insgesamt können die Resultate dieser Arbeit für die Entwicklung von Arzneistoffen hilfreich sein und gleichzeitig zu einem besseren Verständnis der physiologischen und potentiell pathophysiologischen Bedeutung des NPY beitragen.

CHAPTER 1

Introduction

1.1 Neuropeptide Y

1.1.1 Sequence and Secondary Structure

Neuropeptide Y (NPY) which was first isolated from extracts of porcine brain in 1982 [1] is a 36 amino acid peptide amide. It shows a high similarity to pancreatic polypeptide (PP) and peptide YY (PYY). These peptides are also frequently referred to as pancreatic polypeptide (PP) hormone family [2, 3]. The unusually high number of five tyrosine residues (Y) in position 1, 20, 21, 27 and 36 gave reason for the peptide's name.

A comparison of the sequences of the pancreatic polypeptide hormone family members shows several general characteristics (figure 1). First, all peptides have the same chain length of 36 amino acids and are C-terminally amidated. Second, there are several conserved sequence positions, which are: Pro at position 2, 5 and 8, Gly at position 9, Ala at position 12, Tyr at positions 20 and 27, as well as the C-terminal pentapeptide Thr³²-Arg-Xaa-Arg-Tyr³⁶.

The three-dimensional structure of the avian (turkey) pancreatic polypeptide (aPP) has been shown by Blundell and co-workers in 1983. Accordingly, crystalline aPP has a so-called "hairpin-like" structure [4]. Residues 1-8 form a type II proline helix followed by a loop (residues 9-14), which is connected to an α -helix (amino acids 15-32). The four C-terminal amino acids are arranged flexibly

in a loop. Hydrophobic interactions of the highly conserved residues of the proline helix and the amphiphilic α -helix lead to the hairpin-like structure (figure 2). Because of the of the high sequence homology, a similar three-dimensional structure is suggested for the pancreatic polypeptide family members [2].

NPY sequences

NPY human: YPSKPDN**PGED**APAEDMARYYSALRH**Y**INLITR**QRY**

NPY pig YPSKPDN**PGED**APAEDLARYYSALRH**Y**INLITR**QRY**

NPY rat YPSKPDN**PGED**APAEDMARYYSALRH**Y**INLITR**QRY**

PYY sequences

PYY human YPIK**PEAPGED**ASPEELNRYYSALRH**Y**LNLVTR**QRY**

PYY pig YPAK**PEAPGED**ASPEELSRYYASLRH**Y**LNLVTR**QRY**

PYY rat YPAK**PEAPGED**ASPEELSRYYASLRH**Y**LNLVTR**QRY**

PP sequences

PP human APLE**FPVYPGD**NA**TPEQMAQYAADLRRY**INMLTR**PRY**

PP pig APLE**FPVYPGD**DA**TPEQMAQYAAELRRY**INMLTR**PRY**

PP rat APLE**FPMPGD**YA**THEQRAQYETQLRRY**INTL**TRPY**

FIGURE 1: Sequences of members of the pancreatic polypeptide hormone family: NPY, PYY, PP.

The high content of α -helical structures could be confirmed by comparative circular dichroism measurements of different pancreatic polypeptides, of neuropeptide Y and of their fragments. This indicated that the peptides from different species that have been investigated so far are built up in a similar manner [2, 5, 6]. Moreover, ^1H NMR investigations in various solvents, including water, dimethylsulfoxide, and trifluoroethanol confirmed the conformation of the α -helical segment of NPY [7, 8], which is in agreement with the model of Allen et al. [9] that has been obtained by homology modeling.

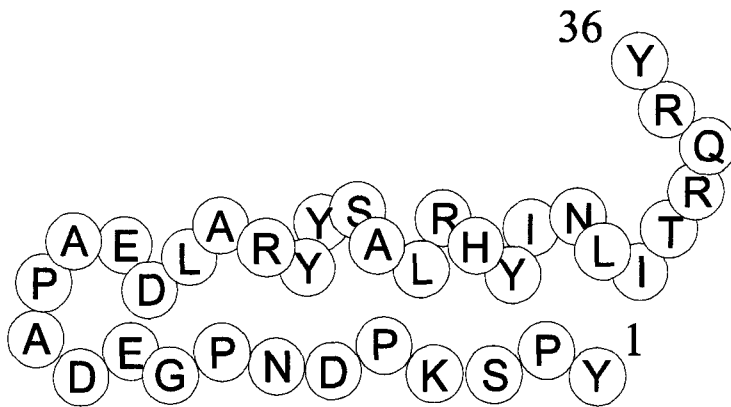


FIGURE 2: Sequence and schematic structure of the hairpin folded pNPY

1.1.2 Areas of Occurrence and Activity

NPY is predominantly located within neurons of the central and sympathetic nervous systems [10, 11]. It is one of the most abundant neurohormones in brain and is localized in high concentrations within the hypothalamus [12]. A variety of biologic effects have been attributed to NPY due to its widespread distribution throughout the CNS. It induces a robust increase in food intake and has marked effects on peripheral metabolism, such as decrease of brown adipose tissue thermogenesis and increase of lipoprotein lipase activity that promotes white fat lipid storage. NPY has also profound effects on secretion of lutenising hormone as well as on growth hormone and insulin release. These observations suggest an important role of NPY in the pathophysiology of obesity and diabetes. It has also been reported that NPY facilitates learning and memory retention processes [13], modulates locomotor behaviors [14, 15], produces hypothermia [16, 17], inhibits sexual behavior [18], shifts the circadian rhythms [19, 20], modulates cardiorespiratory parameters [21] and generates anxiolytic effects [22].

In sympathetic nerves NPY is co-localized with noradrenaline. Prejunctionally, NPY regulates its own release and inhibits the release of noradrenaline. Postsynaptically, NPY evokes an increase in blood pressure by direct vasoconstrictory effects or by potentiating those of other vasoconstrictors such as angiotensin II and noradrenaline (for review see, [23-27]).

1.2 NPY Receptors

1.2.1 Subtypes

Five distinct NPY receptors have been cloned, which have been named Y_1 - [28-30], Y_2 - [31-33], Y_4 /PP $_1$ - [34, 35], Y_5 - [36] and y_6 - [37] receptor subtype. Sequence comparisons show that the receptors Y_1 , Y_4 , and y_6 are more closely related to each other than to the receptors Y_2 and Y_5 .

TABLE 1: Neuropeptide Y receptor subtypes

Receptor	Y_1	Y_2	Y_4	Y_5	y_6
Endogenous Ligands	NPY	NPY	PP	NPY, PP	NPY
Amino acids	384	381	375	455	371
Signal transduction	cAMP inhibition Ca^{2+} mobilization	cAMP inhib. Ca^{2+} , K^+ channel	cAMP inhib.	cAMP inhib. Ca^{2+} mobil.	
Major occurrence	periphery hypothalamus	cortex, hippocampus	intestine, colon	hypothalamus	not in human
Related action	vasoconstriction, anxiety, food intake?	memory, epilepsy, secretion, food intake?	gastro- intestinal regulation	food intake?	

Among the cloned receptors, the Y_1 -, Y_2 -, Y_4 -, and Y_5 -receptors represent fully defined subtypes, while no functional correlate of the cloned y_6 -receptor has been reported to date. The receptors have been identified and characterized using a

range of truncated and substituted peptide analogues in various functional and binding assays (Table 2). Whereas NPY shows high affinity to Y_1 -, Y_2 -, Y_5 - and Y_6 -receptors, PP (pancreatic polypeptide) has been found to be the endogenous ligand of the Y_4 -receptor, which is also called PP_1 -receptor [38]. The existence of a receptor subtype at which PYY is considerably less active than NPY has been reported for several model systems. The evidence for the existence of this receptor subtype remains circumstantial as it has not yet been cloned and no specific ligands have been described. Therefore, binding sites at which PYY is at least ten-fold less potent than NPY are proposed to be referred to as putative Y_3 -receptors [39].

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. While their distribution appears to be species specific, all subtypes belong to the large superfamily of G-protein-coupled, heptahelical receptors [40]. Their signal transduction pathways appear to be similar, and no clear and consistent alignment of a specific receptor subtype with a distinct transduction pathway has been identified. NPY receptors act via pertussis toxin sensitive G-proteins in almost every cell type studied, i.e. via members of the G_i and G_o family. In a small number of cases pertussis toxin insensitive responses to NPY have been found, mostly involving presynaptically localized receptors [41 - 45], while it remains unclear whether this is more likely due to a distinct signaling mechanism or to failure of pertussis toxin to fully inactivate its substrates. The typical signaling response of NPY receptors found in almost all tissues and cell types is the inhibition of adenylyl cyclase, whereas inhibition of Ca^{2+} channels, activation or inhibition of K^+ channels and mobilization of Ca^{2+} from intracellular stores is only found in some [46]. Further responses appear to be induced in some cases, like activation of phospholipase A_2 and D and activation of a tyrosine kinase.

TABLE 2: Characterization of neuropeptide Y receptor subtypes

Subtype	Agonist order of potency	Selective agonists	Selective antagonists
Y ₁	NPY (0.2 nM) ≥ PYY (0.7 nM) >> PP (> 100 nM)	[Pro ³⁴] NPY ^a	BIBP3226 GR231118 ^b
Y ₂	NPY (0.7 nM) ≈ PYY (0.7 nM) >> PP (> 1000 nM)	NPY ₁₃₋₃₆ ^c NPY ₃₋₃₆ ^d	-
Y ₄	PP (0.5 nM) > NPY ^e ≈ PYY ^e	PP [Pro ³⁴] NPY ^a	-
Y ₅	NPY (0.6 nM) ≥ PYY (1 nM) ≥ PP ^f	[Pro ³⁴] NPY ^a PYY ₃₋₃₆ ^d	-
y ₆ ^g	NPY ≈ PYY > PP ^h	not investigated	not investigated

a Selective relative to Y₂ ([Leu³¹, Pro³⁴] NPY, [Pro³⁴] PYY and [Leu³¹, Pro³⁴] PYY behave in the same way).

b Also has high affinity for Y₄-receptor [33].

c Selective relative to Y₁ and Y₅ (PYY₁₃₋₃₆ behaves in the same way).

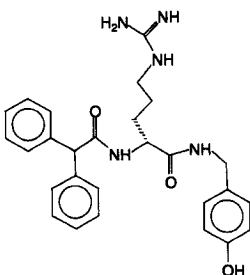
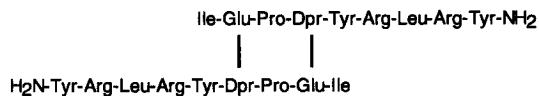
d Selective relative to Y₁ (PYY₃₋₃₆ behaves in the same way).

e While some investigators have detected low nanomolar affinity others have reported values to be greater than 1 μM, particularly in rats.

f While rat PP has low affinity at both the rat and human Y₅-receptor (230 nM), human PP has higher affinity in both species (4 nM).

g No functional protein is expressed in primates due to a truncation in the sixth transmembrane domain.

h Limited available data are controversial; the affinity values in parentheses are median values at mammalian receptors.

BIBP 3226**GR 231118**

Dpr: 2,3-diaminopropionic acid

1.2.2 The Y₂-Receptor

The Y₂-receptor is the predominant NPY receptor subtype in the (rat) brain and particularly numerous in the hippocampus [47].

TABLE 3: Quantitative autoradiographic distribution of Y₂-receptors in normal human brain, post mortem [according to 48].

Brain region	Relative intensity of the signal detected
Frontal cortex	++++
Temporal cortex	+++
Entorhinal cortex	++
Hippocampus	++++
Dentate gyrus	±
Basal ganglia	+
Hypothalamic nuclei	+
Thalamic nuclei	+
Amygdaloid nuclei	++
Substantia nigra	++
Brainstem	++

Many Y₂-receptor mediated effects have been linked to suppression of transmitter release. For instance, the release of glutamate from terminals synapsing on to rat hippocampal CA1 neurons is inhibited by activation of Y₂-receptors. Peripheral Y₂-receptors are associated with suppression of transmitter release from sympathetic, parasympathetic and sensory C-fibers (table 4).

Y₂-receptors are characterized by their propensity to bind also N-terminally truncated analogs of NPY and PYY [50 - 53] (table 2). Accordingly, the C-terminal part of the ligand is of crucial importance for binding at the Y₂-receptor; especially the C-terminal hexapeptide is the key to Y₂-receptor recognition. The hairpin loop of NPY and PYY is not essential for binding but seems to help to create a steric conformation of the C-terminal hexapeptide amide favorable for Y₂-receptor recognition [3, 51, 52].

TABLE 4: Effects associated with Y_2 -receptors [49].

Effect	Target
Suppression of transmitter release	Sympathetic nerve fibers
Suppression of transmitter release	Parasympathetic nerve fibers
Suppression of transmitter release	Sensory C-fibers
Antisecretory effect	Rat intestine
Inhibition of lipolysis	Canine lipocytes
Vasoconstriction	Certain blood vessels
Inhibition of mucociliary activity	Rabbit maxillary sinus
Enhanced memory retention	Rat hippocampus
Suppression of glutamate release	Rat hippocampus
Suppression of noradrenaline release	Rat locus coeruleus
Inhibition of consumatory behavior	Rat hypothalamus
Inhibition of adenylate cyclase	Transfected cells
Elevation of intracellular calcium	Transfected cells

The relative potencies of various NPY analogs have been shown to differ in several tissues proposed to contain Y_2 -receptors. This indicates the existence of at least two Y_2 -receptor subtypes [52]. Moreover, a PYY preferring receptor with characteristics similar to the Y_2 -receptor has been proposed in the intestine [53], which may also indicate heterogeneity among Y_2 -receptors. Finally, the human Y_2 mRNA which is about 4 kb was observed by Northern hybridization in several brain subregions (table 3), but interestingly not in any peripheral tissues that had been investigated [31, 32, 33]. This suggests that the Y_2 -receptor that has been originally identified in the periphery is distinct from the cloned receptor in the brain. However, there are indications for Y_2 -receptor heterogeneity, including the potential existence of a peripheral and a central Y_2 -receptor subtype. This includes the possibility that the potential peripheral subtype might also be expressed in the brain.

Structurally, the cloned Y_2 -receptor consists of 381 amino acids and has the typical heptahelix receptor features including potential glycosylation sites in the amino-terminal part, two extracellular cysteins that may form a disulphide loop,

and a single cysteine in the cytoplasmic tail that probably serves as an attachment site for palmitate (figure 3 and 4).

```

↓1                aminoterminal                49↓
MGPIGAEADENNQTVEEMKVEQYGPQTPRGELVPDPEPELIDSTKLEIVQVV
  transmembrane1 72↓ cytopl. ↓82 transmembrane2 104↓
LILAYCSIIILLGVIGNSLVIHVVIKFKSMRTVTNFFIANLAVADLLVNTLCL
  extracellular1 124↓ transmembrane3 146↓ cytopl.
PFTLTYTLMGEWKMGPVLCHLVPYAQGLAVQVSTITLTVIALDRHRCIVYHL
  166↓ transmembrane4 187↓ extracellular2
ESKISKRISFLIIIGLAWGISALLASPLATFREYSLIEIIPDFEIVACTEKWP
  214↓ transmembrane5      240↓ cytoplasmatic
GEEKSIYGVVYSLSSLLILYVLPGLIISFSYTRIWSKLNHVSPGAANDHYH
  268↓ transmembrane6  291↓ extracell. ↓304
QRRQKTTKMLVGVVVVFAVSWLPLHAFQLAVDIDSQVLDLKEYKLIPTVPHI
transmembrane7 ↓328      carboxyterminal
IAMCSTFANPLLYGWMNSNYRKAFLSAFRCEQRLDAIHSEVSVTFKAKKNLE
  381↓
VRKNSGPNDSFTEATNV

```

FIGURE 3: Sequence of the human Y_2 -receptor [33] with alignment of the potential extracellular, cytoplasmatic and transmembrane segments (marked in gray). Potential glycosylation site (NQT), extracellular cysteine residues potentially forming a disulphide bridge and carboxyterminal cysteine residue potentially palmitoylated are marked in bold.

The human and rat receptors share 94 % sequence identity [54], the human and bovine proteins 94.5 % [55]. The amino acid sequence reported by Rose et al. [31] differs at one position from that reported by Gehlert et al. [33] and Gerald et al. [32]. The two reported rat clones [54] differ at two amino acid positions. These differences are probably allelic.

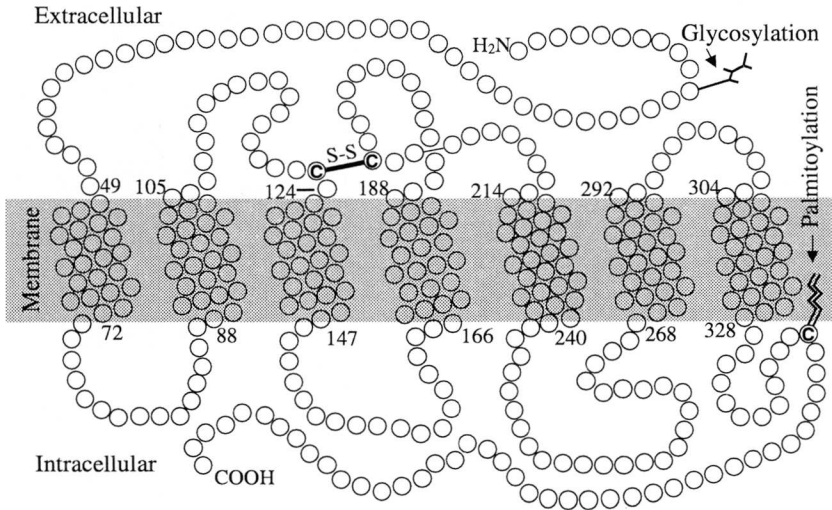


FIGURE 4: Model of the human Y_2 -receptor with alignment of the potential extracellular, cytoplasmatic and transmembrane segments. Each circle represents a single amino acid. Possible disulphide bridge, glycosylation and palmitoylation sites are displayed.

1.3 Structure-Affinity Relationships

Neuropeptide Y binds to at least four different receptor subtypes. Each receptor mediates specific biological effects and is located in certain cell membranes. Ligands that selectively bind to certain receptors would be interesting tools to localize each receptor with radioactive or fluorescent analogues (chapter 2), and to characterize the individual role of each receptor *in vivo* and under physiological conditions (chapter 4 and 5). Furthermore, selective ligands could be used as lead compounds for the development of drugs that specifically act on one receptor subtype. To obtain subtype selective ligands it is necessary to learn about the ligand-receptor interaction, i. e. to know which are the parts of the ligand and the receptor, that are important for affinity and activity.

The different receptors usually recognize the ligand in a slightly different mode, for example either by binding the same ligand in a different conformation, different three-dimensional orientation, or by interacting with different side chains. Thus, there are two possible approaches to study the ligand-receptor relationship that provide access to complementary information: the determination of the binding prerequisites of the ligand and of the receptor.

Besides facilitating the development of subtype selective ligands this structural data may give input in molecular modeling studies to establish three-dimensional models of the ligand-receptor complex.

1.3.1 Ligand

It is important to understand the function of each part of the ligand, which best is performed by so called structure/activity or structure/affinity studies: modified ligands are tested for their affinity and activity, and the biological information is correlated with the structural data. In order to understand the activity of bioactive peptides, segments (N- or C-terminal) are usually synthesized first and can lead to smaller, still biologically relevant peptides. For NPY, for example the C-terminal dodecapeptide still maintains some activity at the Y_2 -receptor [56]. The next steps frequently include so called „scans“. In the case of the 36-mer peptide NPY, 36

analogues have been required for the L-alanine scan with each position singly exchanged by L-alanine to obtain [Ala¹] NPY (Tyr at position 1 replaced by Ala), [Ala²] NPY, etc. to [Ala³⁶] NPY [57] (chapter 5). In general, L-Ala scans lead to the identification of the important side chains of peptides, because Ala causes minimal influence on the conformation, but misses especially the properties of trifunctional amino acids. Further frequently performed scans of full length peptides or of the biologically active peptide segment include D-amino acid scan (no change of charge, polarity, etc. but change of orientation of important side chains and of conformation), glycine scan (only spacer left), proline scan (looking for turns) and phenylalanine scan (role of hydrophobicity) [56, 58]. Furthermore, constrained analogues like cyclopeptides or peptides with conformationally restricting amino acids are required. Turn or helix inducing building blocks are furthermore frequently used [59]. The structure of rigid, small peptides can be identified best with 2D-NMR techniques. In the case of NPY, the bioactive conformation of NPY at the Y₂-receptor could be identified with a C-terminal cyclic peptide (chapter 3), that showed full activity and of which the structure had been solved by 2D-NMR [60]. Conformational studies of larger and less constrained analogues can be obtained by circular dichroism spectroscopy. Although this technique does not provide the three dimensional structure of the molecules, it is a fast and convenient method to compare conformations of related peptides and help to distinguish between direct effects and indirect, conformationally induced ones [61].

1.3.2 Receptor

Due to the lack of x-ray structural data, the knowledge of the three dimensional structure of G-protein-coupled, heptahelical receptors is based on hydropathy plots, the comparison with bacteriorhodopsin and cryoelectron microscopy [review: 62]. The hydropathy plots suggest that all members of this family contain seven hydrophobic domains of 20 - 25 amino acids, which are believed to represent the transmembrane regions. Based on structural similarities with the extensively characterized protein bacteriorhodopsin, for which electron diffraction data are

available [63], these regions are predicted to be α -helices and to be orientated to form a ligand binding pocket. Among the G-protein-coupled, heptahelical receptors themselves only rhodopsin has been structurally characterized by cryoelectron microscopy and the seven-transmembrane bundle could be confirmed [64, 65]. However, the resolution is too low to give detailed information. Furthermore, protease digestion studies and detailed immunological mapping have provided evidence for the seven-transmembrane model, including direct evidence that the N-terminal sequence is extracellular and the C-terminal sequence intracellular.

To learn about the structure/affinity or structure/activity relationship of the receptor and its ligand, which means to determine the parts of the receptor required for ligand binding, the same strategies as for the characterization of the ligand binding sites can be performed. The exchange of single amino acids in the receptor sequence has to be performed by site-directed mutagenesis, because large proteins with potential modifications like glycosylation and palmitoylation cannot be chemically synthesized. Because of the high experimental efforts of this technique it is not possible to perform scans of the complete receptor sequence, which on the other hand is not necessary as the binding pocket of the receptor should be located in the extracellular region of the receptor. However, site-directed mutagenesis of single sequence positions of interest can be used to determine their importance for ligand binding. In the case of NPY this was used to investigate the binding sites of NPY and the nonpeptide antagonist BIPB 3226 at the human Y_1 -receptor and to establish a detailed model of the interaction of the ligand-receptor interaction [66]. The problem of site-directed mutagenesis compared to a scan performed with a peptide is that most frequently mutants with loss of function or binding are generated and it becomes impossible to distinguish between indirect (conformational) and direct effects. So the effect of a single mutation is always questionable (for review see [67]). Either a number of ligands should be tested with the mutant to identify the important functional groups, or a set of mutations should be investigated. Using molecular modeling, the binding site that is suggested by the data obtained by mutagenesis can be hypothesized and used to forecast a next set of mutated receptors. These then lead to a further optimization of the receptor model [66, 68]. The mutational analysis approach of

course is not restricted to point mutations. Chimeric receptors can also be expressed and investigated to obtain further specific information.

Moreover, anti-receptor antibodies against hormone receptors have been shown to be valuable tools, not only for mass determination by SDS-PAGE/Western blot and receptor purification by affinity chromatography on antibody-columns. If the binding site of a monoclonal antibody is known, or if the antibodies are obtained against receptor fragments, then localization of the functional groups by competition with ligands is possible [69].

1.3.3 Crosslinking

Investigations of molecular interactions between ligands and their receptors are mainly performed indirectly starting either from the ligand by using synthetic modifications, or from the receptor by applying mutational analysis. An alternative approach is crosslinking which combines the investigation of receptor and ligand at the same time with regard to parts crucial for affinity or activity. In this approach, the ligand is used to label that distinguished part of the receptor that it interacts with. Cross-linking of the ligand with the receptor via a linker molecule is a further option. However, this method is not necessarily suited for identifying the binding site, as bifunctional reagents will often cross-link the ligand to the receptor at a certain distance of the binding site, depending on the structure of the cross-linker.

A method to overcome this systematic problem is the use of a ligand with built-in photoreactive amino acid that generates a highly reactive species, for example a carbene, nitrene or biradical. The exact knowledge of the ligand and the essential amino acids of peptide hormones, however, is of great significance [70]. Photocrosslinking using a ligand-bound photoreactive amino acid has been used successfully to characterize hormone receptors. The central CCK receptor and the neurokinin 1 receptor were labeled using a *p*-benzoyl-Orn(propionyl) analogue and *p*-benzoylphenylalanine analogue of the native ligand, respectively, and also a photoreactive analogue of the nonpeptide antagonist [71].

Photoactivatable amino acids can be introduced in the ligand by solid phase synthesis exchanging a certain amino acid of the native sequence. This provides a simple access to photoactivatable ligand analogues without major structural

modifications. It is difficult, however, to develop an amino acid analogue that meets all requirements for a perfect photoaffinity label:

- ease of synthesis
- chemical stability prior to photoactivation
- smooth photolysis at wavelengths long enough to cause no photochemical damage to the proteins
- high reactivity of the photogenerated intermediate with no tendency to intramolecular rearrangements to a less reactive compound
- high crosslinking yields
- uniformity and stability of the crosslinking products
- low tendency of the intermediate to react with the solvent water
- neglectable structural and chemical variation of the unmodified ligand

Especially analogues of tyrosine or phenylalanine like *p*-(3-trifluoromethyl)-diazirinophenylalanine or *p*-benzoylphenylalanine just slightly modify the ligand when introduced in exchange against tyrosine and provide favorable photochemical properties [72] (figure 5).

