Neuropeptide Y and orexins
key factors in the hypothalmic regulation of food intake

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Publication Date:
2000

Permanent Link:
https://doi.org/10.3929/ethz-a-004109291

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Neuropeptide Y and Orexins: 
Key Factors in the Hypothalamic Regulation of Food Intake

A dissertation submitted to the 
Swiss Federal Institute of Technology Zurich 
for the degree of 
Doctor of Natural Sciences

presented by

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2000
Meinen Eltern
TABLE OF CONTENTS

TABLE OF CONTENTS I
ABBREVIATIONS V
SUMMARY 1
ZUSAMMENFASSUNG 5

CHAPTER 1 11

On the Role of Neuropeptides in the Hypothalamic Regulation of Food Intake (Review) 11
1.1 Abstract 12
1.2 Peptides are molecules of growing importance 13
1.3 Neuropeptides: key position in the regulation of food intake 14
1.3.1 Hypothalamic regulation of food intake 14
1.1.2 Obesity, a question of genes and environment 25
1.4 From the peptide to the target in medicinal chemistry 27
1.5 Structure-activity studies on selected neuropeptides with respect to their role in obesity 31
1.1.1 Leptin 32
1.1.2 POMC and α-MSH 33
1.1.3 CART 35
1.1.4 Neuropeptide Y 35
1.1.5 AGRP 38
1.1.6 Orexin A and B 39
1.1.7 MCH 40
1.1.8 Galanin 41
1.6 Conclusion 42
1.7 References 44
### CHAPTER 2

Novel Analogs of Neuropeptide Y with Preference to the Y1-Receptor

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Abstract</td>
<td>68</td>
</tr>
<tr>
<td>2.2 Introduction</td>
<td>69</td>
</tr>
<tr>
<td>1.3 Experimental Procedures</td>
<td>71</td>
</tr>
<tr>
<td>1.3.1 Materials</td>
<td>71</td>
</tr>
<tr>
<td>1.1.2 Peptide synthesis</td>
<td>72</td>
</tr>
<tr>
<td>1.1.3 Cell culture</td>
<td>73</td>
</tr>
<tr>
<td>1.1.4 Binding assays</td>
<td>73</td>
</tr>
<tr>
<td>1.1.5 Circular dichroism spectroscopy</td>
<td>74</td>
</tr>
<tr>
<td>1.1.6 cAMP Enzyme-immunoassay</td>
<td>74</td>
</tr>
<tr>
<td>1.4 Results</td>
<td>76</td>
</tr>
<tr>
<td>1.4.1 Peptide synthesis and testing</td>
<td>76</td>
</tr>
<tr>
<td>1.1.2 NPY/PP-chimeric peptides</td>
<td>76</td>
</tr>
<tr>
<td>1.1.3 Analogs with replacements at position 6-8 of NPY</td>
<td>77</td>
</tr>
<tr>
<td>1.1.4 Analogs with replacements in the turn-segment of NPY</td>
<td>78</td>
</tr>
<tr>
<td>1.1.5 Exchanges within the α-helix</td>
<td>79</td>
</tr>
<tr>
<td>1.1.6 Functional studies</td>
<td>80</td>
</tr>
<tr>
<td>1.5 Discussion</td>
<td>83</td>
</tr>
<tr>
<td>1.6 References</td>
<td>87</td>
</tr>
</tbody>
</table>

### CHAPTER 3

Novel Short and Selective Peptide Ligands for the NPY Y₁- and Y₄-Receptor

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Abstract</td>
<td>94</td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>95</td>
</tr>
<tr>
<td>3.3 Results</td>
<td>97</td>
</tr>
<tr>
<td>3.3.1 Structure-affinity relationship</td>
<td>97</td>
</tr>
<tr>
<td>1.1.2 Circular Dichroism</td>
<td>99</td>
</tr>
<tr>
<td>1.1.3 Mathematical analysis of binding data</td>
<td>103</td>
</tr>
<tr>
<td>1.4 Discussion</td>
<td>106</td>
</tr>
</tbody>
</table>
CHAPTER 4

Studies of the Human, Rat, and Guinea Pig Y4 Receptors Using Neuropeptide Y Analogues and Two Distinct Radioligands

4.1 Abstract
4.2 Introduction
4.3 Methods
4.3.1 Cloning
4.3.2 Peptides
4.3.3 Binding
4.4 Results
4.5 Discussion
4.6 References

CHAPTER 5

On the Synthesis of Orexin A: A Novel One-Step Procedure to Obtain Peptides With Two Intramolecular Disulfide Bonds

5.1 Abstract
5.2 Introduction
5.3 Experimental Procedures
5.3.1 Materials
5.3.2 Synthesis Methods
IV Table of contents

5.3.3 Control of complete oxidation and correct folding of orexin A 146
5.4 Results 146
5.4.1 Synthesis of orexin A 146
5.4.2 Control of complete oxidation and correct folding of orexin A 153
5.5 Discussion 153
5.6 References 157