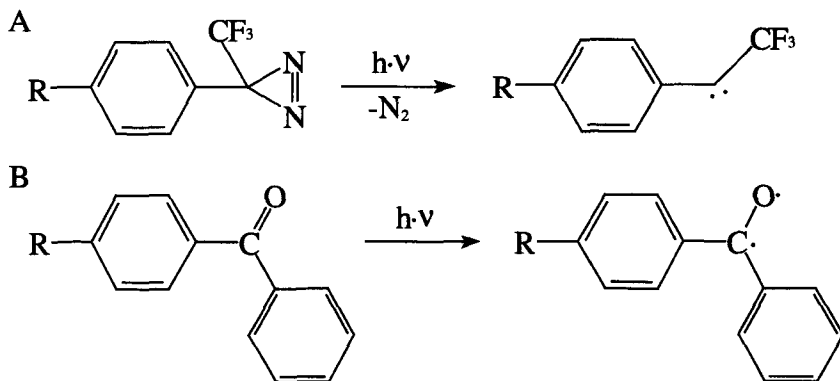


FIGURE 5: Photoactivatable analogues of phenylalanine: **A** (*Tmd*)*Phe* reacting via carbene, **B** *Bpa* reacting via biradical

To facilitate the detection of the generated ligand-receptor-complex, the peptides have to be modified by probes, which allow the direct detection of minimal quantities like radioactive isotopes [73] or fluorescent labels [74]. Another possibility is the indirect detection by introduction of affinity labels like biotin [75, 76]. These labels show a high affinity to other molecules [(strept)avidin in the case of biotin], which can be modified by secondary groups to produce and to amplify a detectable signal. The affinity labels can be used for purification and isolation purposes, too [77].

The possible purification of the ligand-receptor complex may give access to further detailed information about the ligand-receptor binding mode: mass spectrometry or Edman degradation following enzymatic digestion of the ligand-receptor complex can be used to identify the parts of the receptor at which the crosslinking occurred. Thus, direct determination of the receptor sequence positions involved in ligand binding is made possible (figure 6).

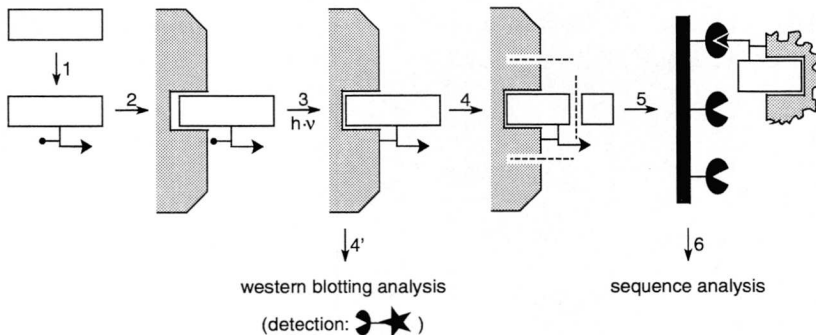


FIGURE 6: Scheme for a photocrosslinking experiment with subsequent characterization. **1** Modification of the peptide with a photoactivatable group [•] and an affinity label [▶]; **2** Incubation of the peptide with the binding molecule, reversible binding; **3** Irradiation and formation of the covalent bond; **4** Degradation of the peptide-receptor complex; or **4'** Western blotting analysis and specific detection by a covalently coupled secondary signal [★] shows the mass of the binding molecule; **5** Purification/isolation by affinity chromatography; **6** Sequence analysis by mass spectrometry or Edman degradation shows the crosslinked site of the receptor.

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CHAPTER 2

Fluorescent Labeled Analogues of Neuropeptide Y for the Characterization of Cells Expressing NPY Receptor Subtypes

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2.1 Abstract

Porcine neuropeptide Y (NPY), a 36 amino acid hormone of the pancreatic polypeptide family, and subtype selective analogues have been synthesized by solid phase peptide synthesis. The peptides were labeled with Cy3TM, a commercially available fluorescent marker based on a cyanine dye, by solid phase strategy. During the cleavage a partial fragmentation of the fluorescent marker occurred. This has been investigated by means of HPLC and electrospray mass spectrometry. The labeled analogues of NPY showed high affinity to the NPY receptor subtypes Y₁ and Y₂. Thus, Cy3-NPY, Y₁-selective Cy3-[Pro³⁴] NPY and Y₂ selective Cy3-[Ahx⁵⁻²⁴] NPY were used to label SK-N-MC- and SMS-KAN-cells, which are stably expressing the Y₁- (SK-N-MC) and the Y₂-receptor subtype (SMS-KAN). The binding of the labeled analogues to the receptors was reversible and specific. The photoactivatable analogue, [(Tmd)Phe²⁷] NPY, which showed high affinity to both receptor subtypes was labeled with Cy3 in solution. Whereas the fluorescent labeling of the cells with analogues without photoactivatable amino acid was reversible, successful photocrosslinking could be investigated by the irreversible staining of the cells using Cy3-[(Tmd)Phe²⁷] NPY. These subtype selective analogues are exciting tools to trace receptors in tissues and to identify the pharmacologically characterized subtypes without radioactivity.

2.2 Introduction

Neuropeptide Y (NPY) is a member of the pancreatic polypeptide family and one of the most abundant neurohormones in the mammalian peripheral and central nervous system [1]. It is a neuronal and endocrine messenger involved in many physiological processes. As a sympathetic co-transmitter it is mediating vasoconstriction through direct effects or by amplification of other vasoconstrictors [2-4]. Furthermore, NPY plays an important role in regulating processes such as food uptake, memory retention and anxiolysis [2-4]. Neuropeptide Y that consists of 36 amino acids displays a high homology with the pancreatic polypeptides in both sequence and 3D-structure [5,6].

The existence of two NPY receptor subtypes (Y_1 , Y_2) has been shown by binding studies using analogues or segments of NPY [7,8]. Both subtypes belong to the G-protein coupled hormone receptor family. A third receptor subtype named Y_4 has been cloned recently [9]. Further subtypes are speculated due to pharmacological investigation [10].

Different receptor subtypes frequently are characterized by different affinity of segments or ligand fragments in different cells or tissues. Thus, each receptor subtype recognizes specific parts of the hormone. The structure affinity/activity relationships are investigated by systematic changes in the primary and secondary structure of the hormone. Frequent analogues are C- or N-terminal segments, centrally truncated analogues [11] or analogues with D-amino acid-, L-alanine- or further single amino acid replacements [12]. Important parts of the hormone for high affinity to the Y_1 - and the Y_2 -receptor could be identified and, finally, led to highly selective compounds. Thus, the replacement of Glu³⁴ by the turn-inducing amino acid Pro results in a loss of affinity to the Y_2 -receptor [8]. Examples for Y_2 selective analogues are NPY 2-36, NPY 3-36 [13] and the centrally truncated [Ahx⁵⁻²⁴] NPY (Ahx = 6-aminoheptanoic acid) [14].

Affinity of ligands is usually investigated by a binding assay using membrane preparations of the cells, which selectively express one of the receptor subtypes, or with the cells themselves. Radiolabeled NPY (³H- or ¹²⁵I-NPY) is used as a tracer to compete with the test compounds.

In order to avoid radioactivity and to allow a simple distinction between receptor subtypes, we labeled NPY, [Ahx⁸⁻²⁰] NPY [11] and the subtype selective analogues [Pro³⁴] NPY, [Ahx⁸⁻²⁰][Pro³⁴] NPY and [Ahx⁵⁻²⁴] NPY with Cy3TM (Fig.1), a commercially available fluorescence dye. The advantage of Cy3 compared to other dyes is its very favorable light quantum yield, which allows a very sensitive detection. The labeled analogues were used for the specific and reversible staining of cells expressing Y₁- or Y₂-receptors, which was investigated by fluorescence microscopy. Moreover, a specific but irreversible staining was obtained by incubating the cells with a Cy3-labelled photoactivatable analogue, Cy3-[(Tmd)Phe²⁷] NPY (TmdPhe = 4'-(3-trifluoromethyl)-3H-diazirine-3-yl-phenylalanine) and subsequent irradiation

2.3 Experimental Procedures

2.3.1 Peptide Synthesis, Analysis and Purification

All peptides were prepared by solid phase synthesis using the Fmoc-strategy (Fmoc = 9-fluorenylmethoxycarbonyl-) on a robot system (Syro, MultisynTech, Bochum) [15]. L-H-(Tmd)Phe-OH was prepared as described previously [16] and N-terminally protected with Fmoc-OSu (OSu = N-hydroxysuccinimide) according to [17] to be used in solid phase synthesis. During synthesis of [(Tmd)Phe²⁷] NPY all proceedings, however, have been performed in the absence of light. All peptides except of [(Tmd)Phe²⁷] NPY were labeled with Cy3TM as they were still covalently attached to the resin, followed by cleavage with trifluoroacetic acid [15]. The modification of [(Tmd)Phe²⁷] NPY with Cy3TM was carried out in solution and completed by purification on a Sephadex G-25 column. Characterization was achieved by reversed-phase-HPLC (column C-18, 3 × 125 mm, 5 μ, gradient: 25 % acetonitrile to 75 % acetonitrile in water/trifluoroacetic acid (100:0.1) within 34 min, flow 0.6 ml/min) (Fig. 2) and electrospray mass spectrometry. The peptides were purified by preparative HPLC on a nucleosil 6 μ C-18 column (25 × 100 mm) with a flow rate of 28 ml/min (gradient: 34 % acetonitrile to 39 % acetonitrile in water/trifluoroacetic acid 100:0.1 within 30 min).

2.3.2 Membrane Preparation

SMS-KAN cells were grown in 50 % nutrient mixture Ham's F12/50 % Dulbecco's modified Eagle medium (Gibco) with 15 % fetal calf serum, 2 mM glutamine and non-essential amino acids at 37 °C and 5 % CO₂ until they were confluent. SK-N-MC cells were grown under the same conditions using MEM Earle's Salts medium with 10 % fetal calf serum. The medium was decanted. The cells were washed with phosphate buffered saline (PBS) and treated with 0.02 % EDTA in PBS for 2 min. The EDTA solution was removed and the cells were suspended in Tris buffer, pH 7.5 with 0.1 % Bacitracin and 50 µM Pefabloc SC. The cell suspension was homogenized 15-times using a potter (Braun-Melsungen, Germany) at maximum speed. After centrifugation at 4 °C for 10 min at 800 × g, the supernatant was decanted and centrifuged at 4 °C for 30 min at 16500 × g. The supernatant was decanted and the pellet resuspended in 30 ml Hepes buffer (25 mM Hepes, 25 mM CaCl₂, 1 mM MgCl₂, pH 7.4, 0.1 % bacitracin and 50 µM Pefabloc SC), homogenized 15-times with a potter and recentrifuged at 4°C for 30 min at 16500 × g. The pellet was resuspended in 15 ml Hepes buffer without protease inhibitors. An aliquot was used to determine protein concentration and inhibitors were added accordingly. Aliquots of the membrane suspension of 1 ml were stored at -80 °C.

2.3.3 Receptor Binding

The membrane preparation was diluted in incubation buffer (MEM/25 mM Hepes, 1 % bovine serum albumin, 50 µM Pefabloc SC, 0.1 % bacitracin, 3.75 mM CaCl₂). 200 µl of the suspension containing 20 µg protein were incubated with 25 µl 1,2 nM ³H-propionyl-NPY (3,18 TBq/mmol) and 25 µl of solutions of the Cy3 labeled analogues in increasing concentrations to give a total volume of 250 µl. After 3 h at room temperature the incubation was terminated by centrifugation of the samples for 10 min at 3,000 × g and 4 °C. The pellets were washed with PBS, resuspended in PBS, mixed with scintillation cocktail and radioactivity was determined. Non-specific binding was defined in the presence of 1 µM NPY.

2.3.4 Visualization and Crosslinking

Cells were prepared and resuspended in incubation medium (0.1 % bacitracin, 50 μ M Pefabloc SC, 3.75 mM CaCl₂ and 1 % bovine serum albumin) to give a final concentration of 1.25×10^6 million cells/ml. Cy3 labeled analogues of NPY were added to 100 μ l of the cell suspension with a final concentration of 10^{-8} M. Unspecific staining was determined by adding NPY (final concentration 10^{-6} M). The cell suspension was incubated at room temperature for 3 h, followed by centrifugation for 10 min at $800 \times g$. The cells were washed and resuspended in PBS. 20 μ l were transferred to a cell counting chamber and detected in a phase contrast fluorescence microscope and a filter of 430 nm (irradiation: 340-380 nm).

The cell suspension containing Cy3-[(Tmd)Phe²⁷] NPY was exposed to a 180 W high pressure mercury lamp (366 nm) for 15 min after incubation in order to photolyze the crosslinker; staining was detected as described above. Irreversible staining was determined by resuspension of the cells in incubation buffer containing NPY in a concentration of 10^{-6} M, incubation, and investigation of the cells by fluorescence microscopy.

2.4 Results and Discussion

2.4.1 Synthesis and Characterization of Cy3 labeled NPY Analogues

The subtype specific analogues of NPY were prepared by solid phase synthesis using Fmoc strategy. The photoactivatable analogue was synthesized by the replacement of tyrosine at position 27 by 4'-(3-trifluoromethyl)-3H-diazirine-3-yl-phenylalanine ((Tmd)Phe) [18]. Preparation of Fmoc-(Tmd)Phe-OH as well as synthesis, cleavage, labeling with Cy3 and purification was carried out in the absence of light.

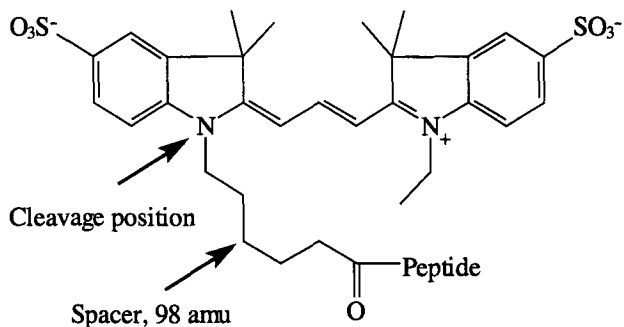


FIGURE 1: Structure of Cy3 labeled peptides.

NPY and [(Tmd)Phe²⁷] NPY have been labeled in solution according to the protocol of the manufacturer. This led to uncompleted modification (20 % labeling yield). Thus, the chromatogram of Cy3-[(Tmd)Phe²⁷] NPY showed two peaks with a ratio of 80:20. The first one represented non-labeled [(Tmd)Phe²⁷] NPY, which was identified by coinjection of [(Tmd)Phe²⁷] NPY. Additional detection at 552 nm (absorption maximum of the dye), revealed Cy3-[(Tmd)Phe²⁷] NPY as the second peak. The low labeling yield which also means a high loss of [(Tmd)Phe²⁷] NPY and the resulting need for purification as well as the possible labeling in various positions of the peptide and the double labeling are the main disadvantages of this procedure.

In order to avoid the problems of synthesis in solution NPY, [Ahx⁸⁻²⁰] NPY and the subtype selective analogues [Pro³⁴] NPY, [Ahx⁸⁻²⁰][Pro³⁴] NPY and [Ahx⁵⁻²⁴] NPY were labeled with Cy3 prior to the cleavage from the resin.

The main advantage of the labeling while the peptide is still covalently attached to the resin is the unambiguity of the modification position as only the N-terminus is deprotected. Moreover, we expected a higher labeling yield. Unfortunately, the yield was only slightly increased up to approximately 30 % due to a partial destruction of the dye under the acidic conditions of the cleavage process.

This was investigated by analytical HPLC and MS. The NPY analogues modified on the resin showed similar chromatogram patterns. As shown in Fig. 2, three products were obtained. The first peak represented the unmodified peptide, which was shown by co-injection of the peptide and by MS. The Cy3-labelled analogue was identified as the second peak by additional detection at 552 nm. The third peak, which was not found in the chromatogram of the analogues modified in solution, represented neither the educt nor the desired product. To identify this byproduct we investigated the purified peaks by electrospray mass spectrometry. The mass of the educt (HPLC-peak 1), the one of the educt plus 98 amu (HPLC-peak 2) and the one of the peptides dimer plus 98 amu (HPLC-peak 3) was found. A Cy3 labeled product could not be detected, which is in contrast to the HPLC investigation with detection wavelength 552 nm.

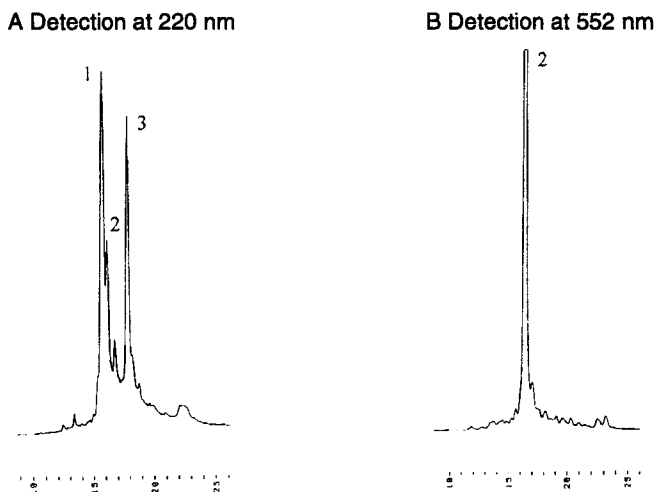


FIGURE 2: HPLC investigation of Cy3-NPY at 220 nm (A) and 552 nm (B). Peak 1 represents unlabelled NPY (RT: 15,5 min), peak 2 Cy3-NPY (RT: 16,1 min) and peak 3 the dimer with the spacer (RT: 17,6 min).

These results can only be explained by a fragmentation of the dye, which occurs both under cleavage conditions as well as under MS conditions (Fig.1).

According to our investigations the dye is cleaved from its spacer, which links the dye covalently to the peptide (Fig. 1). The spacer with the mass of 98 amu remains at the peptide and forms, under cleavage conditions, the peptides' dimer (HPLC-peak 3). This fragmentation is supported by protonation of the connecting nitrogen atom. Thus, preparative HPLC was required to obtain pure Cy3-labelled analogues of NPY. The instability of the Cy3-peptide complex suggests that Cy3-labelled peptides should not be stored in solution due to a potential cleavage of the dye.

2.4.2 Receptor Binding

Although this dye has a remarkable size, the Cy3-labelled analogues showed high affinity to the Y_1 - and the Y_2 -receptor. For Cy3-NPY, we found $K_i = 1.59$ nmol/l (Y_1) and $K_i = 0.58$ nmol/l (Y_2), whereas NPY shows $K_i = 0,17$ nmol/l (Y_1) and $K_i = 0.04$ nmol/l (Y_2).

The size of the label and its hydrophobicity only slightly reduced the affinity of the ligands to the receptors. This is most probably due to the labeling position at the N-terminus, which has been shown to be of minor importance for the ligand/receptor recognition. The hydrophobicity may lead to unspecific staining of cells, which could be reduced by additional washing steps.

2.4.3 Visualization and Crosslinking

The cells expressing either the Y_1 - (SK-N-MC) or the Y_2 -receptor (SMS-KAN) have been incubated with the labeled peptides. In all assays a control probe was handled in parallel but with an excess of unlabelled NPY to prove specificity and to exclude the possibility of unspecific staining. Whereas the cells incubated only with Cy3 peptides were successfully stained as demonstrated by phase contrast fluorescence microscopy (Fig. 3), the cells of the control probe could not be seen under identical conditions.

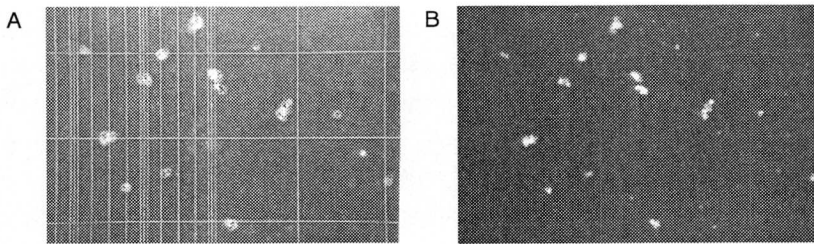


FIGURE 3: Photo (A) and fluorescence (B) of SK-N-MC cells, stained with Cy3-[Pro³⁴] NPY.

The staining of SMS-KAN cells (expressing the Y₂-receptor) with the Y₁ specific Cy3-[Pro³⁴] NPY as well as the staining of SK-N-MC cells (expressing Y₁-receptor) with the Y₂ specific Cy3-[Ahx⁵⁻²⁴] NPY failed, as expected (Table 1).

Subsequent incubation of the marked cells with a 10⁻⁶ M solution of NPY for 3 h completely removed staining. In contrast, the use of Cy3-[(Tmd)Phe²⁷] for incubation followed by irradiation at 366 nm led to specific and irreversible staining. Specificity was shown as no labeling was found in the presence of an excess of unlabelled NPY. Successful photocrosslinking was confirmed by incubation of the stained cells with 10⁻⁶ M NPY after visualization, which did not lead to a significant loss in the number of marked cells.

Each change of the structure of the ligand may influence its affinity to the receptor. Since both receptor subtypes are not considered to recognize the N-terminus of NPY [7, 12, 13, 19-21] a modification at this part of the peptide was expected to be most suitable for modifications. This was confirmed by successful specific and reversible staining of the cells with Cy3 labeled analogues of NPY. Moreover, specific and irreversible staining was obtained by crosslinking. In addition, the crosslinking efficiency of Cy3-[(Tmd)Phe²⁷] NPY might be quantified via the ratio of stained and unstained cells after crosslinking.

TABLE 1: Characterization of SK-N-MC and SMS-KAN Cells with Cy3 Labeled Neuropeptide Y Analogues.

Analogue	SK-N-MC (Y ₁)	SMS-KAN (Y ₂)
Cy3-NPY	reversibly stained	reversibly stained
Cy3-[Ahx ⁸⁻²⁰] NPY	reversibly stained	reversibly stained
Cy3-[Pro ³⁴] NPY	reversibly stained	no staining
Cy3-[Ahx ⁸⁻²⁰][Pro ³⁴] NPY	reversibly stained	no staining
Cy3-[Ahx ⁵⁻²⁴] NPY	no staining	reversibly stained
Cy3-[(Tmd)Phe ²⁷] NPY	irreversibly stained	irreversibly stained

Due to the easy procedure (fast, no radioactivity, no special safety requirement), Cy3 labeled peptides are an interesting tool for the fast qualitative investigation of the expression of receptor subtypes in both existing and new cell lines. Furthermore, it may be useful for a fast screening of tissue preparations in which the existence of a receptor subtype is expected as well as for the control of crosslinking experiments.

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CHAPTER 3

The Bioactive Conformation of Neuropeptide Y Analogues at the Human Y₂-Receptor

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Abbreviations:

ϵ Ahx = 6-aminohexanoic acid

Fmoc = fluoren-9-ylmethoxycarbonyl

NPY = neuropeptide Y

aPP = avian pancreatic polypeptide

Orn = ornithine

PP = pancreatic polypeptide

TBTU = 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-tetrafluoroborate

HOBt = 1-hydroxybenzotriazole.

K25-E29 = [Lys²⁵-Glu²⁹] NPY (Ac-25-36)

E25-K29 = [Glu²⁵-Lys²⁹] NPY (Ac-25-36)

K26-E30 = [Lys²⁶-Glu³⁰] NPY (Ac-25-36)

K27-E31 = [Lys²⁷-Glu³¹] NPY (Ac-25-36)

E27-K31 = [Glu²⁷-Lys³¹] NPY (Ac-25-36)

O27-D31 = [Orn²⁷-Asp³¹] NPY (Ac-25-36)

K28-E32 = [Lys²⁸-Glu³²] NPY (Ac-25-36)

O28-D32 = [Orn²⁸-Asp³²] NPY (Ac-25-36)

K31-E34 = [Lys³¹-Glu³⁴] NPY (Ac-25-36)

O31-E34 = [Orn³¹-Glu³⁴] NPY (Ac-25-36)

K30-E34 = [Lys³⁰-Glu³⁴] NPY (Ac-25-36)

O30-E34 = [Orn³⁰-Glu³⁴] NPY (Ac-25-36)

k30-E34 = [D-Lys³⁰-Glu³⁴] NPY (Ac-25-36)

K29-E34 = [Lys²⁹-Glu³⁴] NPY (Ac-25-36)

O29-E34 = [Orn²⁹-Glu³⁴] NPY (Ac-25-36)

k29-E34 = [D-Lys²⁹-Glu³⁴] NPY (Ac-25-36)

K28-E34 = [Lys²⁸-Glu³⁴] NPY (Ac-25-36)

k28-E34 = [D-Lys²⁸-Glu³⁴] NPY (Ac-25-36)

K27-E34 = [Lys²⁷-Glu³⁴] NPY (Ac-25-36).

3.1 Summary

Several attempts to investigate the bioactive conformation of neuropeptide Y have been made so far. As cyclic peptides are much more rigid than linear ones, we decided to synthesize cyclic analogues of the C-terminal dodecapeptide amide NPY Ac-25-36. Cyclization was performed by side chain lactamization of ornithine or lysine and glutamic or aspartic acid. Affinity of the 19 peptides ranged from K_i of 0.6 nM to >10 000 nM. We found that the size, the position, the orientation, the configuration and the location of the cycle plays an important role for receptor recognition. Circular dichroism studies have been performed to characterize the secondary structure of each peptide. Receptor binding studies were carried out at human neuroblastoma cell lines SK-N-MC (Y_1) and SMS-KAN (Y_2) and on rabbit kidney membranes (Y_2).

The pharmacological and spectral data showed, that the α -helix content is not the predominant factor for high Y_2 -receptor affinity. Instead, the location and the size of the hydrophobic lactam bridge, and the conserved C-terminal tetrapeptide (Arg-Glu-Arg-Tyr) seem to be the main parameters.

Using molecular dynamics the structures of four cyclic peptides (i, i+4) have been investigated and compared with the previously published NMR structure of one of the cyclic peptide analogues. Significant differences have been found in the overall 3D-fold of the peptides. The distances between the N- and the C-terminus allow to discriminate between peptides with high binding affinity and those with low binding affinity, because of the correlation that we found with the measured affinity. Thus, this study suggests that a turn-like structure and the orientation of the C-terminus towards the N-terminus play a major role for high affinity to the Y_2 -receptor of cyclic dodecapeptides.

None of the cyclic segments exhibits significant affinity to the Y_1 -receptor. Thus, these results support the hypothesis of a discontinuous binding site of NPY at the Y_1 -receptor.

Keywords:

Neuropeptide Y, cyclopeptide, circular dichroism, structure affinity relationships, modeling

3.2 Introduction

Neuropeptide Y (NPY) is a 36-mer peptide amide. It is one of the most abundant neurohormones in the mammalian peripheral and central nervous system. Receptor competition binding studies using analogues or segments of NPY revealed that at least six receptor subtypes (Y_1 - Y_6) exist (Grundemar *et al.*, 1997). The Y_1 - (Herzog *et al.*, 1992; Krause *et al.*, 1992; Larhammar *et al.*, 1992), Y_2 - (Rose *et al.*, 1995; Gerald *et al.*, 1995; Gehlert *et al.*, 1996a) Y_4 /PP $_1$ - (Bard *et al.*, 1995; Lundell *et al.*, 1995) Y_5 - (Gerald *et al.*, 1996) and Y_6 - (Weinberg *et al.*, 1996) receptors have been cloned recently and it could be shown, that all belong to the G-protein coupled hormone family (Beck-Sickinger, 1996). Whereas NPY shows high affinity to Y_1 -, Y_2 -, Y_5 - and Y_6 -receptors, PP (pancreatic polypeptide) has been found to be the endogenous ligand of the Y_4 -receptor, which is also called PP $_1$ -receptor (Gehlert *et al.*, 1996b)

The Y_1 -receptor subtype is particularly found in the peripheral nervous system. Moreover, Y_1 -receptors have been identified in the coronary vessels (guinea pigs, rats) and veins (guinea pigs). All available evidence suggests that the peripheral blood pressure elevating effects of NPY are mediated by Y_1 -receptors. Centrally, it is believed that the Y_1 -receptor mediates the NPY-induced effects as stimulation of luliberin and the anxiety-relieving sedative effect in rat.

Y_2 -receptors are predominantly localized on the presynaptic membrane of postganglionic, sympathetic neurons of the peripheral nervous system. In addition, Y_2 -receptors have been identified in the proximal tubuli of kidneys and in the parasympathetic neurons. Centrally, the Y_2 -receptor is more dominant than the Y_1 -receptor subtype. In the hippocampus of rats only the Y_2 -receptors are found as well as at the nerve ends of stimulating neurons which are synaptically bound to the hippocampus CA1 neurons (Dumont *et al.*, 1995). Y_2 -receptors mediate the inhibitory effects of NPY on, amongst others, ion excretion in the rat small intestine, adrenaline release in the mesenteric arteries and bronchial contraction in guinea pig. One of the most important activities of NPY as shown with NPY knock-out mice is the control of neural excitability as NPY suppresses glutamate release from neurons in the hippocampus (Erickson *et al.*, 1996). Furthermore, elevation of memory retention in rats and modulation of other neurotransmitters

have been reported (for recent reviews, see Grundemar *et al.*, 1993; Colmers *et al.*, 1994; Beck-Sickinger & Jung, 1995). Whereas Y_5 -receptors are suggested to play an important role in feeding (Gerald *et al.*, 1996), little is known about distribution and function of Y_6 -receptors.

NPY was isolated from pig brain and sequenced in 1982 (Tatemoto *et al.*, 1982). It belongs to the pancreatic polypeptide hormone family together with the intestinal hormone peptide YY and the pancreatic polypeptide (PP). Because of the high sequence similarity of all members of this family a similar tertiary structure has been suggested. The first structure has been obtained through the work of Blundell and co-workers, who performed X-ray analysis of avian (turkey) pancreatic polypeptide (aPP) (Blundell *et al.*, 1981). According to molecular dynamic simulations of NPY based on the aPP structure (Allen *et al.*, 1987; MacKerell, 1988) residue 1-8 form a type II proline helix, followed by a loop (residue 9-14) which is bound to an amphiphilic α -helix (amino acid 15-32). The four C-terminal amino acids are supposed to be arranged flexibly. Hydrophobic interactions between the two helices are responsible for the typical hairpin like PP-fold (Glover *et al.*, 1985).

Attempts to increase Y_1 -receptor affinity of NPY analogues by stabilizing the α -helical conformation, which was achieved by an exchange of certain amino acids (Grundemar *et al.*, 1992) or by side chain lactamization (Bouvier & Taylor, 1992), have not been successful. However, centrally truncated analogues which bind to the Y_1 -receptor with high affinity have been developed (Kirby *et al.*, 1995, 1997; Rist *et al.*, 1995). Nevertheless it is still unclear whether the Y_1 -receptor recognizes a discontinuous binding site, or whether only the C-terminus is involved in receptor binding of agonists and the N-terminus serves to stabilize folding.

The Y_2 -receptor subtype can be distinguished from the Y_1 -receptor by its ability to bind C-terminal segments such as NPY 13-36 (Wahlestedt *et al.*, 1986) and NPY 18-36 (Boublik *et al.*, 1989). Furthermore, centrally truncated analogues were found to be sufficient for full Y_2 -receptor activity (Beck *et al.*, 1989; Krstenansky *et al.* 1989; Dumont *et al.*, 1993). One of the most potent discontinuous compounds is [ϵ Ahx⁵⁻²⁴] NPY, in which the N-terminal tetrapeptide NPY 1-4 is linked to the C-terminal dodecapeptide NPY 25-36 by ϵ Ahx (Beck *et al.*, 1989). It has been

demonstrated that the N-terminal segment can strongly be modified and consequently serves to stabilize the conformation of the C-terminus by hydrophobic interactions. Moreover the C-terminal tetrapeptide is very sensitive to substitutions and therefore very crucial for high Y_2 -receptor affinity (Beck-Sickinger *et al.*, 1990a). Since analogues with a lack of helicity like [Pro²⁰] NPY, (Fuhlendorff *et al.*, 1990) or a lack of amphiphilicity (Jung *et al.*, 1991) show significantly reduced binding, the amphiphilic α -helix has been suggested to be a prerequisite for binding.

In order to characterize the C-terminal binding site of NPY, we synthesized nineteen cyclic analogues of the dodecapeptide NPY Ac-25-36 (Table 1). They were obtained after substitutions of two amino acids of the natural sequence by residues with one side chain containing an amino group (lysine or ornithine) and one with a carboxyl group (glutamic or aspartic acid). This led to a high diversity of cyclic peptides which differ in length, position, orientation, and configuration of the lactam bridge.

Receptor affinity of the analogues was determined at human neuroblastoma cell lines SK-N-MC (Y_1 -receptor subtype) and SMS-KAN (Y_2 -receptor subtype), for some of the compounds Y_2 -receptor affinity was tested on rabbit kidney membrane preparation. Circular dichroism as well as molecular modeling investigations were performed to extract information about the folding of the cyclic peptides.

3.3 Materials and Methods

3.3.1 Materials

Fmoc-protected amino acids, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-tetrafluoroborate (TBTU) and 4-(2',4'-Dimethoxyphenyl-hydroxymethyl)-phenoxy resin were obtained from NovaBiochem (Läufelfingen, Switzerland), aminomethylated polystyrene from Rapp Polymere (Tübingen, Germany), diisopropylcarbodiimide and thiocresol from Aldrich, 1-hydroxybenzotriazole (HOBt), diisopropylethylamine, trifluoroacetic acid, thioanisole and piperidine from Fluka and dimethylformamide (p. a. grade), diethyl ether, acetonitrile and *tert*-butyl alcohol from Merck. Hepes, Tris, bovine serum albumin, bacitracin, gentamycin and pentamethylsulfonylfluorid were purchased from Sigma, polyethylenimin and Fmoc-aminomethyl dimethoxyphenoxy valeric acid from Serva, sodium pyruvate, minimum essential medium with Earle's salts and Ham's F12/50 % Dulbecco's modified Eagle medium from Gibco, fetal calf serum, glutamine and non-essential amino acids from Boehringer Mannheim. EDTA and all salts for buffer preparation were either from Fluka or from Merck. ^{125}I -Bolton Hunter NPY was ordered from Anawa (Zürich, Switzerland) and specific activity was 81 TBq/mM. ^3H -propionyl-NPY (specific activity of 3.2 TBq/mmol) was purchased from Amersham.

3.3.2 Peptide Synthesis, Purification and Analysis

The linear peptides were synthesized by automated multiple solid phase peptide synthesis using a robot system (Syro, MultiSynTech, Bochum). In order to obtain a peptide amide, 5-(4'-aminomethyl-3',5'-dimethoxyphenoxy)-pentanoyl-alanyl-aminomethyl resin or 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin was used. The polymer matrix was polystyrene-1%-divinylbenzene (30 mg; 15 μmol). The side chain protection was chosen as follows: Tyr(*tert*.-butyl), Glu(*tert*.-butyl), Arg(2,2,3,5,5-pentamethyl-chromansulfonyl), His(trityl), Gln(trityl), Asn(trityl), Thr(*tert*.-butyl), and Lys(*tert*.-butyloxy-carbonyl). Double coupling procedures were performed with diisopropylcarbodiimide/1-hydroxy-benzotriazole activation, 10-fold excess and a coupling time of 40 min.

The N-terminus of the each peptide was acetylated while it was still bound fully protected to the resin. The linear peptide amides were cleaved with trifluoroacetic acid/ thioanisol/thiocresol within 3 h, collected by centrifugation and lyophilized from water.

For cyclization the unprotected but N-terminal acetylated dodecapeptide amides were dissolved in N, N dimethylformamide at a concentration of 2 mM. 1-Hydroxybenzotriazole and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-tetrafluoroborate were added to a final concentration of 6 mM each. After addition of diisopropylethylamine (1% v/v) the solution was shaken for 2 h. Then the solvent was removed at reduced pressure. The residues were dissolved in trifluoroacetic acid and precipitated from cold diethyl ether. The products were collected by centrifugation and resuspended twice in diethyl ether. After the lyophilization from water, the crude cyclic peptides (18-20 mg) were dissolved in methanol/piperidine (9:1, 1 ml) and shaken for 1 h to remove trifluoroacetylation. Again, they were precipitated from cold diethylether. Finally they were dissolved in trifluoroacetic acid and purified as described above.

The peptides were analyzed and purified to homogeneity higher than 94 % by reversed-phase HPLC on nucleosil C-18 columns (analytical (Merk-Hitachi): 5 μ m, 3 x 125 mm; preparative (Waters): 6 μ m, 25 x 300 mm). Acetonitrile/water mixed with 0.1% trifluoroacetic acid was used as eluent. The peptides were analyzed with a flow rate of 0.6 ml/min and using a gradient of 15-60 % acetonitrile in 30 min. Retention times of the different cyclic analogues ranged from 16 to 24 min. For preparative HPLC 17-23 % acetonitrile was used.

Correct mass was identified by ion-spray mass spectrometry (API III, Sciex, Toronto).

(Analytical data shown in Table 1)

3.3.3 Circular Dichroic Investigations

CD spectra were measured over 250-190 nm on a Jasco J720 spectropolarimeter at room temperature in nitrogen atmosphere. The peptides were dissolved in 2,2,2-trifluoroethanol/water (9/1) at concentrations of 0.13-0.18 mM. Peptide concentrations were derived from quantitative amino acid analysis. All measurements were performed three times using a sample cell with path length of 0.2 mm. Response time was set to 2 s at a scan speed of 20 nm/min, a sensitivity range of 10 mdeg, a band width of 2.0 nm and a step resolution of 0.1 nm. High-frequency noise was reduced by the means of a low-path Fourier-transform filter. The CD spectra of pure solvents were subtracted from the peptide solution to eliminate interference from cell, solvent and optical equipment. The CD data are given as mean residue ellipticities $[\Theta]_m$.

Helicity values as shown in Table 1 have been calculated by means of the protein secondary structure estimation program SSE-388 with JASCO J-700 for Windows (Yang *et al.*, 1986).

3.3.4 Membrane Preparation

For preparation of rabbit kidney membranes adult male white New Zealand rabbits were killed by intravenous injection of pentobarbital via an ear vein, the kidneys were rapidly removed and the cortex was dissected. Tissue was homogenized in 50 vol. ice-cold Tris/HCl (pH 7.4 at 37° C) with apolytron and the homogenates centrifuged at 50 000 x g for 10 min. Pellets were washed by resuspending in 50 vol. buffer and incubated for 40 min at room temperature. Then the membranes were centrifuged and washed twice. The resulting membranes were resuspended in 125 vol. of the above Tris/HCl buffer containing 5 mM MgCl₂, 0.1 mg/ml soybean trypsin inhibitor, 0.1% serum albumin and 0.25 mg/ml bacitracin (Beck-Sickinger *et al.*, 1990b). For preparation of SMS-KAN- and SK-N-MC-cell membranes SMS-KAN-cells were grown in 50 % nutrient mixture Ham's F12/50 % Dulbecco's modified Eagle medium with 15 % fetal calf serum, 2 mM glutamine, non-essential amino acids, 1 % gentamycin at 37 °C and 5 % CO₂ until they were confluent. SK-N-MC cells were grown under the same conditions using MEM Earle's Salts medium containing 1 mM sodium pyruvate with 10 % fetal calf

serum. Membrane suspensions were prepared as described previously (Ingenhoven & Beck-Sickinger, 1997).

3.3.5 Receptor Binding Studies

For binding studies membrane suspensions (100 μ l) obtained from SMS-KAN (Y_2) and SK-N-MC (Y_1) -cells were 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 μ l for 2h at room temperature as described recently (Beck-Sickinger *et al.*, 1994; Ingenhoven & Beck-Sickinger, 1997). For binding studies at rabbit kidney membranes, 230 μ l membrane solution containing about 100 μ g protein were used to give a final volume of 240 μ l and incubation was carried out at 21° C for 90 min (Beck-Sickinger *et al.*, 1990b). Non-specific binding was defined in the presence of 1 μ M NPY. Half-maximal inhibition of the specific binding of the 125 I-BH-NPY and the [3 H] NPY of two to three separate experiments is given as the K_i value (mean SEM). Eleven concentrations of the competitors were chosen to bracket the K_i value and to cover six orders of magnitude. All competitor curves could be fitted with a monophasic binding profile suggesting a single binding site.

3.3.6 Modeling

Four different cyclic peptides (E27-K31, K28-E32, O27-D31, O28-D32) were chosen as representatives of the whole set. Their K_i value range from 0.6 nM to 3949 nM, thus covering the full spectrum of the differences in K_i at the Y_2 -receptor. Their models were built starting from the mean structure of the cyclic peptide K28-E32 that has been determined by NMR (Rist *et al.*, 1996). The resulting model was then refined by means of molecular dynamics refinement consisting in energy minimization followed by simulated annealing (Dyson *et al.*, 1994). Molecular mechanics and dynamics were performed using the AMBER4.1 program (Cornell *et al.*, 1995) and the standard parm94 parameter set for all atoms. The dynamics was carried out in vacuum using the distance dependent dielectric function with the dielectric multiplicative constant of 1, in order to

simulate solvation. The amide bond of the lactam bridge was treated as a normal trans peptide bond using distance and torsional constraints. Each peptide was fully minimized by 1000 steps of steepest descent followed by a conjugate gradient minimization procedure until the rms gradient of the potential energy was less than 0.05 kcal/mol.Å. The minimized complex was then submitted to simulated annealing. The temperature was raised from 10 to 900 K during the first picosecond, kept at 900 K during 2 ps and finally re-cooled to low temperature during 12 ps. The structure resulting from simulated annealing was re-submitted to the minimization protocol described before. The simulation as well as the minimization have been analyzed using the CARNAL module implemented in AMBER4.1 and a comparison with the NMR structure has been performed. The quality of the geometry of the final structure has been assessed by means of PROCHECK (Laskowski *et al.*, 1993).

All calculations were run on a CRAY J90. Graphic display and analysis were achieved with the SYBYL6.2 molecular modeling package (TRIPOS, USA).

3.4 Results

3.4.1 Synthesis of Cyclopeptides and Circular Dichroism Investigations

Nineteen dodecapeptides have been obtained by solid phase peptide synthesis applying Fmoc-strategy and subsequent cyclization of the partly protected precursor peptide in solution, which have been divided into three sets according to their sequence type of cycle (Figure 1 a-c).

In the first set of peptides (Figure 1a) the C-terminal tetrapeptide Arg-Glu-Arg-Tyr is conserved. All lactambridges represent the (i, i+4)-type, that means four peptide bonds of the backbone are involved in the cycle.

The analogues K25-E29, K26-E30, K27-E31, K28-E32 (Figure 2a, 1a) differ in the position of the bridge. Among these peptides the highest α -helical content is exhibited by K25-E29, in which already the N-terminal amino acid is part of the cycle. The shifting of the lactambridge towards the C-terminus is accompanied by a decrease of α -helicity. This leads to the suggestion that the (i, i+4) spacing builds the first turn of the α -helix which is extended in the direction of the C-terminus.

The peptide E25-K29 shows the highest helicity of all synthesized analogues, followed by E27-K31. They are very similar to K25-E29 and K27-E31 (Figure 2b, 1a), respectively, there is only a difference in the orientation of the lactambridge. In E25-K29 and E27-K31 the glutamic acid residue is located near the N-terminus and lysine near the C-terminus. It has been reported that in such cases the interaction of the lactambridge with the helix dipole enhances helicity (Housten *et al.*, 1995), which could be confirmed by our studies.

Two peptides (O27-D31, O28-D32) in which the amino acids ornithine and aspartic acid have been used for cyclization showed a tremendous loss of α -helicity by CD spectroscopy (Figure 2c, 1a). Compared to K27-E31 and K28-E32, respectively, their lactambridges are shortened by only two methylene groups.

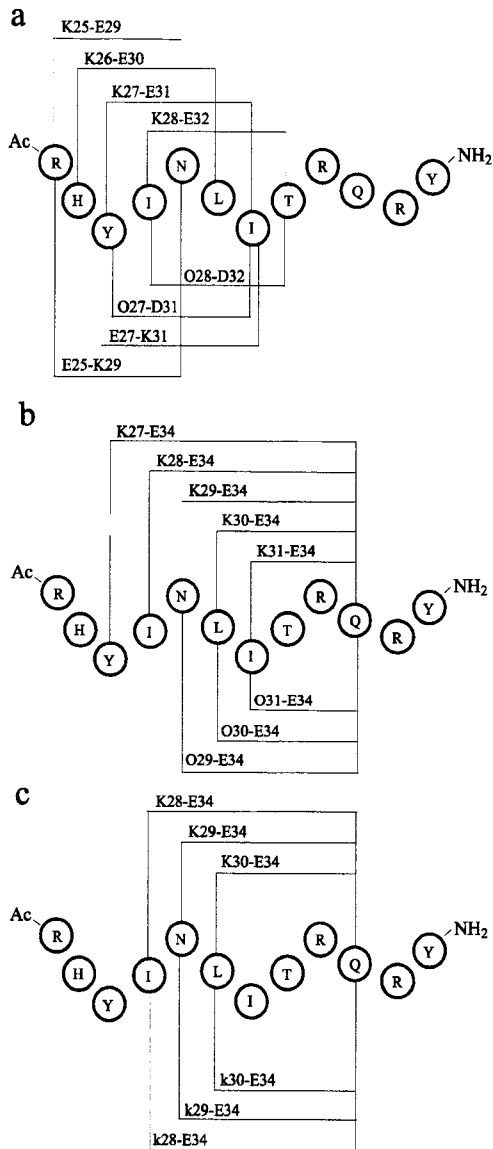


FIGURE 1: Scheme of the three set of cyclic dodecapeptides (a, b, c). The lactambridges are marked and the replaced amino acids are written above each bridge. (Standard one letter code is used for the natural amino acids, k for D-lysine (D-Lys) and O for ornithine.

The influence of the ring-size could be studied by the second set of cyclic peptides (Figure 1b). As the natural amino acid Gln³⁴ is replaced by Glu³⁴ for all of the peptides, the orientation of the lactambridge has not been varied. In the series K27-E34, K28-E34, K29-E34, K30-E34, K31-E34 (Figure 2d, 1b) the side chain of Lys is used for cyclization. Therefore the peptides differ only in the number of amino acids within the cycle. By far, K30-E34 which represents the Lys/Glu (i, i+4)-type exhibits the highest α -helical content. This has been expected, as one turn is built up by 3.6 residues in a common α -helix. Reduced helicity is observed for K29-E34 which is (i, i+5) spaced, followed by K31-E34 of the (i, i+3)-type. The latter two peptides show very similar positive Cotton effects at 194 nm. Regarding the negative peaks at 208 nm and 222 nm, however, a considerable loss of intensity is detected for K31-E34. While the CD-spectra of K28-E34 is very similar to the one of K29-E34, in K27-E34 helicity has found to be totally destroyed.

The three peptides O31-E34, O30-E34, O29-E34 (Figure 2e, 1b) are similar to K31-E34, K30-E34, K29-E34, however, instead of lysine an ornithine residue has been placed in their sequence. For that reason, their lactambridge is shortened by one methylene group. Again the (i, i+4)-type O30-E34 shows the highest α -helical content. Larger (O29-E34) as well as smaller cycles (O31-E34) lead to a loss of helicity. Comparing the two cyclopeptides K30-E34 and O30-E34, the shorter lactambridge leads to a decrease of helicity, which is indicated by a main lack of intensity at 194 nm in the CD-spectra.

In order to investigate the importance of the configuration for lactamization, three peptides containing a D-Lys have been synthesized (k28-E34, k29-E34, k30-E34) (Figure 2f, 1c). Compared to the corresponding analogues with an L-Lys, a strong decrease in intensity of all cotton effects, that is typical for an α -helical conformation has been detected. Interestingly, the symmetries of the CD-spectra are not strongly disturbed.

3.4.2 Structure-Affinity-Relationship

Affinity of NPY, the linear C-terminal segment and the cyclic analogues has been tested at human neuroblastoma cell lines SK-N-MC (Y_1), SMS-KAN (Y_2) and partially at rabbit kidney membrane preparation (Y_2) as shown in Table 1. Comparable K_i values have been determined for both assays.

In the first set of analogues (Figure 1a), which contains only L-amino acids, lactamization is restricted to the position 25-32 and all cycles consist of the same number of peptide bonds (representing the (i, i+4)-type). Here, binding to the Y_2 -receptor varies from K_i of 0.6; 1.0 nM to K_i of 3949 nM, which are four orders of magnitude. Comparing the peptides containing Lys/Glu and the ones, which are bridged by Orn/Asp, the most dramatic differences in affinity could be observed. In the case of K28-E32 the shortening of the lactambridge by two methylene groups O28-D32 causes a 5000-fold decrease in affinity. A 150-fold loss was noticed for O27-D31 showing a K_i of 152.9 nM. In both cases reduced α -helical conformation was detected in the CD spectra of the peptides with Orn/Asp bridge. Other peptides of the (i, i+4)-type (K25-E29, K26-E30, K27-E31, K28-E32) only differ in the position of the cycle. Interestingly, along with the shift of the bridge in the direction of the C-terminus affinity of the peptides increases, although helicity decreases (Table 1). Thus, no correlation between helicity and binding affinity was found which leads to the hypothesis that the general 3D-fold is more important than the simple secondary structure. Furthermore, no correlation between HPLC retention times helicity and affinity was detected, which also supports this idea.

In order to verify this hypothesis molecular modeling techniques have been applied to the cyclic peptides of this set (i, i+4) whose affinity to the Y_2 -receptor varies from 0.6 nM to 3949 nM without any clear correlation with the measured helicity. The conformation of the structures resulting from the molecular dynamics refinement procedure has been analyzed in terms of general fold by means of the measurement of distances corresponding to medium- and long-range NOEs of the NMR experiment performed on the cyclic K28-E32 (Rist *et al.*, 1996). Overall, twenty-nine distances were measured. Eleven of them are of the sequential H^N - H^N and $H^{C\alpha}$ - H^N type corresponding to medium-range NOEs. The other eighteen distances include those between N-terminal and C-terminal backbone hydrogens,

those between N-terminal C β and C-terminal N α hydrogens and the one N-terminal C β and aromatic hydrogens of Tyr36. They correspond exactly to long range NOEs and serve for the judgement of the relative position of the C-terminus to the N-terminus. The conformation of the analyzed peptides shows an α -helical character of the C-terminus induced by the cyclization in agreement with the CD-measurement. The sequential H N -H N and H $^{C\alpha}$ -H N distances for the different peptides are in the same range as the ones of the NMR structure (Figure 3a). Figure 3a shows that there is not a general correlation between these distances and the affinity, with the only exception of distance d7 where a drop in distance corresponds with a decreased affinity. The smallest relative difference between the distance d7 measured for the poor binder and this found for the excellent binder is ca. 18%. The distances between N-terminal and C-terminal hydrogens corresponding to the long-range NOEs turn out to be of great interest and significance. Figure 3b shows the distances d17, d18 and d21 that correspond to the distances between N-terminal and C-terminal backbone hydrogens. The trend that can be depicted is that the distances observed for the strong binder are shorter than these of the weak binder. The smallest relative difference in distance between subnanomolar and submicromolar/ micromolar binder for d17 is ca. 40% while the one of d18 and of d21 is 16% and 30%, respectively.