CHAPTER 6 161

Structure-Affinity studies of truncated Orexin A and B Analogs at OX₁ and OX₂ receptors 161
6.1 Abstract 162
6.2 Introduction 163
6.3 Experimental Procedures 164
6.3.1 Materials 164
6.3.2 Peptide Synthesis 165
6.3.3 Cell Culture, Binding Assays and Functional Assay 166
6.3.4 Circular Dichroism 166
6.4 Results and Discussion 168
6.4.1 Synthesis of the Peptides 168
6.4.2 Biological and Structural Characterization of the Peptides 168
6.4.3 Full length analogs 169
6.4.4 N-terminal Fragments 169
6.4.5 C-terminal Fragments 171
6.5 Conclusion 174
6.6 References 176

PUBLICATIONS AND ORAL PRESENTATIONS 180
CURRICULUM VITAE 183
ABBREVIATIONS

AGRP, Agouti related protein
Ahx, 6-Aminohexanoic acid
Aib, Aminoisoburyric acid
ARC, arcuate nucleus
b, bovine
BBB, Blood-brain barrier
BHK, Baby hamster kidney
BMI, Body mass index
Bpa, p-Benzoylphenylalanine
BSA, Bovine serum albumin
cAMP, Cyclic adenosine monophosphat
CART, Cocaine- and amphetamine regulated transcript peptide
CD, Circular dichroism spectroscopy
Cha, Cyclohexylalanine
CHO, Chinese hamster ovary
CNS, Central nervous system
CRH, Corticotropin-releasing hormone
DIC, N,N'-Diisopropylcarbodiimide
DIPEA, N,N-diisopropylethylamine
DMF, Dimethylformamide
ESI-MS, Electrospray ionization mass spectrometry
Fmoc, fluorenlymethoxy carbonyl
gp, guinea pig
h, human
HOBt, 1-Hydroxy benzotriazole
ICV, Intracerebroventricular
JAK, Janus kinase family
LHA, Lateral hypothalamic area
MC, Melanocortin
MCH, Melanin-concentrating hormone
α-MSH, α-Melanocyte-stimulating hormone
Nal, Naphtyl-alanine
NPY, Neuropeptide Y
OXY, Oxytocin
p, porcine
PFA, Perifomical area
PKA, Protein kinase A
Pmc, 2,2,5,7,8-pentamethylchroman-6-cyclopropanoic acid
POMC, Pro-opiomelanocortin
PP, Pancreatic polypeptide
PVN, Paraventricular nucleus
PYY, Peptide YY
r, rat
RTKs, Receptor tyrosine kinases
SON, Supraoptic nucleus
STAT, Signal transducers and activators of transcription
TFA, Trifluoracetic acid
TRH, Thyrotropin-releasing hormone
Trt, Trityl
VMN, Ventromedial nucleus
SUMMARY

In our days obesity and overweight are beginning to replace undernutrition and infectious diseases as the most significant contributor to the reduction of public health. This asks for a broader understanding of the regulation of food intake and energy homeostasis in human body, in order to encounter this tendency adequately and, where necessary, with sufficient medical treatment. The understanding of overweight and obesity as a willful misconduct with respect to food intake, has recently been extended by a genomically determined approach to understand the individual regulation of energy expenditure and food intake. The superior operating center for energy homeostasis is localized in the hypothalamic region of the central nervous system. A big series of neurotransmitters, mainly neuropeptides, are acting and interacting there in a redundant system (chapter 1). Leptin and insulin, two peripheral adiposity signals, circulate in the blood at levels proportional to the body fat content and enter the central nervous system in proportion to their plasma level. They evolve a wide range of action in the hypothalamus via specific receptors. Deficiency of either peptide induces food intake, whereas central administration into the brain does the opposite. Neuropeptide Y (NPY) and the orexins A and B are three of the important neuropeptides, which transmit the action of leptin and insulin in the hypothalamus. NPY acts in human via at least three different receptor subtypes. They all belong to the superfamily of heptahelical G-protein coupled receptors and are referred to as Y₁<sup>-</sup>, Y₂<sup>-</sup> and Y₅-receptor. Furthermore, pancreatic polypeptide binds to Y₄-receptors. The food intake stimulating effect of NPY is exerted – as known so far – via a team-play of Y₁- and Y₅-receptors. Involvement and function of these two receptor subtypes in signal transduction of NPY-induced food intake can be investigated best by a set of highly receptor subtype selective agonists and antagonists. Whereas for the Y₅-receptor selective agonists and antagonists are known, for the Y₁-receptor only selective antagonists are reported and it still lacks a highly selective agonist. This work presents the first NPY-based analogs with Y₁-receptor preference and information about the role of specific amino acid positions of NPY for binding to the Y-receptor subtypes. Amongst the tested analogs, [F<sup>7</sup>,
P^{34}pNPY has been found to show highest Y₁-receptor affinity, with subnanomolar affinity to the Y₁-receptor, and $K_i$ values of 32 nM and 34 nM at the Y₂- and Y₅-subtype, respectively. In addition, variations of position 6, especially [R⁶, P^{34}]pNPY and variations within positions 20-23 of NPY were found to result in analogs with Y₁-receptor preference (chapter 2). With respect to antagonist, several Y₁-receptor selective ligands are known. One of the mostly applied peptide antagonist at the Y₁-receptor, a homodimer called GR231118, turned out to be a potent agonist at the Y₄-receptor as well. In order to discriminate between Y₁-receptor antagonism and Y₂-receptor agonism, systematic variation of the monomeric sequence of GR231118 was performed with regard to hydrophobicity, size, charge, acidity and orientation of the side-chain of the newly introduced amino acids (chapter 3). This resulted in a set of peptides with increased Y₁-receptor selectivity, which provided promising information for the development of new, highly selective Y₁-receptor antagonists. Dimerization of the most interesting sequences by disulfide coupling showed significant increase in receptor affinity (chapter 3).