Table 1. Affinity to the Y $_2$ -receptor of cyclic analogues of Ac-25-36 NPY investigated on rabbit kidney membranes and SMS-KAN-cell membranes. Analytical data (electrospray mass spectra) and helicity according to circular dichroism.(Standard one letter code is used for the natural amino acids, k for D-lysine (D-Lys) and O for ornithine (Orn). Position of the lactam bridge is marked in bold)

Name of analogue	Sequence	K $_j$ (nM) rabbit kidney	K $_i$ (nM) SMS-KAN	Helicity (%)	Molecular mass (amu) experimental	Molecular mass (amu) theoretical
	RHYINLITRQRY	10.2	7.1	42.2		
K25-E29	K HYI E LITRQRY	22.9	24.7	67.7	1642.9	1642 \pm 0.4

Name of analogue	Sequence	K _i (nM) rabbit kidney	K _i (nM) SMS-KAN	Helicity (%)	Molecular mass (amu) theoretical	Molecular mass (amu) experimental
E25-K29	E HYIKLITRQRY	63.7		87.3	1642.9	1643 ± 0.3
K26-E30	R K YINEITRQRY		6.0	65.2	1662.9	1662 ± 0.4
K27-E31	RH K INLETRQRY	5.5	4.5	49.1	1636.9	1636 ± 0.8
E27-K31	RH E INL K TRQRY	0.8		75.6	1636.9	1637 ± 0.2
O27-D31	RH O INL D TRQRY	152.9		14.5	1608.8	1609 ± 0.4
K28-E32	RHY K NLIERQRY	0.6	1.0	36.0	1698.9	1699 ± 0.8
O28-D32	RHY O NLIDRQRY	3949.0		24.1	1670.9	1671 ± 0.5
K31-E34	RHYINL K TRERY		111.2	21.7	1671.9	1672 ± 0.8
O31-E34	RHYINL O TRERY		305.9	24.8	1657.9	1657 ± 0.3
K30-E34	RHYIN K ITRERY		42.9	44.5	1671.9	1672 ± 0.5
O30-E34	RHYIN O ITRERY		41.2	30.2	1657.8	1658 ± 0.3
k30-E34	RHYIN k ITRERY		>10 000	18.0	1671.9	1672 ± 0.8
K29-E34	RHY I KLITRERY		1576.5	27.2	1670.9	1670 ± 0.5
O29-E34	RHY I OLITRERY		741.2	14.6	1659.9	1657 ± 0.8
k29-E34	RHY i kLITRERY		>10 000	11.6	1670.9	1671 ± 0.3
K28-E34	RHY K NLITRERY		1255.5	16.9	1671.9	1672 ± 0.8
k28-E34	RHY k NLITRERY		>10 000	5.0	1671.9	1671 ± 0.9
K27-E34	RH K INLITRERY		60.9	7.5	1621.9	1621 ± 0.3

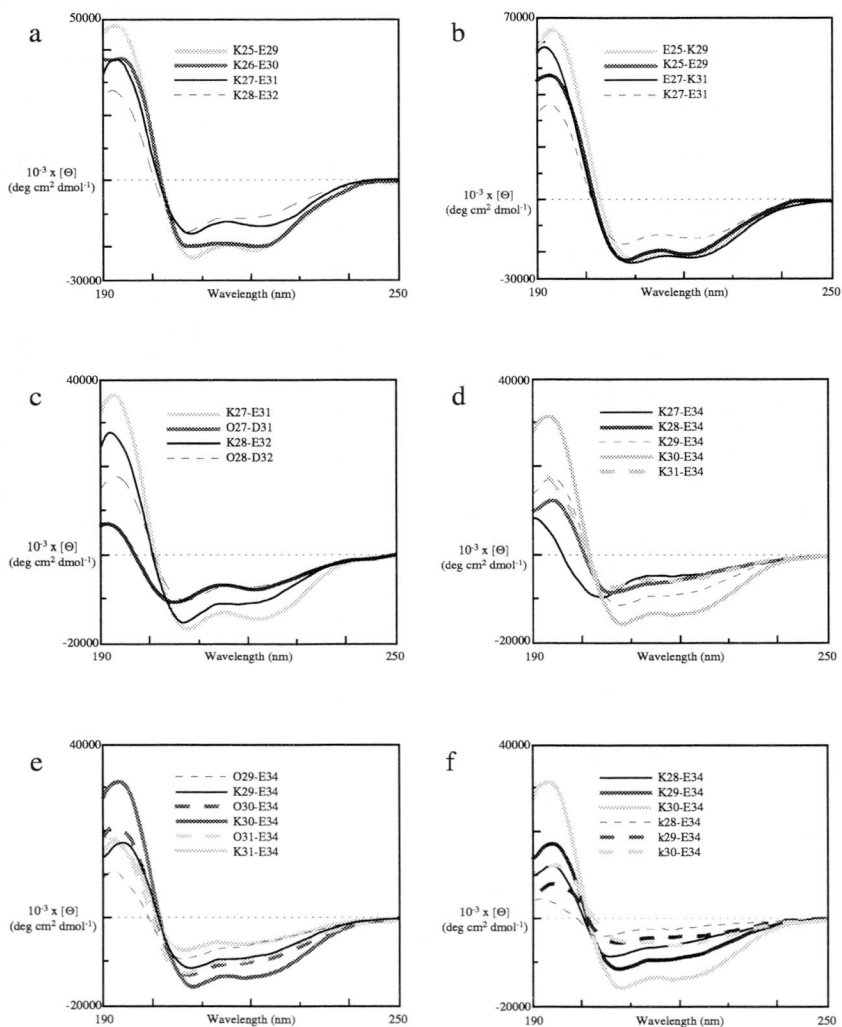


FIGURE 2: CD spectra of the cyclopeptides. (Peptides are named according to the amino acids at the position of the cycle using standard one letter code, k for D-lysine (D-Lys) and O for ornithine.

The distances between N-terminal C β and C-terminal N α hydrogens and between N-terminal C β and aromatic hydrogens of Tyr³⁶ are shown in Figure 3c as d13 and d22. The profile of the curves shows that an increase of the distance corresponds to a decrease in binding affinity. In the case of d13 the smallest relative difference in distance between subnanomolar and submicromolar /micromolar binder is ca. 60% while the one of d22 is located around 75%.

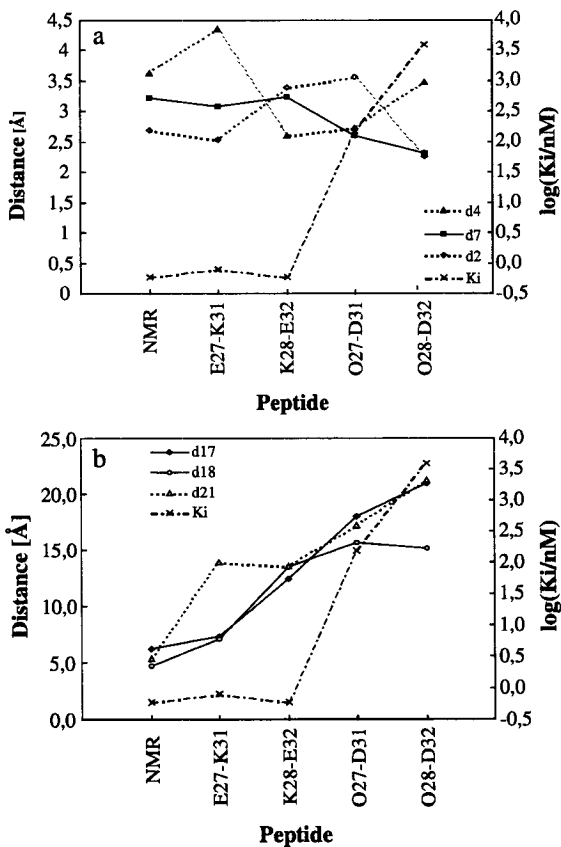
Thus, the distances d17, d21, d13 and d22 can be considered relevant for the affinity, because of the shown trend and of the significant difference in their measured values. The significance of d7 and d18 is lower compared to d17, d21, d13 and d22 because of the smaller difference in their measured values.

Summarizing the analysis of the modeling results points out that excellent binder (E27-K31, K28-E32) tend to have a closer hairpin-like conformation with reduced distance between N- and C-terminus, while poor binder (O27-D31, O28-D32) are characterized by a more open conformation indicated by longer distances between N- and C-terminus. In the case of the better Y₂-receptor binding peptides the N-terminus covers one side of the C-terminal helix in the same way as the full length NPY (Rist *et al.*, 1996), while the open conformation found for the less potent peptides does not allow this coverage. This situation is illustrated by the comparison of the 3D-model of the NMR mean structure of K28-E32 to that of O28-D32 (Figure 4). This result supports the idea of the general hairpin-like fold playing a major role for binding and a certain distance between N-terminus and C-terminus being needed in order to achieve higher binding affinity.

In the second set of cyclic peptides Glu³⁴ is the C-terminal part of the lactambridge, while the position of Lys varies between 27-31, and that of Orn between 29-31. Here, best Y₂-receptor binding has been found for O30-E34 and K30-E34 (K_i 41.2 and 42.9 nM). Their cycles are of the (i, i+4)-type, which is most favorable for α -helix stabilization. Surprisingly the only peptide, which shows no α -helical secondary structure (K27-E34) still maintained some Y₂-receptor affinity (K_i 60.9 nM). This compound is characterized by its large cycle, which spans 6 amino acids within the lactambridge.

Regarding the cycles of the (i, i+5)- and (i, i+6)-type helicity decreases by enlarging them. Compared to K27-E34, which is of the (i, i+7)-type, binding is reduced to the extend of two magnitudes.

CD-studies reveal that cycles of the (i, i+3)-type, represented by the analogues K31-E34 and O31-E34 still maintain a small amount of helical character. The symmetry and the ratio of intensity $[\theta]_{194}/[\theta]_{222}$ that is around 2, indicate a helical part in secondary structure. However, both showed only minimal Y_2 -receptor affinities.



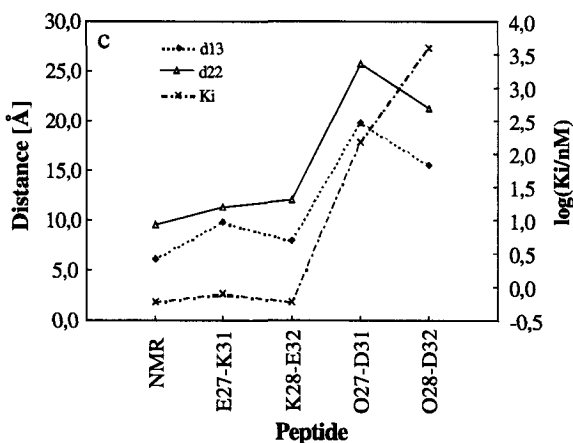


FIGURE 3: Distances and K_i values (in nM) in comparison. The values of the distances were measured for the NMR-structure of K28-E32 (Rist et al. 1996) and for the models resulting from the refinement procedure described in methods. The AMBER nomenclature is used for the hydrogen atoms and the amino acid position is given in brackets. a) Distance d2 is a representative member of the sequential $H^{C\alpha}\cdots H^N$ distances [d2: HA(4) \cdots H(5)]. Distances d4 and d7 correspond to the distances between the sequential $H^N\cdots H^N$ [d4: H(6) \cdots H(7); d7: H(8) \cdots H(9)]. The profile showed by d7 represents the only exception among the group of the sequential $H^N\cdots H^N$ distances. b) Distances d17, d18 and d21 correspond to the distances between N-terminal and C-terminal backbone hydrogens [d17: HA(2) \cdots H(12); d18: H(3) \cdots H(11); d21: H(4) \cdots H(12)]. c) Distances d13 and d22 are representatives of the distances between N-terminal C_β and C-terminal N_α hydrogens [d13: HB2(1) \cdots H(12)] and between N-terminal C_β and aromatic hydrogens of Tyr36 [d22: HB2(1) \cdots HE1(12)] respectively.

(Peptides are named according to the amino acids at the position of the cycle using standard one letter code and O for ornithine)

In the third set of peptides the incorporation of D-Lys leads to a dramatic loss in α -helicity. Furthermore no cyclic peptide which contains a D-Lys showed any Y_1 - or Y_2 -receptor affinity ($K_i > 10\,000$ nM). Comparing the peptides, which only differ in the configuration of lysine, it becomes evident that the D-configured amino

acid destroys the α -helical secondary structure. Additionally, the resulting orientation of the lactambridge induces a conformation that is not favorable for any high receptor affinity.

3.5 Discussion

In order to evaluate the role of the secondary structure of the C-terminal segment NPY Ac-25-36 for Y_1 - and Y_2 -receptor recognition, we performed a systematic approach by synthesizing cyclic analogues of this segment. Since short linear peptides exhibit no pronounced tertiary structure, cyclization was performed to obtain more constrained peptides. The cycles were built by side chain lactamization of lysine or ornithine and aspartic or glutamic acid. Besides the length of the lactambridge, the peptides vary in size, position, configuration, orientation and location of the cycle. In the present investigation we studied in particular the conformational properties of each cyclic analogue. Additionally we revealed the importance of the C-terminal tetrapeptide Arg-Gln-Arg-Tyr for Y_2 -receptor affinity. None of the peptides showed significant Y_1 -receptor binding capacities.

Compared to the linear segment NPY Ac-25-36, cyclization leads to a number of compounds, which show strongly increased Y_2 -receptor affinities.

Regarding the peptides which exhibit high affinity ($K_i < 10$ nM), conspicuously the C-terminal tetrapeptide is not involved in the cycle. This fact manifests the sensitivity of these residues which was already found by the L-Ala-scans of [ϵ Ahx⁵⁻²⁴] NPY (Beck-Sickinger *et al.*, 1990a; Kirby *et al.*, 1993) as well as of full length NPY molecule (Beck-Sickinger *et al.*, 1994). A further common feature for all analogues with high Y_2 -receptor affinity is a cycle of the Lys/Glu (i, i+4)- or Glu/Lys (i, i+4)- type. The resulting lactambridges keep up the optimal length to stabilize one turn of an α -helix (Houston *et al.*, 1995). CD studies revealed, that all these analogues showed a typical α -helical secondary structure, however, the various spectra differed in the intensity of the CD bands. α -Helicity seems to be a prerequisite for binding but there is no apparent relationship between helix content and binding affinity. Important for binding properties is the position of the bridge being extended over the hydrophobic or hydrophilic site of the amphiphilic helix. In the case of the three most potent analogues the bridge is located at the hydrophobic face of the helix. In the native hormone, the C-terminal α -helical conformation is stabilized by hydrophobic interactions between Pro⁸ and Tyr²⁰ as well as between Pro⁵ and Tyr²⁷. The six methylene groups of the Lys/Glu lactam bridge in the analogues K28-E32, E27-K31 and K27-E31 also increased the hydrophobicity of the amphiphilic α -helix and could be suggested to take over the role of the N-terminus in natural NPY.

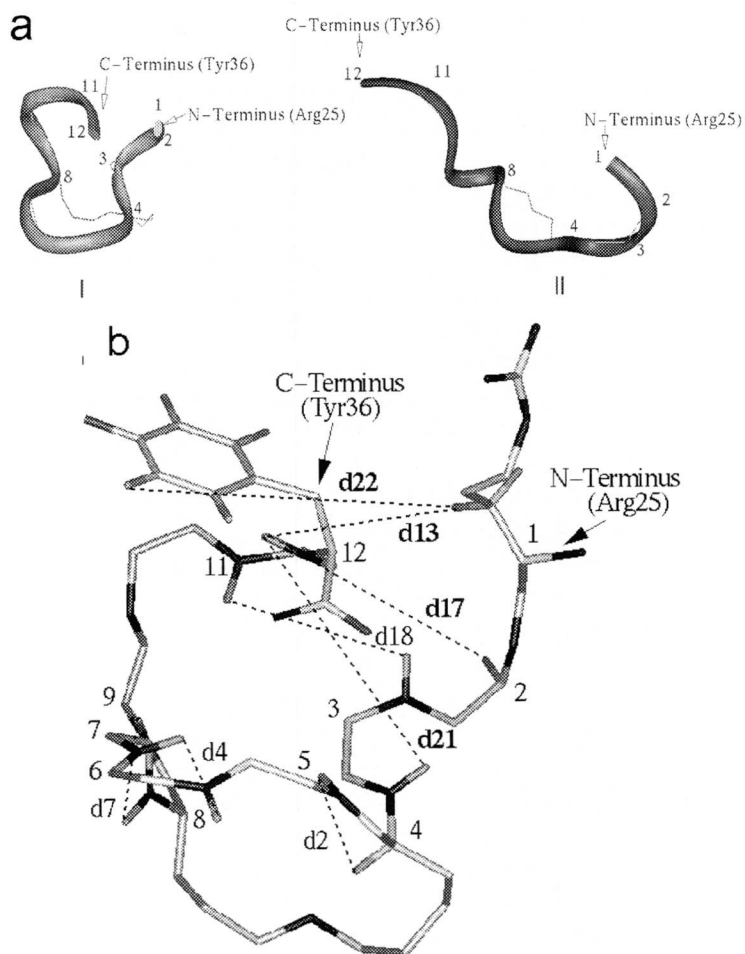


FIGURE 4: Comparison of the conformation of cyclic NPY analogues. a) The relative position of the C-terminus to the N-terminus is shown by a ribbon representation of the NMR mean structure of K28-E32 (I) and of the model of O28-D32 (II). The lactam bridge as well as the C α trace are shown as a thin line. The amino acids involved in long range interactions (Rist et al. 1996) are labeled. The picture was prepared by means of program InsightII (Biosym Inc., San Diego, CA). b) Schematic drawing of K28-E32 indicating the distances reported in Figure 3. The most relevant distances are marked in bold and the corresponding amino acids are numbered.

The cyclization on the hydrophilic face of the helix as for K26-E30, K25-E29 and E25-K29 make evident that α -helicity plays a minor role for Y_2 -receptor interaction. Here, increased helicity is accompanied by a decrease in binding capacity. Although E25-K29 (K_i 63.7 nM) exhibits by far the highest α -helical content, it is 10 times less potent than K26-E30 (K_i 6.0 nM). The replacement of Arg²⁵, in order to create cycles of the (i, i+4) type, forces lactambridges that are most efficient in stabilizing α -helical secondary structure, but obviously they do not favor tight receptor binding. Similar effects have been obtained recently for cyclopeptides based on des-AA7-24[Gly⁶] (Kirby *et al.*, 1997).

According to the Ala scan, the loss of charges due to the substitution of polar Arg²⁵ should not induce reduced binding. Moreover, Leban *et al.* (1995) synthesized a decapeptide [Tyr³², Leu³⁴] NPY 27-36 which distinctly exhibits higher Y_2 -affinity in rat brain than [Tyr³², Leu³⁴] NPY 25-36 still containing Arg²⁵. Replacement of Asn²⁹ seems to be more critical. Particularly in the decapeptide mentioned above Asn is involved in stabilizing the active conformation by hydrogenbonds to the backbone. In addition, loss of affinity for analogues of NPY Ac-25-36 has been reported for peptides with replacement of Asn against Ala, Gly or Phe (Schnorrenberg, 1992).

The carbonyl group of a lactambridge is very similar to the oxygen found in the functional side chains of Asn and Gln respectively, although it contains further methylene groups and the flexibility of the carbonyl group is uncommonly restricted by the bridge giving at the same time a higher directionality. Nevertheless, K25-E29, with a K_i of 22.9; 24.7 nM, exhibits a three-fold higher affinity than E25-K29 whose carbonyl group seems to be too far away to contribute to the stabilization. These facts allow to speculate, that the lactambridge in E25-K29 could be able to imitate Asn²⁹.

The cyclic peptide K30-E34, which exhibits slight binding to the Y_2 -receptor (K_i of 42.9 nM), contains a cycle of the Lys/Glu (i, i+4)-type. As expected, an α -helical structure is found by CD. The corresponding analogue O30-E34, with a lactambridge shortened by one methylene group, shows reduced α -helical content, but Y_2 -receptor affinity is maintained. The replacement of Gln³⁴ by Glu³⁴ and the subsequent lactamization - independent of the created cycle - never leads to an analogue with increased Y_2 -receptor affinity compared to the linear segment

NPY Ac-25-36. We conclude that structural constraints forced by the replacement of Gln³⁴ do not serve to stabilize the active conformation of NPY adopted at the Y₂-receptor. In summary, the data produced by the substitution of Asn²⁹ or Gln³⁴ indicate that flexibility is needed at position 34 while higher directionality is desired at position 29.

According to our results the biological active conformation of NPY required to activate Y₁- and Y₂-receptors is significantly different. In both cases the C-terminal part of NPY plays an important role for receptor interaction (Beck-Sickinger *et al.*, 1994). However, for Y₁-receptor binding, a turn like structure is suggested for the C-terminal tetrapeptide of NPY as Gln³⁴ can be replaced by the turn inducing amino acid Pro. Neither the helix stabilizing amino acid Ala, nor D-Gln, nor D-Pro are tolerated at this position (Beck-Sickinger *et al.*, 1994).

Possibly due to lactamization a turn like structure could be induced. However, none of our cyclic peptides exhibits Y₁-receptor affinity. Either the active conformation is very sensitive and we missed to stabilize it, or the N-terminus of NPY is a part of the binding site at the Y₁-receptor, as frequently suggested. Thus we were able to stabilize the NPY conformation probably adopted only at the Y₂-receptor. Furthermore, our cyclopeptides with high affinity are in agreement with structure-affinity studies (Kirby *et al.*, 1993; Leban *et al.*, 1995; Balasubramaniam *et al.*, 1994, 1996) which contain variation in the C-terminal segment.

These findings together with the data of the NMR experiment and the depiction of distances that are sensitive for the affinity may be used for the design of non-peptide agonists of NPY at the Y₂-receptor.

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CHAPTER 4

Presynaptic Action by Multiple Neuropeptide Y Receptors Modulates GABAergic Transmission onto Parvocellular Neurons of the Paraventricular Nucleus of Rat Hypothalamus

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4.1 Abstract

Neuropeptide Y (NPY) causes hyperphagia and obesity when injected into the paraventricular nucleus (PVN) of the hypothalamus. We studied the mechanism and pharmacology of NPY's actions in the PVN slice with analogues including a novel, centrally-truncated NPY agonist analogue, [ahx⁸⁻²⁰][Pro³⁴] NPY. Application of NPY (500 nM) reduced the GABAergic inhibitory postsynaptic current (IPSC) evoked by focal stimulation in parvocellular neurons by 57 %, while peptide YY (PYY – active at all but Y₃-receptors) inhibited it by 41 % and NPY₂₋₃₆ (“feeding” receptor agonist) inhibited it by 37 %. [ahx⁸⁻²⁰][Pro³⁴] NPY (Y₁ and Y₅) and [ahx⁵⁻²⁴] NPY (Y₂) inhibited the IPSC by 29 % and 22 %, respectively. Human pancreatic polypeptide (hPP- Y₄ and Y₅) inhibited it by 19 %; rat PP (prefers Y₄) was not effective. D-Trp³² NPY (prefers Y₅) inhibited the IPSC by 30 %. NPY, PYY and NPY₂₋₃₆ were effective at nearly all cells tested, the centrally-truncated agonists were effective at about 75 % of cells, but the remainder of the agonists were active at 50 % or less of neurons tested.

The Y₁ antagonists, BIBP3226 and 1229U91 had no effect on the response to NPY itself, but both blocked the effect of the Y₁- and Y₅- preferring agonist, [Leu³¹][Pro³⁴] NPY.

NPY disinhibits parvocellular neurons of the PVN by a presynaptic action on GABA release. Y₁-, Y₅-, and Y₂-receptors all appear to be involved. Multiple NPY receptors appear to coexist on presynaptic terminals, possibly sharing a mechanism of action. The pharmacology of this response resembles that of the induction of food intake, and suggests that a step in eliciting feeding involves the disinhibition of some PVN parvocellular neurons.

Key Words: Neuropeptide Y, Feeding, Y₁-receptor, Y₂-receptor, Y₅-receptor, Y₄-receptor, D-Trp³²NPY, Hypothalamic paraventricular nucleus, Parvocellular neurons, Presynaptic inhibition, GABA release, NPY receptor analogues

4.2 Introduction

Neuropeptide Y (NPY) is the most powerful known neurochemical stimulant of eating behavior, producing a robust response that can greatly exceed an animal's normal capacity (Stanley and Leibowitz, 1985). Clark et al. (1984) first demonstrated that the intracerebroventricular (icv) injection of NPY, and to lesser extent pancreatic polypeptide (PP), elicits eating in satiated rats. When NPY is injected directly into the hypothalamic paraventricular nucleus (PVN), it produces a stronger eating response at lower doses than when given icv (Stanley and Leibowitz, 1984, 1985). Although the area of the hypothalamus most sensitive to NPY's stimulation of eating is located outside the PVN, in the neighboring perifornical hypothalamus (PFH), the action of NPY in the PVN is independent of this site, suggesting multiple sites of NPY action within the hypothalamus (Stanley, 1993). Although NPY-knockout mice eat normally (Erickson et al, 1996), and knockout of either Y_1 - or Y_5 -receptors has little effect on natural or NPY-induced eating (Marsh et al, 1998, Pedrazzini, 1998), NPY nonetheless appears to be important in natural feeding, since treatment of adult rats with NPY antisense mRNA suppresses eating in proportion to the reduction in NPY expression (Akabayashi et al., 1993).

Initial pharmacological studies on the feeding behavior induced by NPY suggested that the response was mediated by an NPY receptor whose agonist structure-activity relationship resembled that of the Y_1 -receptor, but which was more sensitive to an NPY fragment, NPY₂₋₃₆, than the Y_1 -receptor, and thus designated as " Y_1 -like" (Stanley et al., 1991, Wahlestedt and Reis, 1993). More recent evidence has suggested the involvement of a different NPY receptor subtype. Messenger RNA encoding the recently-cloned Y_5 -receptor has been localized with *in situ* hybridization to several hypothalamic regions, specifically, the PVN, lateral hypothalamus (LH), supraoptic nucleus (SON) and arcuate (ARC) nucleus (Gerald et al., 1996) all of which are associated with the central regulation of food intake. However, radioligand binding or *in situ* hybridization studies have also reported the presence of other NPY receptors, including Y_1 and Y_4 , in the PVN and related hypothalamic areas (Corp, 1996, Bard et al, 1995). Icv injection of NPY leads to an increase in the neuronal expression of the immediate early

gene *c-fos* and its protein product in the PVN and other sites shown to be related to feeding (Li et al., 1994; Lambert et al, 1995; Xu et al., 1995), with parvocellular neurons of the PVN consistently reported to be *fos*-positive in response to NPY, suggesting the activation of parvocellular PVN neurons by NPY.

We have studied the sites and mechanisms of action for NPY and related agonists, including several centrally-truncated analogues which lack the disadvantage of NPY's poor washout from tissue (Klapstein and Colmers, 1997, McQuiston and Colmers, 1996), in electrophysiological recordings of synaptic responses in parvocellular PVN neurons in slices of the adult rat hypothalamus. NPY and related agonists presynaptically suppress synaptic inhibition mediated by GABA_A receptors in these neurons, without any evidence of postsynaptic actions. The pharmacology of the NPY response is consistent with a mixed population of NPY receptors, including Y₁-, Y₅- and Y₂-receptors. It appears that different receptor subtypes coexist on presynaptic GABA terminals, and probably share a presynaptic mechanism.

4.3 Methods

4.3.1 Preparation of Brain Slices

Hypothalamic slices were prepared from brains of male Sprague-Dawley rats (28-35 days old). Rats were sacrificed according to a protocol approved by the University of Alberta Health Sciences Laboratory Animal Welfare Committee. The brain was rapidly removed and placed in ice-cold (4° C) artificial cerebrospinal fluid (aCSF) containing in (mM) 124 NaCl, 3 KCl, 1.3 MgSO₄, 1.4 NaH₂PO₄, 26 NaHCO₃ and 10 glucose saturated with 95 % O₂- 5 % CO₂ (carbogen). A block of tissue containing the hypothalamus was dissected and cut into coronal slices (400 µm) with a vibratome (TPI, St Louis, and MO). Slices of hypothalamus containing PVN were obtained just caudal to the optic chiasm. Slices were maintained in carbogenated aCSF at 32 °C for 1 h before recording.

4.3.2 Electrophysiological Recording

Single hypothalamic slices were transferred into a recording chamber and perfused, submerged in aCSF at $34 \pm 0.5^\circ$ C. Recordings were made from neurons in parvocellular area of the PVN using the "blind" whole cell patch clamping technique (Blanton et al, 1989; McQuiston and Colmers, 1992). Glass electrodes were pulled on a two-stage puller (PP-83, Narishige) from borosilicate glass (WPI, Sarasota, FL) and had initial resistances in the bath of 5-6 M Ω when filled with an internal solution of the following composition (in mM): 135 potassium gluconate, 2 KCl, 5 HEPES, 5 MgATP, 0.3 NaGTP, 1.1 BAPTA, pH adjusted to 7.25 with KOH, osmolality 284-290. We routinely included neurobiotin (0.02 %; Vector Laboratories, Burlingame, CA) in the pipette solution for subsequent identification of the neurons. Electrodes were connected to the headstage of an Axoclamp 2A amplifier (Axon, Burlingame, CA) used either in the bridge current clamp or continuous single electrode voltage clamp mode.

Parvocellular neurons were identified by their position in the slice and by their electrophysiological properties. Neurons we chose for this study generally had properties similar to the non-bursting, low-threshold spike (LTS) neurons described by Hoffman et al (1991), although we did not classify them further in this study. Parvocellular neurons could be readily differentiated from magnocellular neurons on the basis of action potential shape and amplitude, resting potential and input resistance. These cells normally rested between -40 and -55 mV, their input resistances, estimated from their slope conductance between about -90 and -60 mV were 295 ± 13 M Ω , and they showed no sign of bursting behavior. Neurons were routinely held in voltage clamp at -50 to -60 mV, somewhat negative to their normal resting potential of between -40 and -55 mV. Neurons were only studied if their holding current and access resistance remained stable in voltage clamp for 10 - 15 minutes prior to any other manipulations.

Synaptic potentials were recorded upon stimulation via a sharpened, monopolar tungsten electrode, which was positioned in the perifornical area after the neuron was acquired, and repositioned within this region if necessary to elicit optimal responses. Pairs of stimuli (10 - 20 V, 100 - 200 μ sec, 80 ms interstimulus interval) generally elicited both inward and outward synaptic currents at the

holding potential. Outward currents were sensitive to bicuculline (10 μM) and picrotoxin (50 μM), thus indicating mediation by GABA acting at GABA_A receptors. Inward synaptic currents could be blocked by 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione (NBQX, 1 μM , gift of Novo Nordisk) and DL-2-amino-5-phosphonovaleric acid (APV; 50 μM) and therefore were mediated by glutamate acting at AMPA and NMDA receptors. At least three control records were taken at 5 min intervals to ensure the stability of synaptic responses prior to drug application. Each control record was a digital average of 3 successive responses elicited at 10 second intervals. To ensure the stability of recordings, and determine if changes occurred to subthreshold postsynaptic conductances, the membrane current response to voltage ramps (from -90 mV to -50 mV over 2 sec) were assessed just prior to drug application and during the peak effect.

4.3.3 Synthesis and Preparation of a Centrally-truncated Peptide Analogue

Fmoc-protected amino acids, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-tetrafluoroborate (TBTU) and 4-(2',4'-Dimethoxyphenyl-hydroxymethyl)-phenoxy resin were obtained from NovaBiochem (Läufelfingen, Switzerland), aminomethylated polystyrene from Rapp Polymere (Tübingen, Germany), diisopropylcarbodiimide and thiocresol from Aldrich, 1-hydroxybenzotriazole (HOBt), diisopropylethylamine, trifluoroacetic acid, thioanisole and piperidine from Fluka and dimethylformamide (p. a. grade), diethyl ether, acetonitrile and tert. butyl alcohol from Merck.

The peptides were synthesized by automated multiple solid phase peptide synthesis using a robot system (Syro, MultiSynTech, Bochum). In order to obtain the peptide amides, 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin was used. The polymere matrix was polystyrene-1 %-divinylbenzene (30 mg; 15 μmol). The side chain protection was chosen as follows: Tyr(tert.-butyl), Asp(tert.-butyl), Arg(2,2,3,5,5-pentamethyl-chromansulfonyl), His(trityl), Gln(trityl), Asn(trityl), Thr(tert.-butyl), Ser(tert.-butyl), and Lys(tert.-butyloxy-carbonyl). Double coupling procedures were performed with diisopropylcarbodiimide/1-hydroxybenzotriazole activation, 10-fold excess and a coupling time of 40 min.

The peptide amides were cleaved with trifluoroacetic acid/ thioanisole/thiocresol within 3 h, collected by centrifugation and lyophilized from water. The peptides were analyzed and purified to homogeneity higher than 94 % by reversed-phase HPLC on nucleosil C-18 columns (analytical (Merk-Hitachi): 5 μm , 3 x 125 mm; preparative (Waters): 6 μm , 25 x 300 mm). Acetonitrile/water mixed with 0.1 % trifluoroacetic acid was used as eluent. The peptides were analyzed with a flow rate of 0.6 ml/min and using a gradient of 25 to 75 % acetonitrile in 30 min. For preparative HPLC 17-23 % acetonitrile was used. Correct mass was identified by ion-spray mass spectrometry (API III, Sciex, Toronto).

4.3.4 Membrane Preparation

SMS-KAN cells (Y_2 -receptor-expressing) were grown in 50 % nutrient mixture Ham's F12/50 % Dulbecco's modified Eagle medium with 15 % fetal calf serum, 2 mM glutamine and non-essential amino acids at 37° C and 5 % CO_2 until they were confluent. SK-N-MC cells (Y_1 -receptor-expressing) were grown under the same conditions using MEM Earle's Salts medium with 10 % fetal calf serum, BHKrY5 cells (Y_5 -receptor-expressing) in DMEM (4.5 g/l glucose) with 10 % fetal calf serum, 1 % PENStrep and 1 mg/ml Geneticin. The medium was decanted. The cells were washed with phosphate buffered saline (PBS) and treated with 0.02 % EDTA in PBS for 2 min. The EDTA solution was removed and the cells were suspended in Tris buffer, pH 7.5 with 0.1 % bacitracin and 50 μM Pefabloc SC. The cell suspension was homogenized 15-times using a Potter (Braun-Melsungen, Germany) at maximum speed. After centrifugation at 4° C for 10 min at 800 \times g, the supernatant was decanted and centrifuged at 4° C for 30 min at 16500 \times g. The supernatant was decanted and the pellet resuspended in 30 ml HEPES buffer (25 mM HEPES, 25 mM CaCl_2 , 1 mM MgCl_2 , pH 7.4, 0.1 % bacitracin and 50 μM Pefabloc SC), homogenized 15-times with a Potter and recentrifuged at 4° C for 30 min at 16500 \times g. The pellet was resuspended in 15 ml HEPES buffer without protease inhibitors. An aliquot was used to determine protein concentration and inhibitors were added accordingly. Aliquots of the membrane suspension of 1 ml were stored at -80° C.

All cell culture media and supplements were purchased from Gibco, Pefabloc SC from Serva and Bacitracin from Sigma.

4.3.5 Receptor Binding

Membrane preparations of SK-N-MC, SMS-KAN or BHKrY5 cells were diluted in incubation buffer (MEM/25 mM Hepes, 1 % bovine serum albumin, 50 μ M Pefabloc SC, 0.1 % bacitracin, 3.75 mM CaCl_2). 200 μ l of the suspension containing 20 μ g protein were incubated with 25 μ l 1.2 nM ^3H -propionyl-NPY (3.18 TBq/mmol; Amersham) and 25 μ l of solutions of the analogues in increasing concentrations to give a total volume of 250 μ l. After 1.5 h at room temperature the incubation was terminated by centrifugation of the samples for 10 min at 3,000 \times g and 4° C. The pellets were washed with PBS, resuspended in PBS, mixed with scintillation cocktail and radioactivity was determined. Nonspecific binding was defined in the presence of 1 μ M NPY.

All agonists and antagonists were dissolved in warmed carbogenated aCSF just prior to use and applied via bath perfusion. Peptides kept at -20° C as concentrated aliquots until immediately prior to use. NPY was purchased from Dr. S. St.-Pierre (Université de Québec, Montreal); PYY, NPY₂₋₃₆, rat and human PP and D-Trp³² NPY were purchased from Bachem (Torrance, CA). BIBP 3226, a Y₁-receptor-selective antagonist (Rudolf et al., 1994) was purchased from Peninsula Laboratories (Belmont, CA), and 1229U91, a peptidic Y₁-receptor antagonist, was a gift of Dr. Alex Daniels, Glaxo-Wellcome. All chemicals for aCSF were obtained from BDH (Toronto, ON) and all other chemicals were obtained from Sigma (St. Louis).

4.3.6 Immunocytochemistry

At the end of a recording session, slices were immersion-fixed overnight at 4° C in 4 % paraformaldehyde in a 0.1 M phosphate buffer (pH 7.35). After washout of the fixative in 3, 10-minute changes of phosphate buffer, slices were dehydrated in 20 % sucrose in phosphate buffer for 12-24 h. Slices were sectioned in a cryostat (20-25 μ m) and collected in 0.1 M phosphate buffer, pH 7.2. Sections were

incubated in streptavidin-conjugated Texas Red (Molecular Probes, Eugene) diluted (1:50) in 0.1 M phosphate buffer containing 0.3 % Triton X, according to the manufacturer's directions. Sections were viewed and photographed with an epifluorescence microscope (Zeiss) equipped with rhodamine filters.

4.3.7 Electrophysiological Data Analysis

Because the pharmacology of the feeding response in PVN suggested that Y_1 and/or Y_5 -receptors might be involved, we modified an existing, centrally-truncated analogue with broad affinity for NPY receptors, [ahx⁸⁻²⁰]NPY (Rist et al., 1995), by substituting a proline for glutamine at position 34 to make it more Y_1 - and Y_5 -receptor-preferring (Fuhlendorff et al, 1990).

We determined the affinity of this new agonist against NPY on membranes of cells expressing Y_1 -, Y_2 - and Y_5 -receptors (Fig. 1). The IC_{50} for [ahx⁸⁻²⁰][Pro³⁴]NPY binding against [³H]NPY at cloned rat Y_5 -receptors expressed in BHKrY5 cells was 230 ± 33 nM, while the IC_{50} for NPY itself was 1.5 ± 1.9 nM (Fig. 1A). The IC_{50} for [ahx⁸⁻²⁰][Pro³⁴]NPY against NPY at Y_1 -receptors expressed naturally in SK-N-MC cells was lower, 13 ± 3.3 nM, while the IC_{50} for NPY itself was 10-fold less (Fig. 1B). By contrast, the IC_{50} of [ahx⁸⁻²⁰][Pro³⁴]NPY at Y_2 -receptors was greater than 10,000 nM (not illustrated). Thus the molecule has about a 20-fold preference for Y_1 -receptors over Y_5 , very similar to the preference of [Leu³¹][Pro³⁴]NPY for these two receptors (Gerald et al., 1996), but is inactive at Y_2 -receptors.

4.4 Results

4.4.1 Synthesis and Characterization of a Centrally-truncated NPY Analogue

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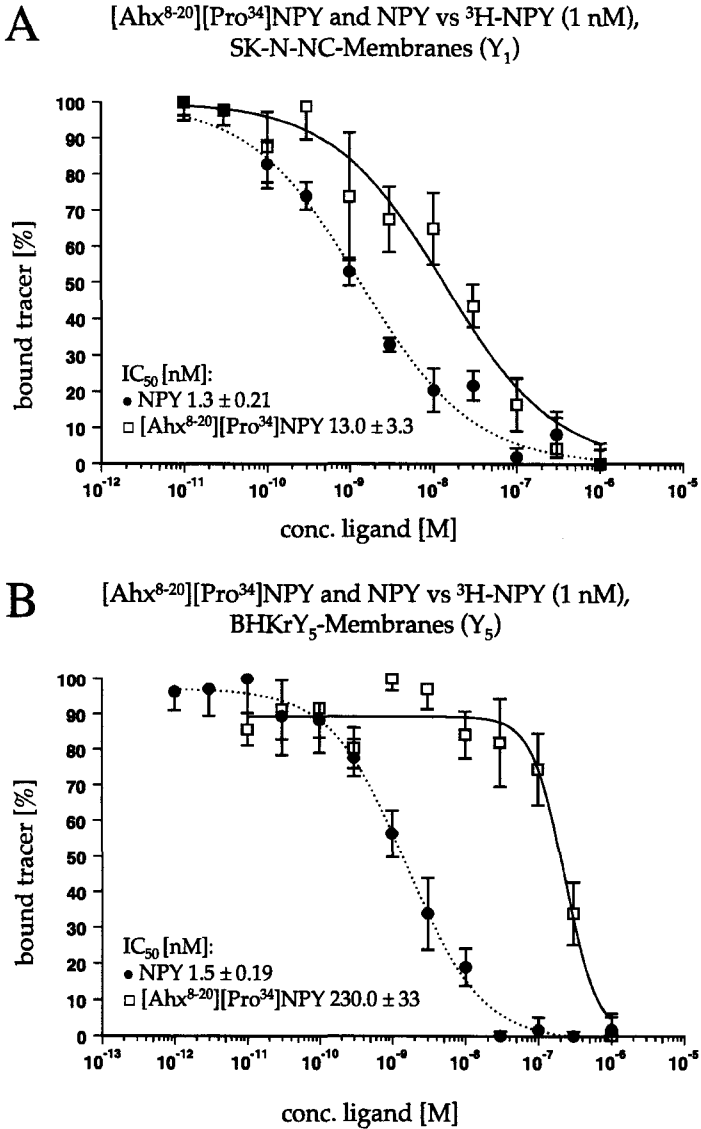


FIGURE 1: Membrane binding displacement curves for NPY and [ahx⁸⁻²⁰][Pro³⁴] NPY against ³H-labeled NPY. **A.** Binding to Y₁-receptors expressed in SK-N-MC cells. **B.** Binding to rat Y₅-receptors expressed in BHKrY₅ cells.

4.4.2 NPY and Related Peptides Reduce Inhibitory Postsynaptic Currents

Recordings were routinely made from parvocellular neurons of the PVN. These cells often had processes extending toward the ventricle, when filled with neurobiotin, fixed and stained (Fig. 2).

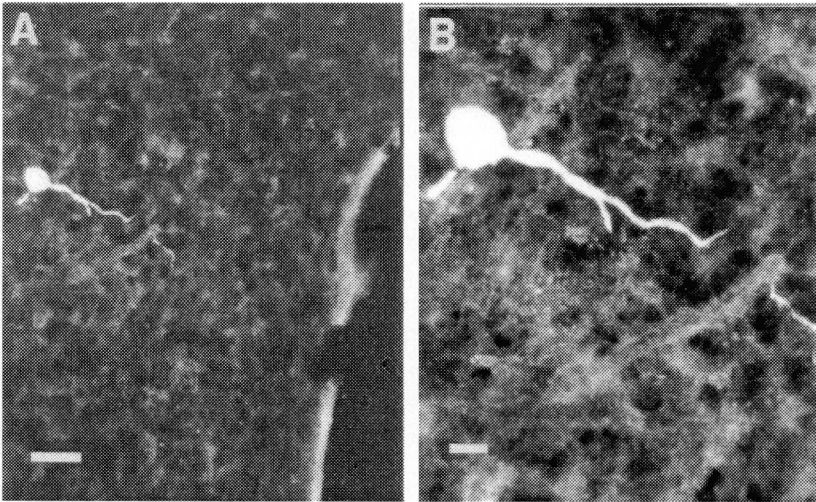


FIGURE 2: A neuron in the parvocellular region of PVN in rat hypothalamus, labeled with neurobiotin via a patch pipette, shown at different magnifications. This neuron had a dominant GABA synaptic response, which was sensitive to NPY. The marker was visualized with biotin-conjugated Texas-Red. The bars in the lower left corner of **A** and **B** represent 50 μm and 10 μm , respectively.

Focal electrical stimulation of the perifornical area elicits mostly inhibitory postsynaptic currents (IPSCs) in PVN parvocellular neurons. These IPSCs reversed at between -60 to -65 mV, and were always sensitive to application of the GABA_A receptor antagonists, picrotoxin or bicuculline, when tested (Fig. 3A). In some of the neurons, both IPSCs and glutamate-mediated excitatory postsynaptic currents (EPSC) could be seen. In some experiments, we isolated the IPSC by superfusing the slices for 10 min with NBQX (1 μM) and APV (50 μM)

to block AMPA and NMDA receptors respectively. This treatment had a small but measurable effect on the synaptic current (Fig. 3B). However, in most experiments, synaptic currents were elicited without the addition of glutamate blockers, while the cell was held positive to rest at -40 mV. At this potential, the IPSC is an outward current, and the EPSC is a (relatively small) inward current under the experimental conditions used.

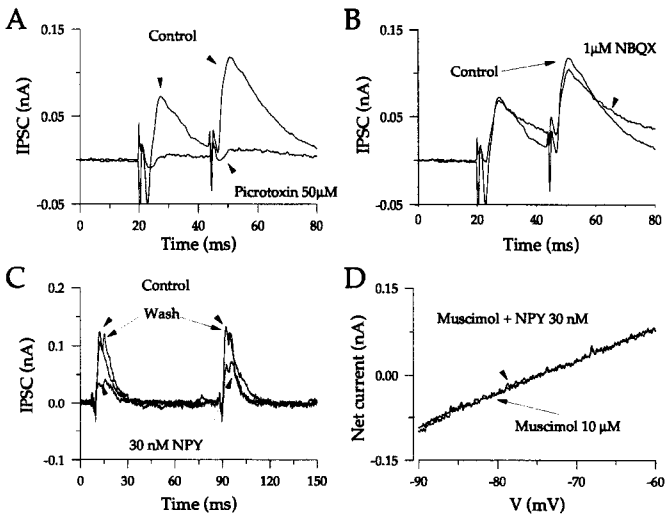


FIGURE 3: NPY reduces $GABA_A$ receptor-mediated inhibitory postsynaptic currents in parvocellular PVN neurons by a presynaptic action. **A.** The $GABA_A$ receptor blocker, picrotoxin ($50 \mu\text{M}$) inhibits most of the synaptic current evoked by electrical stimulation in a parvocellular PVN neuron **B.** In the same neuron as in **A**, application of the AMPA-receptor antagonist, NBQX ($1 \mu\text{M}$), only slightly alters the synaptic current evoked by electrical stimulation of perifornical area. **C.** The application of NPY (30nM) reversibly reduces the amplitude of the $GABA_A$ IPSC in a parvocellular neuron. Synaptic responses in control, in the presence of 30nM NPY and after 30' washout of NPY are shown superimposed. **D.** NPY does not affect the change in membrane conductance caused by application of the $GABA_A$ agonist, muscimol ($10 \mu\text{M}$) to the cell in **C**. Shown superimposed are the net (muscimol – control) membrane current responses to slow voltage ramps acquired in the absence and presence of 30nM NPY.

Bath application of NPY caused a reversible reduction in the amplitude of IPSCs (Fig. 3C). The peak of the effect was generally observed within the first 5 min after completing the 3-min NPY application. NPY (500 nM) reduced the IPSC amplitude by 57.09 ± 3.62 ($n=7$, $P < 0.0001$, paired-t test). The inhibition reversed within 25-40 min of commencing washout. The action of NPY appeared to be entirely presynaptic, as no changes were observed either in the holding current or the membrane current response to the voltage ramp, nor did NPY cause any changes in the postsynaptic response of the cell to the GABA_A agonist, muscimol (10 μ M; $n = 3$, Fig. 3D). Initial experiments on magnocellular neurons of the PVN (identified by location and physiological properties, $n=8$) indicated they were insensitive to the actions of NPY, and were therefore not further studied here.

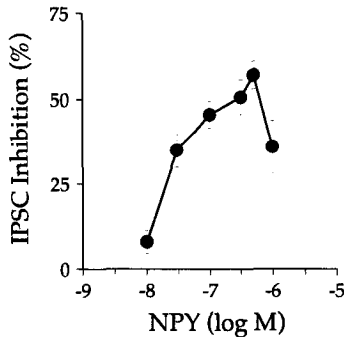


FIGURE 4: Concentration-response curve for the NPY-mediated inhibition of the IPSC in parvocellular PVN neurons. The EC_{50} , calculated from all concentrations of NPY below 1 μ M, was 28 nM. Data are all from cells in which the effect of NPY reversed substantially on washout.

To determine the apparent affinity of NPY at the presynaptic receptor, we tested NPY in the concentration range from 10 nM to 1 μ M. (Fig. 4). NPY was less effective at 1 μ M than at 500 nM, giving the concentration-response curve an inverted “U”-shape. Because of this, the data at 1 μ M were not used to calculate the EC_{50} . The EC_{50} , determined from the dose-response data was about 28 nM. Unlike many other related agonists (see below), NPY inhibited the IPSC in essentially all neurons tested (Fig. 5).

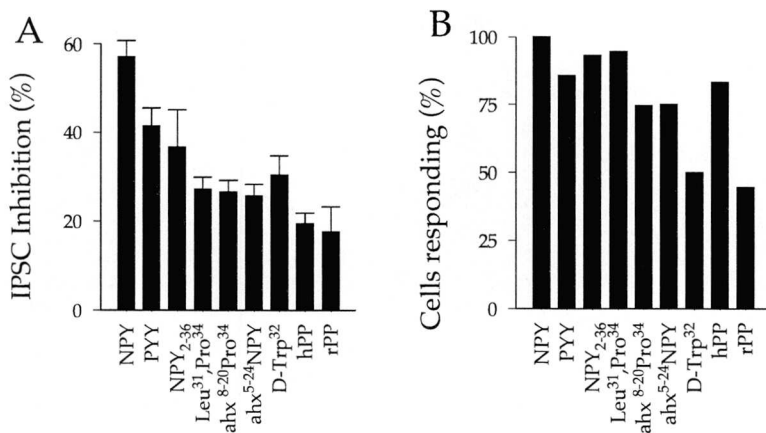


FIGURE 5: Structure-activity relationship of NPY and related agonists on the inhibition of the IPSC in rat parvocellular PVN neurons. **A.** Inhibition of the IPSC. **B.** Percentage of cells responding. All compounds were tested at 500 nM. The effects of all agonists on the IPSC were significant except for that of rPP.

We tested a number of NPY peptide analogues with characterized receptor specificities to identify which receptor, or receptors, mediated the presynaptic response. To facilitate comparison, all peptides were tested at a concentration of 500 nM. Peptide YY (PYY), a closely related peptide, is an agonist at all known NPY receptors, except for Y₃ (Michel et al., 1998, Blomquist and Herzog, 1997). Bath application of PYY (500 nM) inhibited the IPSC with less potency ($41.43 \pm 4.06\%$, $n=3$, $P < 0.01$, unpaired-t test; 12/14 neurons responded; Fig. 5, Fig. 6A) than did NPY. As had been observed earlier in our laboratory (Klapstein and Colmers, 1997), PYY took longer (about 40-45 min) than NPY to wash out. NPY₂₋₃₆, a particularly effective agonist of the feeding response to NPY (Stanley et al., 1992) also inhibited IPSCs by $36.70 \pm 8.44\%$; $n=5$, $P < 0.01$ (paired-t test; Fig. 5, Fig. 6B) in nearly all cells tested (14/15); washout took about 25 - 30 minutes. The Y₁- and Y₅-preferring agonist, [Leu³¹][Pro³⁴]NPY, was also effective, inhibiting the IPSC by $27.20 \pm 2.67\%$ in 18/19 cells ($P < 0.001$).

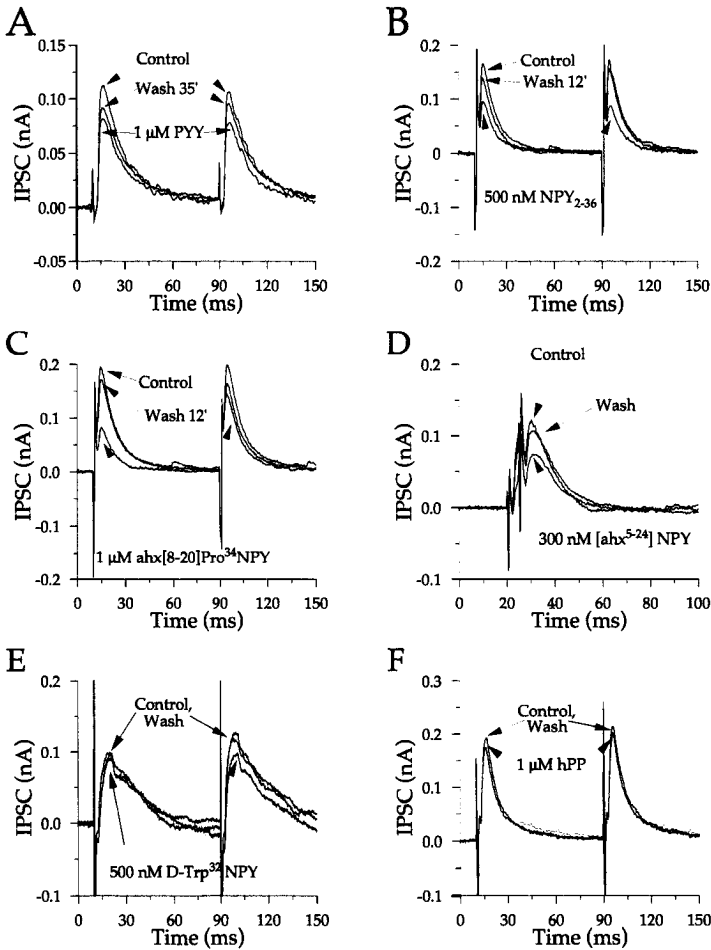


FIGURE 6: Representative effects of different NPY-related agonists on the IPSC in parvocellular PVN neurons. Data shown are only from neurons showing essentially complete washout after drug application. **A** and **B** show the inhibitory effect of the potent orexigenic agents PYY and NPY_{2-36} , respectively; **C** The effect of $[\text{ahx}^{8-20}][\text{Pro}^{34}]\text{NPY}$ NPY, a Y_1 - and Y_5 - selective agonist (see Fig 1); **D** Effect of $[\text{ahx}^{5-24}]\text{NPY}$, a Y_2 - specific agonist. **E** The Y_5 selective agonist $\text{D-Trp}^{32}\text{NPY}$. **F** Human pancreatic polypeptide (hPP).

[ahx⁸⁻²⁰][Pro³⁴] NPY, the centrally-truncated agonist which acts at Y₁- and Y₅-receptors (Fig. 1), was effective in 38 of 51 neurons tested and at 500 nM, it inhibited the amplitude of IPSCs by 26.42 ± 2.60 %; (n=29, p < 0.0001, Fig. 5, Fig. 6C). The Y₂-selective, centrally truncated agonist, [ahx⁵⁻²⁴] NPY, which has little or no activity at Y₁- or Y₅-receptors (Beck Sickinger et al, 1993 and unpublished), reduced the IPSC amplitude by 25.60 ± 2.70 % (n=9; P < 0.0001; Fig. 5, Fig. 6D) in 8 of 12 neurons tested. However, despite our expectations, the washout of these modified agonists took between 20 and 30 minutes, no different from the time needed to reverse the actions of the full-length agonists.

The Y₅-selective agonist, D-Trp³² NPY (Balasubramaniam et al., 1994; Gerald et al, 1996), inhibited IPSCs only in half of the neurons tested (12 of 24, Fig. 5). The extent of inhibition (30.40 ± 4.3 %, n=6, P < 0.001) was comparable to that of [ahx⁸⁻²⁰][Pro³⁴] NPY (Fig. 5, Fig. 6E). Human PP was less effective than the other agonists tested, hPP inhibited the IPSC by 19.38 ± 2.49 % (n=8, P < 0.005; 10/12 cells responded; Fig. 5, Fig. 6F) while rat PP did not inhibit it significantly (17.63 ± 5.62 % n=3, P > 0.05; 4/9 cells responded; Fig. 5).

4.4.3 Y₁ and Other Receptors Contribute to the Action of NPY in Parvocellular Region of PVN

Because Y₁- and Y₅-receptors have been implicated in the feeding response to NPY, (Pedrazzini et al, 1998, Marsh et al, 1998), we hypothesized that Y₁-receptors would contribute to the response in PVN. We therefore examined the actions of NPY alone or in the presence of two different Y₁-receptor antagonists on the same neuron. Briefly, we found that neither antagonist affected the inhibition of the IPSC by NPY itself. Specifically, 100 nM NPY reduced the IPSC by 44.51 ± 6.68 %, and by 44.71 ± 5.32 % in the same neurons in the presence of 500 nM BIBP3226 (n = 7, P > 0.25 paired t-test; Fig. 7). We next tested a different Y₁ antagonist, 1229U91, which has a higher affinity for Y₁-receptors than does BIBP3226 (pK_i = 10.9 vs. 7.2; Hedge et al., 1995; Tedepalli et al., 1996). 500 nM 1229U91 also did not significantly affect the IPSC inhibition caused by 100 nM NPY (36.83 ± 3.34 %, vs 36.95 ± 5.72 % with 1229U91 present; n=8; Fig. 7).

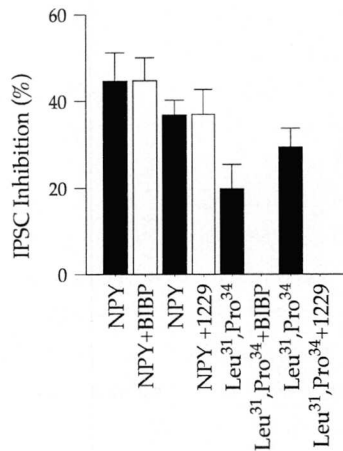


FIGURE 7: The effect of Y_1 selective antagonists on the NPY-mediated presynaptic inhibition of the IPSC in parvocellular neurons. The Y_1 -receptor antagonists, BIBP3226 and 1229U91 (500 nM), do not affect the action of NPY (100 nM), but completely prevent the effect of [Leu³¹][Pro³⁴] NPY. Solid bars represent the effect of NPY or [Leu³¹][Pro³⁴] NPY alone, while the open bars represent the effect of the same agonists in the presence of the respective antagonists. Data are from neurons in which a given agonist was tested in the absence and the presence of an antagonist, and which demonstrated substantial recovery from the agonist under all conditions applicable.

We next hypothesized that more than one NPY receptor subtype (including Y_1) may coexist on the GABA terminals in PVN. We reduced the number of receptors likely to be activated by repeating the above experiments with the Y_1 - (and Y_5 -) preferring agonist, [Leu³¹][Pro³⁴] NPY (500 nM). In this series of experiments, [Leu³¹][Pro³⁴] NPY inhibited the IPSC by $19.8 \pm 5.64\%$, but had no measurable effect when reappplied in the presence of BIBP 3226 ($n = 5$, $P < 0.001$, paired t test; Fig. 7, Fig. 8A). Similarly, 1229U91 (500 nM) also completely blocked the inhibition of the IPSC by 500 nM [Leu³¹][Pro³⁴] NPY in a different series of neurons by ($29.36 \pm 4.35\%$ vs. 0.0% ; $n=5$, $P < 0.001$, paired t test; Fig. 7, Fig. 8B). Thus, Y_1 -selective antagonists inhibit the actions of a Y_1 -preferring

agonist, but not of NPY itself, consistent with a redundancy of NPY receptors at presynaptic GABA terminals innervating parvocellular neurons in the PVN.

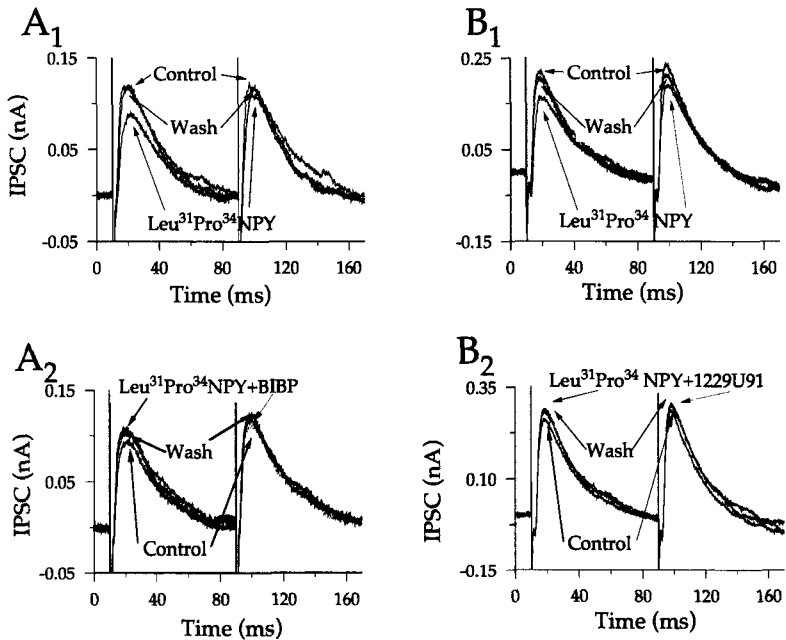


FIGURE 8: Effects of the antagonists BIBP3226 and 1229U91 on the inhibition of the IPSC by $[\text{Leu}^{31}][\text{Pro}^{34}]$ NPY in PVN parvocellular neurons. **A1.** Effect of 500 nM $[\text{Leu}^{31}][\text{Pro}^{34}]$ NPY. **A2.** Effect of $[\text{Leu}^{31}][\text{Pro}^{34}]$ NPY on the same neuron as in A1 in the presence of 500 nM BIBP3226. **B1.** Effect of 500 nM $[\text{Leu}^{31}][\text{Pro}^{34}]$ NPY. **B2.** Effect of $[\text{Leu}^{31}][\text{Pro}^{34}]$ NPY on the same neuron as in B1 in the presence of 500 nM 1229U91. Neither antagonist had any effects by itself in these experiments.

4.5 Discussion

The application of NPY resulted in a prominent decrease in the amplitude of GABA_A receptor-mediated IPSCs evoked in parvocellular neurons of the PVN in slices of adult rat hypothalamus. NPY acts at a presynaptic site to suppress GABA release in the PVN. The pharmacology of this action is similar to that observed for the increases in food intake elicited by injections of NPY and related agonists into the PVN or neighboring regions of the rat hypothalamus *in vivo*. It also appears that multiple NPY receptors can suppress GABA release from the same terminal. The present evidence supports an entirely presynaptic role for NPY in the rat PVN. No changes were observed with NPY application in either the holding current or the response to subthreshold voltage ramps, nor did NPY affect the membrane current response to the GABA_A agonist, muscimol. Unlike in arcuate nucleus neurons (Rhim et al., 1997), in PVN cells there was no evidence for NPY activating a postsynaptic K⁺ current, nor for its having a significant effect on the EPSC of neurons that also received NPY-sensitive IPSCs. A net reduction in GABA release onto PVN parvocellular neurons would be expected to result in increased activity in these cells, consistent with the increase in *c-fos* expression observed *in vivo* in this population of neurons in response to NPY injection (Li et al, 1994, Xu et al., 1995)

4.5.1 NPY Receptor Pharmacology in PVN

NPY is active at an entire family of different receptors (Michel et al., 1998). Our results suggest that the most potent and most frequently effective NPY agonists in PVN are also those with the most potent actions on food intake (Stanley, 1993), but which also have the least receptor specificity. Our structure-activity studies with a panel of agonists were not consistent with a single known receptor subtype. NPY was the most potent and widely active of the agonists, followed by PYY and the "feeding receptor" agonist, NPY₂₋₃₆. The effectiveness of PYY makes participation of Y₃-receptors in the PVN response unlikely. Human PP (an agonist at Y₄- and Y₅-receptors) was less potent, although it affected most cells tested,

while rat PP was not significantly effective, essentially excluding the rat Y₄-receptor.

4.5.2 Centrally-truncated Agonists

The Y₁- and Y₅-receptor-preferring, centrally-truncated agonist that we developed for this study, [ahx⁸⁻²⁰][Pro³⁴] NPY, was somewhat less potent in suppressing the IPSC than either the full-sequence peptides or NPY₂₋₃₆, and was effective at about 75 % of neurons tested, but was about equipotent with the Y₅-selective agonist, D-Trp³², which affected inputs to only half of the neurons tested. The Y₂-receptor-preferring, centrally truncated agonist, [ahx⁵⁻²⁴] NPY (Beck-Sickinger et al., 1993; McQuiston and Colmers; 1996, Klapstein and Colmers, 1997) was also significantly effective, although considerably less potent than the full-length agonists, and affected 75 % of the neurons tested. As this agonist has no significant activity at either Y₁- or Y₅-receptors (Beck-Sickinger et al., 1993, and unpublished results), it suggests the presence of Y₂-receptors at many of the GABA terminals in the parvocellular region. We have no explanation for the finding that the centrally-truncated analogues we tested did not wash out as rapidly as in the hippocampal slice (Klapstein and Colmers, 1997; McQuiston and Colmers, 1996). Overall, the agonist structure-activity profile includes several receptors, specifically, Y₁-, Y₅- and, to a lesser extent, Y₂-, while largely excluding Y₃- and Y₄-receptors.

4.5.3 Antagonist Studies

While testing the hypothesis that Y₁-receptors mediate the response to NPY, we were surprised that the Y₁-receptor antagonists, BIBP3226 or 1229U91 did not affect the response to NPY itself. However, when we tested the hypothesis that Y₁-receptors formed part of a complement of NPY receptor subtypes on the presynaptic terminals using a selective agonist with essentially the same affinity for Y₁-receptors as NPY itself, [Leu³¹][Pro³⁴] NPY, both Y₁ antagonists blocked its effect. These observations are consistent with the hypothesis that Y₁-receptors mediate part of the response to NPY, as suggested for the Y₅-receptor knockout

mice (Pedrazzini et al, 1998) but are also consistent with the hypothesis that Y_1 -receptors coexist on presynaptic terminals with other NPY receptors, such as Y_5 and Y_2 , and that these other receptors also inhibit GABA release there.

Different subtypes of NPY receptors have been previously reported to coexist in somata and terminals of suprachiasmatic nucleus neurons in culture (Chen and van den Pol, 1996). Y_1 - and Y_2 -receptors coexist on cell bodies of acutely-dissociated dentate granule cells, where they both inhibit N-type Ca^{2+} currents, (McQuiston et al, 1996), and on acutely-isolated nodose ganglion cells, where their actions on Ca^{2+} currents are opposite (Wiley et al., 1993). Different NPY receptor subtypes are thus frequently co-expressed in neurons, and sometimes can access identical signaling pathways.

4.5.4 NPY Receptors and Feeding

Evidence links both the Y_5 - and Y_1 -receptors with the feeding response to NPY. Thus, Y_5 -receptors are localized in areas important to the control of food intake, namely the paraventricular nucleus, the lateral hypothalamus and the arcuate nucleus (Gerald et al. 1996). Many Y_1 -receptor-preferring ligands that induce feeding also activate Y_5 -receptors (Gerald et al, 1996), while agonists that are relatively inactive at Y_1 -receptors, such as hPP and PYY₃₋₃₆, and the weak but apparently selective Y_5 agonist, D-Trp³²NPY, nonetheless elicit food intake (Gerald et al., 1996). Y_5 -receptor knockout mice were reported to have an attenuated feeding response to NPY (Marsh et al., 1998). However, these animals ate normally, had only mildly attenuated responses to injections of NPY, and crosses of these with leptin deficient, *ob/ob* mice are no leaner than the *ob/ob* counterparts, while crosses of NPY knockouts with *ob/ob* mice were leaner than *ob/ob* mice (Erickson et al, 1996). Finally, the Y_1 antagonist BIBP3226 is reportedly inactive against NPY-induced feeding (Gerald et al., 1996).

Others however report that both BIBP3226 and 1229U91 block NPY-induced feeding in rats (O'Shea et al, 1997, Kanatani et al, 1996). 1229U91 reportedly inhibits natural feeding, in addition to that induced by NPY, while BIBP3226 was reported to also inhibit feeding induced by galanin and noradrenaline (O'Shea et al., 1997). Furthermore, in Y_5 knockout mice, treatment with 1229U91 entirely

inhibited the remaining feeding response to NPY (Marsh et al., 1998). On the other hand, knockout of the Y_1 -receptor in mice caused only a minor reduction in normal and NPY-induced food intake (Pedrazzini et al., 1998).

The evidence from a number of studies thus suggests that NPY mediates feeding via more than one receptor, including at least Y_1 and Y_5 . This is consistent with the observations on the inhibition of synaptic responses in the PVN observed here. In addition, there is evidence here for a Y_2 -receptor-mediated inhibition of the IPSC in parvocellular PVN neurons. While less evidence exists for the involvement of Y_2 -receptors in appetite stimulation, it is noteworthy that, with the exception of D-Trp³² NPY, the structure-activity relationship for feeding effects published by Gerald et al. (1996) closely matches a Y_2 -receptor profile.

The question remains whether the electrophysiological response to NPY we observed here relates to feeding *in vivo*. The PVN appears to be an important link in the pathway responsible for the regulation of natural eating behavior (Stanley, 1993), and NPY appears to be important in this pathway for several reasons. 1) The parvocellular region of the PVN receives substantial NPY innervation from cells of the arcuate nucleus (Bai et al., 1985; Chronwall et al., 1985), NPY is more concentrated in the parvocellular than in the magnocellular region (Jhanwar-Uniyal et al., 1993), and elevations in NPY levels at the beginning of the natural feeding cycle as well as after periods of food deprivation are restricted to the parvocellular region of the PVN (Beck et al., 1990; Jhanwar-Uniyal et al., 1990). 2) Leptin, the obese gene product whose mutant inactivity results in gross obesity (Zhang et al., 1994), has recently been reported to inhibit activity of the NPYergic arcuate nucleus neurons which innervate PVN (Glaum et al., 1996; Rhim et al., 1997). This suggests that uncontrolled NPY release in the parvocellular region of the PVN can result in significant obesity. This hypothesis was supported in part by the reduced weight gain observed in crosses of NPY-knockout mice with obese, *ob/ob* mice (Erickson et al., 1996), by the reduction in natural feeding observed in animals treated with antisense oligonucleotides directed against NPY (Akabayashi et al., 1994), and recent reports of the roles of Y_1 - and Y_5 -receptors in feeding (Pedrazzini et al., 1998, Marsh et al., 1998). However, the definitive experiments, using selective antagonists to all the NPY receptors, remain to be done.

In summary, our results suggest that several NPY receptors mediate very similar responses in the PVN in an apparently parallel fashion, with a response profile broadly similar to that for stimulating appetite. It is tempting to speculate from this that the natural ligand, NPY, or related ligands that activate multiple NPY receptors with high affinity, are the most active at eliciting the feeding response precisely because they act at more than one NPY receptor. Finally, microinjection experiments (Rattan and Mangat, 1990, Baldwin et al., 1990) have shown that inhibition of the PVN results in feeding, i.e., that activation of the PVN may restrain feeding. While it appears that NPY in fact activates neurons of the PVN, it is tempting to speculate that the PVN neurons indirectly activated by NPY make inhibitory connections onto other neurons in or near the PVN, that inhibition of these downstream neurons may elicit eating behavior.

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CHAPTER 5

Structure-Activity Relationships with Neuropeptide Y Analogues: a Comparison of Human Y₁, Y₂ and Rat Y₂-like Systems

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Keywords:

Neuropeptide Y analogues, rat jejunum mucosa, SK-N-MC, SMS-KAN, structure-activity relationships

Abbreviations:

CF, 4(5)-carboxyfluorescein

CFSE, 4(5)-carboxyfluorescein-N-succinimidylester

DIC, *N,N'*-diisopropylcarbodiimide

DMF, *N,N*-dimethylformamide

FITC, 4(5)-fluorescein isothiocyanate

HOBt, 1-hydroxy-benzotriazole

5.1 Summary

A structure-activity study utilizing 36 synthetic Ala-analogues of the 36-residue oligopeptide neuropeptide Y (NPY) has been performed with mucosal preparations from the rat jejunum (Y_2 -like receptor) and compared with receptor displacement binding in the human neuroblastoma cell lines, SMS-KAN, (Y_2 -receptors) and SK-N-MC cells (Y_1 -receptors). Each amino acid of the natural sequence was replaced by L-alanine, and the four intrinsic alanine residues at position 12, 14, 18 and 23 were replaced by glycine. The purified peptides were characterized by electrospray mass spectrometry, analytical HPLC and amino acid analysis. Binding was investigated using membranes prepared from either SMS-KAN or SK-N-MC cells. The activity of each Ala-NPY analogue was assessed in mucosal preparations of rat jejunum, where NPY and PYY exert antisecretory responses which are Y_2 -like in pharmacology. Fourteen analogues with L-alanine replacements at position 3, 5, 8, 13, 20, 21, 22, 26, 27, 28, 29, 30, 34 and 36 were selected, none of which exhibited any antagonism of NPY responses. An order of agonist potency showed [Ala³] NPY and [Ala³⁰] NPY equipotent with NPY, a 4 - 20-fold loss of activity with [Ala⁵] NPY, [Ala¹³] NPY, [Ala²⁰] NPY, [Ala²¹] NPY and [Ala²²] NPY; a 50 - 100-fold loss of activity, [Ala⁸] NPY, [Ala²⁷] NPY, [Ala²⁸] NPY and [Ala³⁶] NPY, while [Ala³⁴] NPY was inactive. This structure-activity relationship is similar to, but not the same as that observed in Y_2 -expressing SMS-KAN cells.

5.2 Introduction

Neuropeptide Y (NPY) is a 36-residue peptide amide which exhibits similarities with pancreatic polypeptide (PP) and peptide YY (PYY) in both sequence and 3D-structure [1, 2]. NPY was isolated from pig brain and sequenced in 1982 [3] and has been found to be one of the most abundant neuropeptides in the mammalian peripheral and central nervous systems. Competition binding studies using analogues and fragments of NPY initially, and later the cloning of different Y-receptors, has revealed that at least six Y-receptor types (Y₁-y₆) exist [4]. Whereas NPY shows affinity for Y₁-, Y₂-, Y₅- and y₆-receptors, PP is the preferred agonist at the Y₄-receptor (which has been called PP1 [5]).

In addition to their original identification on prejunctional membranes of the postganglionic, sympathetic neuro-effector junction, Y₂-receptors are present in renal proximal tubules, on parasympathetic neurons and erythrocytes from rats. In the CNS, particularly, the hippocampus (CA1 neurons) Y₂-receptors mediate the inhibitory effects of NPY, and Y₂-like inhibitory effects have been described upon anion secretion in the rat small intestine, bronchial smooth muscle contraction in the guinea pig (for recent reviews, see [6 - 8]). The Y₂-receptor was cloned recently [9 - 11] and shown to belong to the G-protein coupled receptor (GPCR) family [12]. Cell lines exclusively expressing the Y₂-receptor have been identified, and the common transduction mechanisms identified as an inhibition of cAMP accumulation (in LN319 astrocytoma cells [13] rat vas deferens [14] and rat jejunum epithelia [15]) or inhibition of N-type Ca²⁺ channel current amplitudes (e.g. SH-SY5Y neuroblastoma cells [16]).

While traditionally the entire NPY molecule is thought to be required for binding to the Y₁-receptor, C-terminal segments of NPY (e.g. NPY(13 - 36)) bind to the Y₂-receptor with only a slightly diminished potency compared with the full length peptide (IC₅₀ 5 - 10 fold [17]). Furthermore, using discontinuous analogues of NPY [18 - 20] and cyclopeptides, some selective Y₂-receptor agonists have been developed [21, 22].

The aim of this investigation was to assess the contribution made by each amino acid side chain of NPY, i) to receptor binding affinity in Y₁- or Y₂-expressing neuroblastoma cells, and ii) to Y₂-like receptor activation in rat jejunal

epithelia, by systematically exchanging single residues of NPY 1 - 36 with L-alanine (Ala). The four Ala residues in native NPY (at positions 12, 14, 18, and 23) were replaced by glycine (Fig. 1). The rat jejunum mucosa is a tissue known to be activated by C-terminal fragments of NPY and PYY, but is not sensitive to Pro³⁴-substituted peptides or to PP [20, 23, 24]. The agonist order of potency in this Y₂-like system has been compared with the peptide order in competition binding assays using human neuroblastoma SMS-KAN cells (Y₂) and SK-N-MC (Y₁). Preliminary functional data has been presented to the British Pharmacological Society [25, 26].

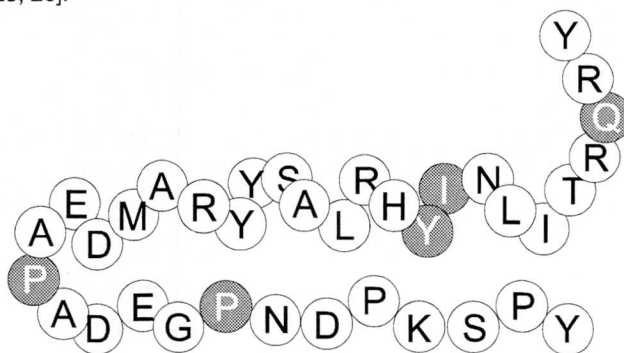


FIGURE 1: Sequence and schematic hairpin fold (the PP-fold) of NPY according to [28]. Residues, whose substitution by L-alanine lead to significant loss of biological activity are marked in dark gray, with white lettering.

5.3 Materials and Methods

5.3.1 Materials

Fmoc-protected amino acids were obtained from NovaBiochem (Läufelfingen, Switzerland), aminomethylated polystyrene from Rapp Polymere (Tübingen, Germany); diisopropylcarbodiimide and thiocresol from Aldrich; 1-hydroxybenzotriazole, trifluoroacetic acid, thioanisole and piperidine from Fluka and dimethylformamide (p. a. grade), diethyl ether, acetonitrile and tert.-butyl alcohol

from Merck. HEPES, Tris, bovine serum albumin (BSA) bacitracin, gentamycin and phenylmethylsulfonylfluoride (PMSF) were purchased from Sigma; polyethylenimine and Fmoc-aminomethyl-dimethoxyphenoxy valeric acid from Serva; sodium pyruvate, minimum essential medium with Earle's salts and Ham's F12/50 % Dulbecco's modified Eagle medium from Gibco; fetal calf serum, glutamine and non-essential amino acids were from Boehringer Mannheim. EDTA and all salts for preparing the buffers were either from Fluka or from Merck. ^{125}I -Bolton-Hunter NPY was from Anawa (Zürich, Switzerland) and specific activity was 81 TBq/mM.

5.3.2 Peptide Synthesis, Purification and Analysis

The linear peptides were synthesized by automated multiple solid phase peptide synthesis using a robot system (Syro, MultiSynTech, Bochum). In order to obtain a peptide amide, 5-(4'-aminomethyl-3',5'-dimethoxy-phenoxy)-pentanoyl-*l*-alanine-aminomethyl resin or 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin was used. The polymer matrix was polystyrene-1 %-divinylbenzene (30 mg; 15 mmol). The side chain protection was tert.-Butyl, tert.-butyloxycarbonyl, trityl and 2,2,3,5,5-pentamethyl-chromansulfonyl. Double coupling procedures were performed with diisopropylcarbodiimide/1-hydroxy-benzotriazole activation, 10-fold excess and a coupling time of 40 min. The peptide amides were cleaved with trifluoroacetic acid/thioanisole/thiocresol within 2 h, collected by centrifugation and lyophilized from water/tert.-butyl alcohol (1:1). The peptides were purified to homogeneity by preparative HPLC on a nucleosil C-18 column 5, 25 x 250 mm (Grom, Ammerbuch) using a Waters 590 system and a flow rate of 28 mL/min. Isocratic conditions varied from 30 % acetonitrile to 37 % acetonitrile in water/trifluoroacetic acid (100:0.1) for the different analogs. The peptides were analyzed by analytical HPLC (Waters 610), electrospray mass spectrometry (API III, Sciex, Toronto) and analytical HPLC and showed that identity was > 95 % for each peptide.

5.3.3 Membrane Preparation and Receptor Binding

SK-N-MC and SMS-KAN-cells were grown in 50 % nutrient mixture Ham's F12/50 % Dulbecco's modified Eagle medium with 15 % fetal calf serum, 2 mM glutamine, non-essential amino acids, 1 % gentamycin at 37°C and 5 % CO₂ until reaching confluence. After removal of growth medium, the cells were washed twice with 50 mM Tris-HCl, pH 7.5. Tris buffer with inhibitors (0.1 % bacitracin and 50 mM Pefabloc SC) is added and the cells removed with a rubber policeman. The cell suspension is homogenized for 10 s with an Ultraturrax, 15 times potted with speed maximum. After centrifugation at 4°C, 10 min, 820 x g, the supernatant was decanted and centrifuged at 4°C, 30 min, 37 000 x g. The resulting pellet was resuspended in 30 ml HEPES buffer (25 mM HEPES, 2.5 mM CaCl₂, 1 mM MgCl₂, pH 7.4) 0.1 % bacitracin, 50 mM Pefabloc SC, homogenized, recentrifuged at 4°C, 30min, 37000 x g and the pellet is resuspended in HEPES buffer without protease inhibitors. An aliquot was used to determine protein concentration and inhibitors were added accordingly. The suspension was resuspended 5 times with a syringe before freezing and aliquots were stored at -80°C. Binding assays were performed as reported previously [24, 25]. In brief, the SMS-KAN membranes were diluted in incubation buffer (MEM/25 mM HEPES, 1% BSA, 50 mM Pefabloc SC, 0.1% bacitracin, 3.75 mM CaCl₂). 200 µl of membrane suspension (containing 20 µg protein) was incubated with 25 µl 1.2 nM ³H-propionyl-NPY (3.18 TBq/mmol) and 25 µl of increasing analogue concentrations (final volume, 250 µl). After 3 h at room temperature the incubation was terminated by centrifugation and pellets were washed and resuspended in PBS, mixed with scintillation fluid and radioactivity was determined. Nonspecific binding was defined in the presence of 1 µM NPY.

5.3.4 Voltage - Clamp Studies with Rat Jejunum Mucosa

Male Sprague-Dawley rats (200 - 300g) were killed and a 6 - 8 cm length of jejunum, removed and placed in oxygenated Krebs-Henseleit (KH) buffer (containing, in mM: NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0 and glucose 11.1). The longitudinal and most of the circular smooth muscle were removed from the underlying mucosae by blunt dissection under a

microscope, as described previously [24]. Mucosal preparations (exposed area, 0.64 cm²) were placed between two halves of Perspex Ussing chambers and bathed in oxygenated (95% O₂ / 5 % CO₂) KH solution (15 ml each side) at 37°C, pH 7.4. These mucosal preparations were voltage-clamped at zero potential (WP Instruments, Sarasota, FL, U.S.A.) and allowed to stabilize for at least 30 min. The short-circuit current (s.c.c.) was monitored continuously on pen recorders throughout. All peptides were added to the basolateral surface and resultant changes in s.c.c. were converted to $\mu\text{A}/\text{cm}^2$. Concentration-response profiles were obtained cumulatively, increasing peptide concentrations being added without intermediate washing. Each peptide response curve was analyzed using the iterative curve-fitting program, Graphpad Prism (version 2.0, Graphpad Software Inc, San Diego, U.S.A.). EC₅₀ values were calculated for each preparation and values were then pooled (mean \pm 1 s.e.m.) within particular groups. Single concentration responses were compared with those of respective NPY (human or porcine, both at 300 nM) and also to the preferred agonist, PYY (100 nM) by one-way ANOVA with Newman-Keuls post testing (significant differences were observed when $p < 0.05$).

5.4 Results

None of the 36 Ala-NPY analogues tested inhibited subsequent NPY, PYY or clonidine responses instead they all, to varying degrees, induced reductions in s.c.c. like NPY (Figs. 2 & 3). A single addition of each Ala-analogue of NPY (300 nM) was made to the basolateral reservoir of mucosal preparations and the resultant reductions in s.c.c. compared with control responses of either human or porcine NPY (both at 300 nM) or PYY (100 nM, data shown in Fig. 2). Subsequent addition of the α_2 -adrenoceptor agonist, clonidine further reduced s.c.c. levels and were not significantly altered when compared with controls in the absence of NPY (data not shown). All of the 36 analogues tested were less potent than NPY (and PYY). One way ANOVA analysis showed statistical reductions in response size compared with PYY (Fig. 2) for Ala-substitutions at positions; 12, 13, 14, 15, 16 and 17 while replacements at position 33, 34, 35 or 36 reduced biological activity even further (to less than 20 % of control responses). [Ala²⁰] NPY, [Ala²²] NPY, [Ala²³] NPY, [Ala²⁷] NPY, [Ala²⁹] NPY and [Ala³²] NPY stimulated responses that were between 20 - 30% of controls. Furthermore, activity levels of 30 - 40% were observed with Ala-replacement at positions 8, 9, 18, 25 and 28. Between 40 - 50% active peptides were those substituted at residues 2, 5, 7, 11, 21, and 26, but none of these changes were significantly different from control NPY responses.

The affinity of each Ala-analogue was tested in binding assays with membranes from the Y1-expressing cell line, SK-N-MC and the Y2-receptor line, SMS-KAN and these results are shown in Table 1 [27] and can be compared with their respective EC₅₀ values in rat jejunum mucosal preparations. Full concentration-response curves (see Fig. 3, where six analogues are shown compared with their respective control NPY curves) were generated for 14/36 Ala-analogues and this pooled data yielded the calculated EC₅₀ values listed in Table 1. In summary, [Ala³] NPY and [Ala³⁰] NPY were equipotent with NPY while a 4 - 20-fold loss of activity was found for [Ala⁵] NPY, [Ala¹³] NPY, [Ala²⁰] NPY, [Ala²¹] NPY, [Ala²²] NPY and [Ala²⁶] NPY. [Ala⁸] NPY, [Ala²⁷] NPY, [Ala²⁸] NPY, [Ala²⁹] NPY and [Ala³⁶] NPY showed a 50-100 fold loss of activity while [Ala³⁴] NPY was predictably, inactive.

5.5 Discussion

Comparing the relative activities of these Ala-NPY analogues with their affinities for either hY₁- or hY₂-receptors (Table 1) shows a greater similarity with the latter receptor type. The Y₁-receptor binding classically exhibits a preference for full length NPY and the significant loss of affinity we observed for Ala-analogues substituted at positions 3, 5, 20, 30 and 36, differed substantially from the functional order in the mucosa. This observation is broadly in agreement with previous studies, which have shown a Y₂-like pharmacological profile in the rat jejunum mucosa [23 - 26]. The EC₅₀ and IC₅₀ orders in jejunum and SMS-KAN cells respectively, were similar for Ala-replacements at positions: 3, 5, 20, 21, 22, 26 and 30. [Ala⁸] NPY, [Ala¹³] NPY, [Ala²⁷] NPY and [Ala²⁸] NPY were 10 to 30 times more potent in binding assays compared with their functional potency.

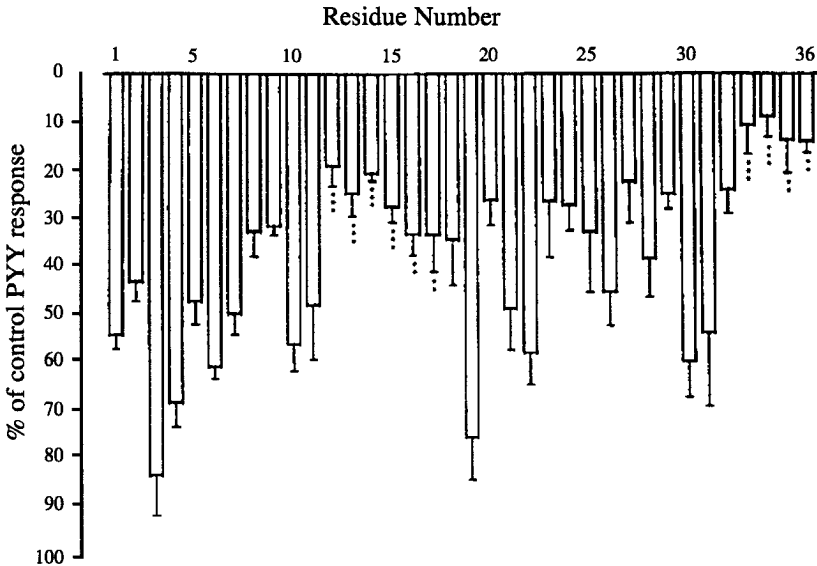


FIGURE 2: Responses to single addition of 300 nM Ala-substituted NPY peptides in rat jejunum mucosal preparations. PYY responses (100 nM) were denoted as 100 % and all other responses calculated as a % of this value from each tissue. Native pNPY and hNPY responses were 57 % and 73 % respectively.

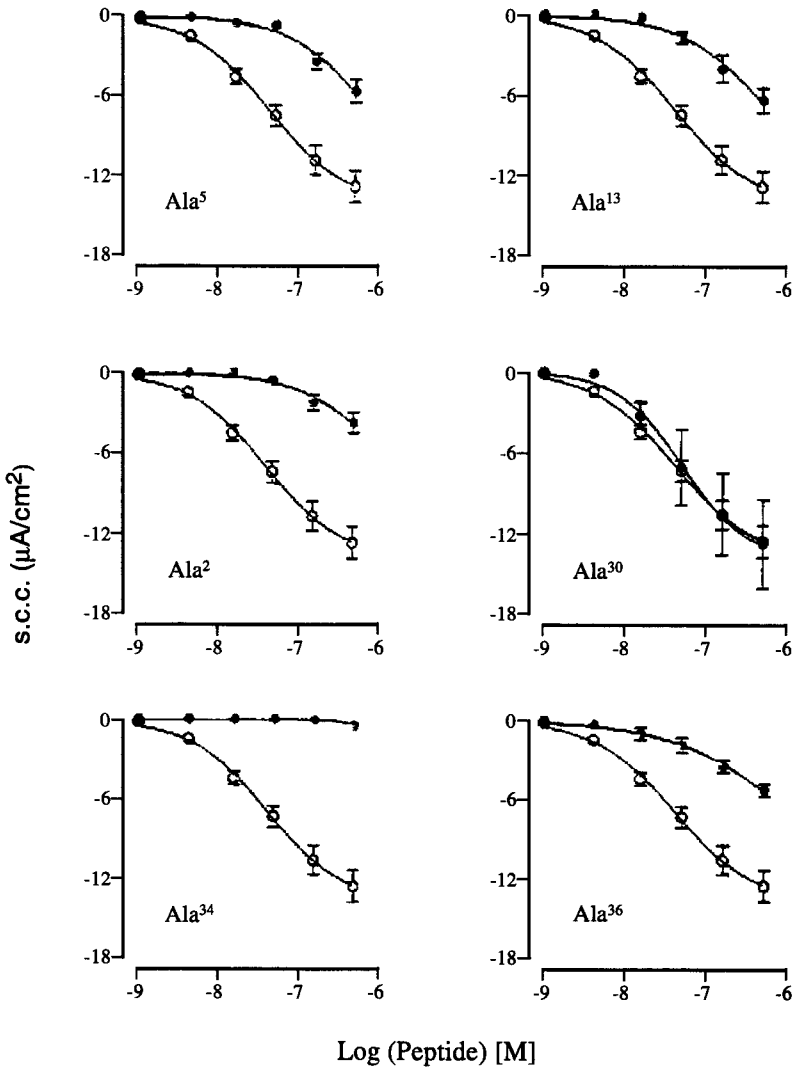


FIGURE 3: Concentration-response curves for six of the fourteen Ala-analogues tested in mucosal preparations from the rat jejunum. Peptide additions were made cumulatively and only to the basolateral reservoir. Subsequent data was pooled to generate the curves shown, and from which EC_{50} values were calculated and listed in Table 1.

Because of the high peptide concentrations required to estimate the maximal responses for some of these agonists we are assuming that they are full agonists (e.g. [Ala⁸] NPY, [Ala²⁷] NPY, [Ala²⁸] NPY, [Ala²⁹] NPY and [Ala³⁶] NPY) there being no indication that they attenuate subsequent NPY responses. [Ala³⁴] NPY was a better tolerated substitution in binding to Y₂-receptors than in functional studies, where it was inactive in the jejunum mucosa, in agreement with previous studies with [Pro³⁴] NPY [20].

Notwithstanding the differences likely to exist between the same receptor types expressed in different species, our data confirm the importance of the C-terminal portion of NPY not only for binding to the Y₁- and Y₂-receptor in human neuroblastoma cell lines, but especially for receptor activation in the rat mucosal Y₂-like assay. Furthermore, we found that the replacement of Pro⁸, Pro¹³, Tyr²⁷, Ile²⁸ and Asn²⁹ results in analogues with high Y₂-receptor affinity, but only 'partial' activity (at the concentrations tested). While the role of Pro⁵ and Pro⁸ is suggested to provide interdigitating hydrophobic side chains with the C-terminal α -helix, Pro¹³ could be the start of the C-terminal α -helical segment of the peptide and together their substitution by Ala resulted in profound loss of biological activity in the Y₂-like assay. The three proline residues have an indirect influence, stabilizing the PP-fold *i.e.* the optimal bioactive conformation necessary for receptor activation ([21] based on the segment 25 to 36).

Previous studies have shown that of the Y₂-receptor binding cyclopeptide NPY analogues, neither Tyr²⁷ nor Ile²⁸ appear to be directly involved in binding. They do however, exert structural constraints such that bridging at position 27 led to a reduction in affinity of three orders of magnitude [22]. Accordingly, this segment of peptide sequence seems to be very sensitive to conformational changes and potentially, the less hydrophobic side chain of alanine (compared with tyrosine or isoleucine) is not sufficient to stabilize the bioactive conformation. Ala-substitution in this region resulted in loss of activity and in Y₁-receptor affinity. In summary the Ala-scan functional data from the rat jejunum more closely resembles the order of potency exhibited by the Y₂-expressing neuroblastoma cell line but there were anomalies, some of which may arise from species differences.

Table 1: Comparison of the IC_{50} and EC_{50} values calculated for different Ala-substituted NPY analogues in three preparations

Peptide	SK-N-MC Y ₁ IC ₅₀	SMS-KAN Y ₂ IC ₅₀	Rat Jejunum Y ₂ -like EC ₅₀	Relative affinity Y ₁ (binding)	Relative affinity Y ₂ (binding)	Relative potency
NPY	0.2	0.08	37.4	1.0	1.0	1.0
[Ala ³] NPY	7.5	0.29	52.9	37.5	3.6	1.4
[Ala ⁵] NPY	228.0	24.0	600.0	1100	300.0	16.0
[Ala ⁸] NPY	31.8	0.75	2000	160.0	9.4	53.5
[Ala ¹³] NPY	7.5	0.07	630.0	375.0	0.9	16.8
[Ala ²⁰] NPY	71.0	1.2	275.0	35.0	15.0	7.4
[Ala ²¹] NPY	5.5	0.24	142.8	27.5	3.0	3.8
[Ala ²²] NPY	3.5	0.25	155.2	17.5	3.1	4.2
[Ala ²⁶] NPY	1.9	0.1	118.3	9.5	1.3	3.2
[Ala ²⁷] NPY	250.0	1.42	1700	1300	17.8	45.5
[Ala ²⁸] NPY	63.5	0.12	1000	320.0	1.5	26.7
[Ala ²⁹] NPY	58.0	1.4	1000	290.0	17.5	26.7
[Ala ³⁰] NPY	38.0	0.1	36.3	200.0	1.3	1.0
[Ala ³⁴] NPY	65.0	6.0	10000	325.0	75.0	> 270.0
[Ala ³⁶] NPY	970.0	780.0	1200	5000	9750	32.1

Values shown in the first three columns are in nM

The rat Y₂-like receptor appears to require a greater proportion of the N-terminal peptide tail and the β-tum (both features characteristic of a Y₁- system) than the human Y₂-receptor, however substitution of the native glutamine at position 34 renders the peptide inactive in the jejunum mucosa. Future functional and binding studies with Ala-substitutions of peptide YY (more likely to be the natural Y₂ ligand at peripheral targets) in human and rat tissues will provide a more complete picture of the subtle differences that may exist between the Y₁- and Y₂-receptor types in these two species.

Acknowledgments

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CHAPTER 6

Characterization of the Human Y_2 -Receptor with Non-Radioactive Approaches

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Abbreviations:

AP, alkaline phosphatase

SA, streptavidin

SDS, sodium dodecyl sulfate

PAGE, polyacrylamide gel electrophoresis

6.1 Introduction

Specific detection of proteins on blots after separation by SDS-PAGE can either be achieved by immunodetection or by introduction of a specific label into the protein of interest by photoaffinity labeling. Autoradiography of radioactive labeled ligands is the most popular detection method used in photoaffinity labeling studies as it provides high sensitivity to detect very small amounts of labeled protein.

Immunodetection of proteins on blots has become a broadly applied detection method after its introduction and is based on specific antibodies and enzyme linked secondary antibodies. The detection reaction catalyzed by the enzyme induces the signal by converting either a staining or a chemiluminescence substrate. Especially chemiluminescence detection is highly sensitive as the chemiluminescence persists up to days, depending on the enzyme and substrate used.

Our aim was to investigate the binding site of neuropeptide Y (NPY) at the Y_2 -receptor by photoaffinity labeling. Neuropeptide Y (NPY) is a 36 amino acid peptide hormone and belongs to the pancreatic polypeptide family [1]. It exhibits several actions on the cardiovascular and the central nervous system [2], which are mediated by at least five receptor subtypes named Y_1 , Y_2 , Y_4 , Y_5 and Y_6 [3]. As the expression rate of NPY receptor subtypes is low and photocrosslinking only introduces a single label per receptor with a probability of 2 to 20 %, ^{125}I -labeled photoactivatable NPY analogues have been required so far.

6.2 Results and Discussion

To overcome the disadvantages of the application of radioactive markers in photoaffinity labeling procedures, we used biotinylated ligand analogues, like $[\text{N}_\alpha\text{-biotinyl-Ahx}_2(\text{Tmd})\text{Phe}^{36}]$ NPY or $[\text{N}_\alpha\text{-biotinyl-Ahx}_2\text{Bpa}^{20}]$ NPY. Detection sensitivity after SDS-PAGE and electroblotting was not sufficient by classical approaches that used alkaline phosphatase (AP) conjugated streptavidin (SA), because femtomolar amounts of biotin had to be detected. Therefore we amplified the signal by subsequent incubation with streptavidin-AP, biotin-AP and AP-conjugated anti-biotin-antibodies, which all are commercially available. The first

step is the binding of streptavidin to the biotinylated ligand-receptor-complex. As streptavidin is able to bind four biotin molecules, there will be unoccupied binding sites available after incubation, if streptavidin is added in excess. In the second step these sites are able to bind biotinylated alkaline phosphatase, which is labeled with three to six biotin residues. Therefore, there are again unoccupied biotin residues after the second incubation, which can be targeted with anti-biotin-antibodies. Accordingly, a single ligand-receptor-complex is conjugated to up to twenty molecules of alkaline phosphatase. This signal amplification increases detection sensitivity significantly and allowed the detection of our ligand-receptor complex. In addition, sensitivity may be further increased using chemiluminescent dyes for staining. We found, that the signal amplification system is based on inexpensive, commercially available compounds and can be used in immunoprobng as well.

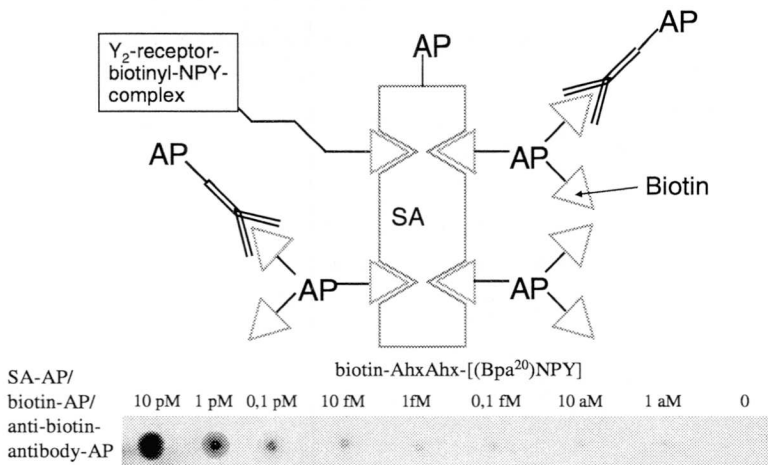


FIGURE 1: Scheme of detection of the receptor-ligand complex and dot-blot detection: SA streptavidin, AP, alkaline phosphatase.

A method for the identification of protein bands in the sub-femtogram range after electrophoresis and electroblotting is described. This method is based on biotin-labeled proteins and does not require radioactive markers. The biotin-label can be introduced either by photoaffinity labeling with biotinylated ligand analogues or

immunodetection with biotinylated antibodies. A signal amplification system based on alkaline phosphatase labeled streptavidin, biotin and anti-biotin antibodies is used to increase sensitivity and detection is performed either by a staining- or a chemiluminescence-reaction.

6.3 References

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CHAPTER 7

Molecular Characterization of the Human Neuropeptide Y Y₂-Receptor

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Abbreviations:

Abbreviations: Ahx, 6-aminohexanoic acid; DATD, *N,N'*-diallyltartardiamide; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl-; ^3H -Tmd27, [N_α -biotinyl-Ahx₂, ^3H -propionyl-Lys⁴, Ahx⁵⁻²⁴, (Tmd)Phe²⁷] NPY; ^3H -Tmd36, [N_α -biotinyl-Ahx₂, ^3H -propionyl-Lys⁴, (Tmd)Phe³⁶] NPY; KLH, keyhole limpet hemocyanin; NPY, neuropeptide Y; (Tmd)Phe, 4-(3-trifluoromethyl)-3*H*-diazirin-3-yl-phenylalanine; OSu, N-hydroxysuccinimide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; PBS, phosphate buffered saline; TBS, Tris buffered saline; Tmd27, [N_α -biotinyl-Ahx₂, Ahx⁵⁻²⁴, (Tmd)Phe²⁷] NPY; Tmd36, [N_α -biotinyl-Ahx₂, (Tmd)Phe³⁶] NPY; Tris, tris(hydroxymethyl)amino-methane; Tween 20, polyoxyethylene-sorbitan monolaurate.

7.1 Abstract

Five neuropeptide Y receptors, the Y_1 -, Y_2 -, Y_4 -, Y_5 - and y_6 -subtypes have been cloned, which belong to the rhodopsin-like G-protein coupled, 7 transmembrane helix-spanning receptors and bind the 36-mer neuromodulator NPY (neuropeptide Y) with nanomolar affinity. In this study, the Y_2 -receptor subtype expressed in a human neuroblastoma cell line (SMS-KAN) and in transfected Chinese hamster ovary cells (CHO-Y2) was characterized on the protein level by using photoaffinity labeling and anti-receptor antibodies. Two photoactivatable analogues of NPY were synthesized, in which a Tyr residue was substituted by the photoreactive amino acid 4-(3-trifluoromethyl)-3H-diazirin-3-yl-phenylalanine ((Tmd)Phe): [N_α -biotinyl-Ahx₂, (Tmd)Phe³⁶] NPY (Tmd36) and the Y_2 -receptor subtype selective [N_α -biotinyl-Ahx₂, Ahx⁵⁻²⁴, (Tmd)Phe²⁷] NPY (Tmd27). Both analogues were labeled with ³H-succinimidyl-propionate at Lys⁴ and bind to the Y_2 -receptor with affinity similar to the native ligand.

A synthetic fragment of the second (E2) extracellular loop was used to generate subtype selective anti-receptor antibodies against the Y_2 -receptor. Photoaffinity labeling of the receptor followed by SDS-PAGE and detection of bound radioactivity and SDS-PAGE of solubilized receptors and subsequent Western blotting revealed the same molecular masses. Two proteins correspondingly have been detected for each cell line with molecular masses of 58 ± 4 kDa and 50 ± 4 kDa, respectively.

7.2 Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide that is widely distributed both, peripherally as well as centrally. Similar to many other neurotransmitters NPY elicits diverse physiological effects, e. g. induction of food intake and potent vasoconstriction (1-4). Accordingly, NPY is a neuronal and endocrine messenger that exerts its effects via several receptor subtypes of which the so-called Y_1 - (5-7), Y_2 - (8-10), Y_4 /PP₁- (11, 12), Y_5 - (13) and y_6 -receptor (14) have been cloned (15). Sequence comparisons show that the receptors Y_1 , Y_4 , and y_6 are more closely related to each other than to the receptors Y_2 and Y_5 . 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. While their distribution appears to be species specific, all subtypes belong to the large superfamily of G-protein-coupled, heptahelical receptors (16). The Y_2 -receptor is the predominant NPY receptor subtype in the brain and particularly abundant in the hippocampus (17). The cloned Y_2 -receptor consists of 381 amino acids (10) and has the typical heptahelix receptor features including a potential glycosylation site in the amino-terminal part, two extracellular cysteins that may form a disulfide loop, and a single cysteine in the cytoplasmatic tail that probably serves as an attachment site for palmitate.

Characterization of the receptor subtypes has been restricted so far to pharmacological experiments and investigations on the mRNA level. Because of possible post-translational modifications like glycosylation and palmitoylation, characterization on the protein level is necessary. A receptor subtype identified on the mRNA level may or may not be functionally expressed. Moreover, since rapid axonal transport has been suggested for the Y_1 -receptor (18) mRNA localization must not necessarily fit with the localization of the mature protein. Characterization and localization of the protein can be achieved by photoaffinity labeling and immunodetection. The first method, photoaffinity labeling, has been widely used for the identification of binding sites in different receptor systems (19-21). The use of this method for determination of molecular masses depends on specific labeling of the receptor. The problem of unspecific labeling may be overcome by constructing a ligand with a built-in photoreactive amino acid that generates a highly reactive, short-living species, for example a carbene (21, 22). To increase the chance of productive coupling reactions, the photoactivatable amino acid should be placed near or within the binding site. To reach maximal specificity we compared the results of the photoaffinity labeling by using two different ^3H -propionylated analogues of NPY (Fig. 1) with built-in photoreactive amino acids: [N_α -biotinyl-Ahx₂, ^3H -propionyl-Lys⁴, (Tmd)Phe³⁶] NPY (^3H -Tmd36) and [N_α -biotinyl-Ahx₂, ^3H -propionyl-Lys⁴, Ahx⁵⁻²⁴, (Tmd)Phe²⁷] NPY (^3H -Tmd27). Centrally truncated NPY analogues like [Ahx⁵⁻²⁴] NPY and Tmd27 additionally have been shown to be selective for the Y_2 -receptor subtype (23).

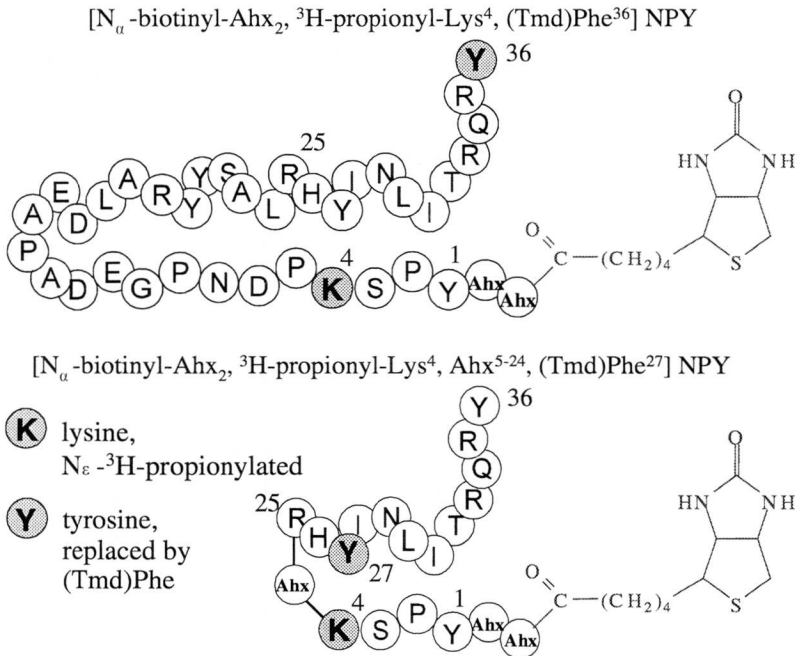


FIGURE 1: Sequence and modified positions of the photoactivatable NPY analogues.

Second, various studies have shown that antibodies produced against hormone receptors are valuable tools (24-27). Anti-receptor antisera can be raised by immunization with purified, enriched receptors (24) or by immunization with receptor fragments (25-27). Molecular mass determination by SDS-PAGE and subsequent Western blotting, receptor purification by affinity chromatography on antibody-columns (26) and investigations of the receptor localization (28) are only a few applications of anti-receptor-antibodies (29). Antibodies specifically recognizing the NPY receptor subtypes could be used to determine the localization pattern and the quantity of the receptor protein expression as well as to determine the topology and function of a receptor subtype. In this study, we compared the molecular masses of human neuropeptide Y Y₂-receptors expressed endogenously in a human neuroblastoma cell line (SMS-KAN) and in Chinese hamster ovary cells (CHO-Y2) that have been transfected with human

Y₂-receptor cDNA (10). To increase the reliability of the results molecular masses are determined by two independent methods, photoaffinity labeling and immunodetection.

7.3 Experimental Procedures

7.3.1 Peptide Synthesis

All peptides were prepared by solid phase synthesis using the Fmoc-strategy (Fmoc, 9-fluorenylmethoxycarbonyl-) on a robot system (Syro, MultisynTech, Bochum) (30). In order to obtain peptide amides, 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin was used for anchoring. The polymer matrix was polystyrene-1 %-divinylbenzene (30 mg; 15 μmol). L-H-(Tmd)Phe-OH was prepared as described previously (31) and N-terminally protected with Fmoc-OSu (OSu = N-hydroxysuccinimide) according to (32). Cleavage of the peptide amides from the resin was achieved with trifluoroacetic acid/thioanisole/thiocresol within 2 h. Cleaved peptides were collected by centrifugation and lyophilized from water. All proceedings including (Tmd)Phe have been performed in the absence of light. Characterization was achieved by reversed-phase-HPLC (column C-18, 3 × 125 mm, 5 μ, flow 0.6 ml/min, gradient: 25 % acetonitrile to 75 % acetonitrile in water/trifluoroacetic acid (100:0.1) within 30 min, Tmd27: 5 % acetonitrile to 50 % acetonitrile in water/trifluoroacetic acid (100:0.1) within 30 min) and electrospray mass spectrometry (SSQ710, Finnigan, San Jose, CA). In order to test the photochemical properties, photoactivatable peptides were irradiated for 10 min with light of 366 nm and re-investigated by HPLC using the same gradient.

Tmd27: retention: 20,85 min; retention after irradiation: 18,11; mass^{calc.}: 2763,5 amu; mass^{exp.}: 2763,8 amu.

Tmd36: retention: 12,40 min; retention after irradiation: 11,29; mass^{calc.}: 4795,37 amu; mass^{exp.}: 4795,80 amu.

Synthetic fragment of second extracellular loop:

Sequence: IFREYSLIEIIPDFEIVAF; retention: 17,3 min; mass^{calc.}: 2313,7 amu; mass^{exp.}: 2314,0 amu.

7.3.2 Peptide Modification

Tmd27 and Tmd36 were labeled with ³H-succinimidyl-propionate (3,59 TBq/mmol; Amersham, Switzerland) at Lys⁴. For each analogue 1 ml of ³H-succinimidyl-propionate solution (37 MBq) in toluene was dried in a nitrogen stream and the analogue was added equimolar in 10 μ l DMF. After addition of 0,1 % DIPEA in DMF the mixture was incubated for 20 h at darkness and room temperature. The complete reaction mixture was diluted in 1 ml 10 % acetonitrile/0.1 % trifluoroacetic acid and loaded onto a handpacked C-18-column (0,5 cm³ bed volume), followed by washing with 5 ml 10 % acetonitrile/0.1 % trifluoroacetic acid. The peptides were eluted with 1 ml 60 % acetonitrile/0.1 % trifluoroacetic acid. Specific activity of ³H-Tmd27 and ³H-Tmd36 was 3,0 and 2,8 TBq/mmol, respectively.

7.3.3 Membrane Preparation and Receptor Binding

Expression cloning of the human Y₂-receptor was performed as described previously (10), cultivation of SMS-KAN cells and membrane preparation was performed according to (33). Displacement of ³H-propionyl-NPY (3,18 TBq/mmol; Amersham, Switzerland) by NPY resulted in K_i = 0,67 nM for both cell lines (33). Binding of the tritiated photoactivatable analogues was assayed as follows. The membrane preparation was diluted in incubation buffer (MEM/25 mM Hepes, 1 % bovine serum albumin, 50 μ M Pefabloc SC, 0.1 % bacitracin, 3.75 mM CaCl₂). 200 μ l of the suspension containing 20 μ g protein were incubated with 25 μ l 8,3 nM solution of the analogues and 25 μ l of solutions of NPY in increasing concentrations to give a total volume of 250 μ l. After 1,5 h at room temperature, the incubation was terminated by centrifugation of the samples for 10 min at 3,000 \times g and 4 °C. The pellets were washed with PBS, resuspended in PBS, mixed with scintillation cocktail and radioactivity was determined.

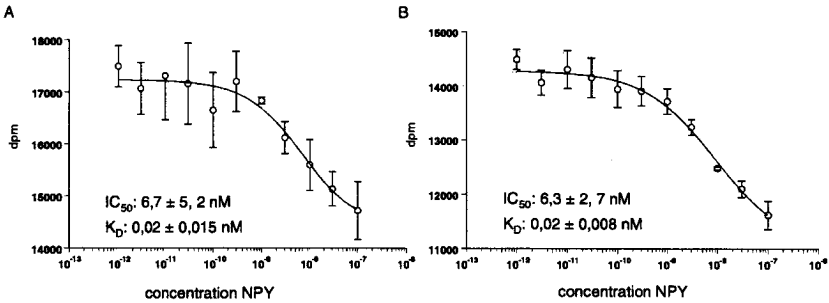


FIGURE 2: Competition assay of $[N_{\alpha}\text{-biotinyl-Ahx}_2, {}^3\text{H-propionyl-Lys}^4, \text{Ahx}^{5-24}, (\text{Tmd})\text{Phe}^{27}]$ NPY (A) and $[N_{\alpha}\text{-biotinyl-Ahx}_2, {}^3\text{H-propionyl-Lys}^4, (\text{Tmd})\text{Phe}^{36}]$ NPY (B) against NPY at CHO-Y2 membranes. For details see Experimental Procedures.

Non-specific binding was defined in the presence of $10 \mu\text{M}$ NPY. K_i^{NPY} and IC_{50} was used to determine K_D^{Analogue} (Fig. 2) according to (34). The K_D^{Analogue} values obtained were similar to K_D of ${}^3\text{H-propionyl-NPY}$ at SMS-KAN cells ($0,018 \pm 0,008 \text{ nM}$).

$[N_{\alpha}\text{-biotinyl-Ahx}_2, {}^3\text{H-propionyl-Lys}^4, \text{Ahx}^{5-24}, (\text{Tmd})\text{Phe}^{27}]$ NPY:

$K_D = 0,02 \pm 0,015 \text{ nM}$

$[N_{\alpha}\text{-biotinyl-Ahx}_2, {}^3\text{H-propionyl-Lys}^4, (\text{Tmd})\text{Phe}^{36}]$ NPY: $K_D = 0,02 \pm 0,008 \text{ nM}$

7.3.4 Photocrosslinking of Y_2 -Receptor Containing Membranes

Membranes were prepared as described above and $500 \mu\text{g}$ protein from this suspension was incubated with 10 nM solution of the photoactivatable analogue for 90 min in 2 ml binding buffer (10 mM Hepes, 150 mM NaCl, 5 mM KCl, 2,5 mM CaCl_2 , 1,3 mM K_3PO_4 , 1,2 mM MgSO_4 , 25 mM NaHCO_3 , 1 % bovine serum albumin, 50 μM Pefabloc SC, 0.1 % bacitracin, pH 7,4). A control probe was handled in parallel, but with addition of NPY in excess ($1 \mu\text{M}$). The incubation was stopped by centrifugation at $2,000 \times g$ for 5 min at $20 \text{ }^\circ\text{C}$. The resulting pellets were suspended in 2 ml cold crosslinking buffer (25 mM Hepes, 2.5 mM CaCl_2 , 1 mM MgCl_2 , 50 μM Pefabloc SC, 0.1 % bacitracin, pH 7.4) centrifuged again for 5 min, resuspended in 2 ml ice-cold crosslinking buffer and transferred to a 6-well cell culture plate on ice. The samples were irradiated for 20 min using a 180 W

high-pressure mercury lamp (366 nm). The membranes were pelleted and washed 3 times with 1 ml of cold binding buffer, dissolved in SDS sample buffer (10 % glycerol, 2.3 % SDS, 1.5 % mercaptoethanol, 30 mM Tris pH 6.8, 0.1 % bromophenol blue, 8 M Urea), treated at 95 °C for 5 min and subjected to SDS-PAGE (12 % polyacrylamide gel,) according to the procedure of Laemmli (35) with *N,N'*-diallyltartardiamide (DATD) as crosslinker (36). The gels were cut into 1,3 mm stripes, incubated in 2 % periodic acid for 60 min at 37 °C with shaking, resuspended in liquid scintillation cocktail and radioactivity was measured.

7.3.5 Preparation of the Conjugates and Immunization

For immunization the peptide from the second extracellular loop (Sequence: IFREYSLIEIIPDFEIVAF) was coupled to keyhole limpet hemocyanin (KLH) (Biotrend, Cologne, Germany). Polyclonal antibodies against the KLH-coupled receptor fragment were obtained by immunization of rabbits (Biotrend, Cologne, Germany). Antibodies were tested for binding and specificity by ELISA and Western blotting (37) and were found to be Y_2 -receptor subtype selective.

7.3.6 Immunoblot

Membranes from SMS-KAN- and CHO-Y2 cells were prepared by incubation at 95°C for 7 min in an urea sample buffer (8 M urea, 63 mM Tris/HCl pH 6.8, 2 % SDS, 5 % mercaptoethanol, 0.01 % bromophenol blue) and were separated on a 12 % polyacrylamide gel according to Lämmlí (35). After blotting for 60 min (350 mA/20 V) in a semi-dry electrophoretic transfer cell (Biorad) onto nitrocellulose membrane in a transfer buffer containing 25 mM Tris/HCl pH 8.3, 150 mM glycine, 20 % methanol, the blots were blocked over night with TBS/Tween (0.5 % Tween 20 in TBS/HCl pH 7.4) containing 1 % BSA. The blots were incubated with antibodies for 90 min with gentle shaking, washed three times with TBS/Tween, incubated for 60 min with alkaline phosphatase-bound secondary antibodies, and washed three times again. Immunoreactivity was detected with 5-bromo-4-chloro-indolyl-phosphate (Sigma, Buchs, Switzerland).

7.4 Results

7.4.1 Synthesis and Analysis of Photoactivatable NPY Analogues

Since NPY-analogues, containing Phe but not Trp are still recognized at the hY_2 -receptor (38), we decided to use (Tmd)Phe instead of other photoactivatable amino acid of larger size as for example p-benzoylphenylalanine, which previously has been used for efficient photocrosslinking of other peptides (39, 40). Fmoc-(Tmd)Phe was coupled manually in twofold excess, all the subsequent cycles were performed using a peptide synthesizer. Preparation of Fmoc-(Tmd)Phe-OH as well as all synthesis, cleavage and purification steps were carried out in the absence of light. The peptide was characterized by means of analytical HPLC and electrospray mass spectrometry. In addition, a small sample of each peptide was dissolved in water and illuminated, which caused a change in the retention time as observed by HPLC. This result confirmed that the side chain of (Tmd)Phe remained intact during the remaining cycles of peptide synthesis after coupling of (Tmd)Phe. Tmd27 and Tmd36 were labeled successfully with ^3H -succinimidyl-propionate. Both peptides contained a single reactive amino group (sidechain of Lys⁴) which facilitated the coupling of a single label per peptide. The labeled peptides, ^3H -Tmd27 and ^3H -Tmd36, showed a high specific activity of 3,0 and 2,8 TBq/mmol, respectively, which suggests a labeling efficiency of at least 90 %.

The binding of both NPY analogues to the Y_2 -receptor was determined in a competition assay against NPY using the labeled analogues as radioactive tracer (Fig. 2). Although both peptides were modified at three positions (N-terminal spacing and biotinylation, propionylation at Lys⁴ and exchange of Tyr against (Tmd)Phe in a single position), their binding properties at the Y_2 -receptor were only slightly reduced compared to ^3H -propionyl-NPY used in the binding assay (^3H -propionyl-NPY: $K_D = 0,018 \pm 0,008$ nM; ^3H -Tmd27: $K_D = 0,02 \pm 0,015$ nM; ^3H -Tmd36: $K_D = 0,02 \pm 0,008$ nM).

7.4.2 Photoaffinity Labeling

SMS-KAN- and CHO-Y2 membranes were crosslinked using the labeled photoactivatable NPY analogues ^3H -Tmd27 and ^3H -Tmd36. For each crosslinking experiment a probe was handled in parallel with addition of $1\ \mu\text{M}$ NPY as control. SDS-PAGE was performed subsequently, followed by cutting of the gels in 1,3 mm slices and determination of the radioactivity of each gel slice. Four sets of experiments have been performed. SMS-KAN membranes were crosslinked using ^3H -Tmd27 and ^3H -Tmd36, respectively, and the same experiments were processed with CHO-Y2 membranes. Two major radioactive protein bands (Fig. 3, Table 1) were found in all cases.

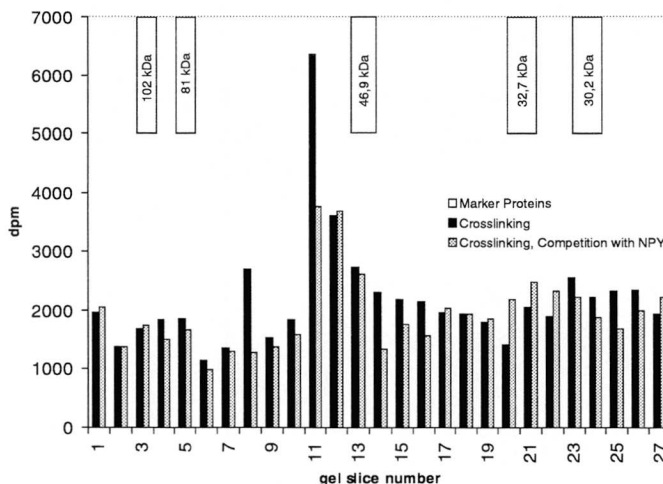


FIGURE 3: Crosslinking experiment of [$\text{N}\alpha$ -biotinyl-Ahx2, ^3H -propionyl-Lys4, Ahx5-24, (Tmd)Phe27] NPY at CHO-Y2 membranes. Control was performed with an excess of NPY. After SDS-PAGE the gel was cut into 1,3 mm slices and radioactivity was measured. A set of marker proteins was run on the same gel. The numbers of the gel slices in which the marker proteins turned up are shown as dotted bars and were used to establish a calibration curve. The curve was used to determine the molecular mass range for the proteins covered by each gel slice.

In parallel, a set of marker proteins always was run on the same gel. The numbers of the gel slices in which the marker proteins turned up were used to establish a calibration curve for each gel. The curves were used to determine the molecular mass range for the proteins covered by each gel slice. Depending on the calibration method the molecular masses obtained for the proteins varied up to 4 kDa. Molecular masses of the receptor proteins detected for SMS-KAN membranes were found to be 54 and 44 kDa using ^3H -Tmd27 for crosslinking and 53 and 44 kDa using ^3H -Tmd36. Photoaffinity labeling on CHO-Y2 membranes led to the identification of two proteins as well with molecular masses of 59 and 46 kDa with ^3H -Tmd27 and 58 and 46 kDa with ^3H -Tmd36. In some cases minor bands in the range of 30 kDa were observed which are presumed to represent partially degraded receptor proteins. Both sequence positions (Tyr²⁷ and Tyr³⁶) exchanged against (Tmd)Phe allowed insertion of the photoactivatable group into the receptor protein upon irradiation at 366 nm. Using both analogues for crosslinking of SMS-KAN membranes, the molecular masses identified by SDS-PAGE (and subtracted by the mass of the photoactivatable NPY analogue used) correspond well in both cell lines. A slight difference was found between the two cell lines for the upper mass (54 versus 58 kDa) which most likely is due to difficulties in exact weight determination by SDS-PAGE.

7.4.3 Immunoblot

Membranes from SMS-KAN- and transfected CHO-Y2 cells were solubilized and proteins separated on a gel followed by Western blotting using the Y₂-receptor selective antibodies. Again, two major protein bands were detected and the marker proteins were used to establish a calibration curve. The molecular masses observed for the Y₂-receptor expressed in SMS-KAN cells were approximately 58 kDa and 54 kDa. For CHO-Y2 membranes slightly lower masses of 57 kDa and 51 kDa were found (Fig. 5, Table 1). Depending on the calibration curve these masses varied up to 4 kDa. In analogy to the photoaffinity labeling experiments, weaker bands the range of 30-40 kDa were found, too, which are believed to represent degradation products of the receptor protein, and which are still recognized by the antibody.

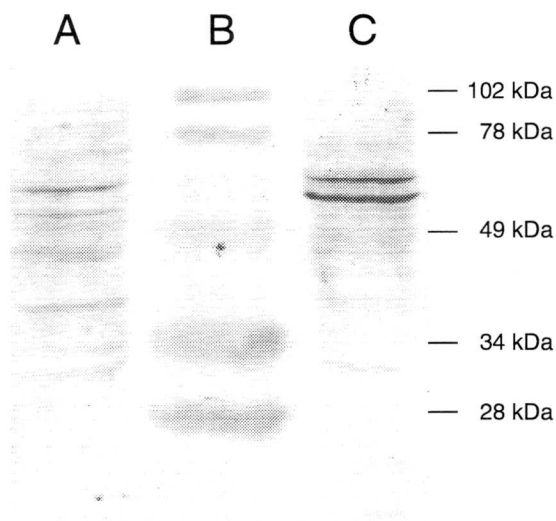


FIGURE 4: Western blot of solubilized CHO-Y2 (A) and SMS-KAN membranes (C). Molecular weight marker is shown in lane (B). Staining was achieved using Y₂-receptor specific antibodies and alkaline phosphatase conjugated secondary antibodies.

7.5 Discussion

We successfully synthesized photoactivatable tritium labeled analogues of NPY with high receptor affinity (³H-Tmd27 and ³H-Tmd36). Using these ligands, photoaffinity labeling of the NPY Y₂-receptor subtype expressed in SMS-KAN- and transfected CHO-Y2 cells identified two labeled proteins with molecular weights of 54/44 kDa and 59/46 kDa, respectively. Y₂-receptor subtype selective antibodies were raised by immunization of rabbits with a synthetic fragment of the second extracellular loop of the Y₂-receptor and subtype selectivity was shown by ELISA and Western blotting. These antibodies were used for Western blotting in order to confirm the molecular mass determination by photoaffinity labeling. Again, two proteins were identified with molecular weights of 58/54 kDa for SMS-KAN- and 57/51 kDa for transfected CHO-Y2 cells. The molecular weight determination is based on a mass calibration curve established for 5-6 marker proteins. The ratio

of the migration of the proteins in the gel and their molecular masses is not linear. Thus a curve has to be fitted to the marker weights, which might cause differences in the molecular weights of the proteins detected, depending on the mathematical model used. In addition to the inherent inaccuracy of SDS-PAGE, the masses determined for the Y_2 -receptor might vary up to 4 kDa. The masses identified by photoaffinity labeling with two different photoactivatable analogues are in good agreement for both cell lines. When compared to the mass determination via Western blotting, the upper masses identified are in good agreement for both cell lines as well, whereas the masses of the lower protein bands seem to differ slightly (Table 1).

Table 1: Molecular masses identified for the NPY Y_2 -receptor subtype

Determination method of molecular mass	Masses of labeled proteins in SMS-KAN cells [kDa]	Mass of labeled proteins in CHO-Y2 cells [kDa]
Crosslinking with $^3\text{H-Tmd27}$	$57 \pm 4 / 47 \pm 4$	$62 \pm 4 / 48 \pm 4$
subtracted by mass of crosslinker	$54 \pm 4 / 44 \pm 4$	$59 \pm 4 / 45 \pm 4$
Crosslinking with $^3\text{H-Tmd36}$	$58 \pm 4 / 49 \pm 4$	$63 \pm 4 / 51 \pm 4$
subtracted by mass of crosslinker	$53 \pm 4 / 44 \pm 4$	$58 \pm 4 / 46 \pm 4$
Western blotting	$58 \pm 4 / 54 \pm 4$	$57 \pm 4 / 51 \pm 4$

This might be explained by the error in mass determination or the different experimental efforts for photoaffinity labeling and Western blotting and the different detection methods that have been applied. Thus, the results of the molecular mass determination of the Y_2 -receptor suggest the same protein masses of 58 and 50 kDa in two different cell lines. SMS-KAN cells are human neuroblastoma cells, which are endogenously expressing the Y_2 -receptor subtype, whereas CHO-Y2 cells have been transfected with human Y_2 -receptor cDNA. The

corresponding molecular masses suggest similar posttranslational modifications in both cell lines. When compared to the mass of the Y_2 -receptor calculated from the sequence (42 kDa), a difference of up to 16 kDa caused by posttranslational processing was observed. Glycosylation alters the migration behavior of proteins in SDS-PAGE in a nonlinear way (41). This means that the exact increase in the mass of the Y_2 -receptor caused by glycosylation cannot be determined exactly by SDS-PAGE, but our results suggest a remarkable glycosylation of the Y_2 -receptor at the consensus sequence of the N-terminus. Preliminary deglycosylation experiments showed a decrease of the molecular masses after application of endoglycosidase F and peptide-N-glycosidase F. Therefore, the two different masses of the proteins identified in each cell line are most likely due to different glycosylation of the Y_2 -receptor.

Previous studies using chemical crosslinking with PYY and NPY analogues in different cells or tissues revealed huge differences in the molecular masses for the Y_2 -receptor. Accordingly, in rat hippocampus and rabbit kidney membranes the Y_2 -receptor was identified as a glycoprotein of 50 kDa (42), which was found also for bovine (43), human (44) and porcine hippocampal membranes (45). The same molecular weight was identified in a renal proximal tubule cell line of mice (46), whereas molecular masses from 39 kDa up to 70 kDa were found in other tissues and species (47-50). These results suggest that NPY Y_2 -receptor subtypes in hippocampal membranes from different species are conserved in their size and different from those in other tissues (43). The existence of at least two different Y_2 -receptor subtypes, located centrally and peripherally, is suggested by Northern hybridization (51). Our approach for molecular mass determination of the Y_2 -receptor subtype identified two glycoproteins of 58 and 50 kDa in SMS-KAN cells as well as in transfected CHO- Y_2 cells, which are believed to represent the same protein with two different amounts of glycosylation. This approach included two different methods for molecular mass determination, specific crosslinking by photoaffinity labeling and Western blotting using Y_2 -receptor selective antibodies, which provides a high reliability of the masses determined.

Both photoactivatable analogues have been shown to allow the formation of a covalent ligand-receptor complex. In the putative receptor binding region the ligands were only slightly modified, which suggests, in combination with the

observed high receptor affinity, a receptor binding mode similar to the native ligand. The biotin label of the photoactivatable analogues, as well as the subtype selective anti-receptor antibodies, will facilitate the purification of the covalent ligand-receptor complex. This will be used to identify the crosslinked positions of the receptor after enzymatic cleavage. Thus, the photoactivatable NPY analogues and anti-receptor antibodies described are valuable tools for identification of the receptor regions involved in ligand binding. Because of the Y₂-receptor subtype selectivity of ³H-Tmd27 and the used antibody, this will be possible even in tissues in which other Y-receptor subtypes are present.

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