Besides the characterization of different receptor subtypes, NPY analogs can further be used for the characterization of species specific differences of a distinct receptor subtype (chapter 4). This was performed on the human, rat and guinea pig Y₄-receptors, using [A³³]pNPY, [A³⁴]pNPY, [A³⁵]pNPY, [A³⁶]pNPY, a set of centrally truncated NPY analogs and $^{125}$I-hPP (human pancreatic polypeptide) or $^{125}$I-PYY (peptide YY) as radioligands. NPY and PYY competed with $^{125}$I-hPP at Y₄-receptors expressed in CHO cells according to a two-site model. This was investigated for guinea pig Y₄ by saturation with either radiolabeled hPP or PYY. The number of high-affinity binding-sites for $^{125}$I-pPYY was about 60% of the receptors recognized by $^{125}$I-hPP. Porcine [Ala³⁴]NPY and [Ahx⁸⁻²⁰]NPY bound to rat Y₄, but not to human or guinea pig Y₄, according to a two-site model. These results suggest that different full agonists can distinguish between different active conformations of the guinea pig Y₄ receptor and that Y₄ receptors may display functional differences in vivo between human, guinea pig, and rat (chapter 4).

Orexin A and B, also known as hypocretin 1 and 2, are two recently discovered hypothalamic neuropeptides, which are importantly involved in the regulation of food intake and in the sleep-wake cycle. Orexin A is a 33 amino acid peptide amid
with two intramolecular disulfide bonds and orexin B a linear 28 amino acid peptide amid. In order to provide orexin A for biological and pharmacological tests, four different synthesis methods for the synthesis of peptides containing two disulfide bonds are compared and optimised regarding to reaction time, purity of the crude peptide and yield of the purified peptide. A new one-step cyclisation method in solution is presented for fast, easy and high yield synthesis of orexin A, based on iodine oxidation in acetic acid/water using S-acetamidomethyl (S-Acm) and S-trityl (S-Trt) as cysteine protecting groups. Furthermore, this work indicates the building of different mono- or bicyclic configurations of orexin A after unselective disulfide formation, indicating the necessity of selective formation of the two disulfide bonds in the synthesis of orexin A (chapter 5). Orexin A and B are endogenous ligands of two closely related G-protein-coupled receptors termed OX1 and OX2. Up to now, no structure-activity relationships of any of the two orexin peptides have been reported, nor any potent subtype selective antagonist or agonist is known to characterize the physiological and pharmacological role of the two receptor subtypes. Structure-affinity relationships of fragments and analogs of orexin A and B were investigated on OX1- and OX2-receptors, expressed in CHO cells, and on SK-N-MC-cells (chapter 6). The results suggest the importance of the almost conserved C-terminal decapeptide of orexin A and B for ligand-receptor interaction. N-terminal fragments completely or almost completely lost binding. Orexin B(18-28), the C-terminal undecapeptide of orexin B, is presented as the first high affinity peptide antagonist at the OX1-receptor. This compound did not alter intracellular calcium concentration, investigated by fluorometric imaging technique, despite very high binding. The C-terminal methionine residue of orexin B was found to be crucial for receptor activation. Interestingly, it could be sufficiently replaced by leucine, the C-terminal residue of orexin A. Binding experiments on SK-N-MC cells, a human neuroblastoma cell line, revealed the existence of an orexin-receptor. Differences in the binding profile of orexin A and B fragments at the OX1-/OX2-receptors and the receptor on SK-N-MC cells give raise to the suggestion of a third orexin receptor subtype (chapter 6).
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Zusammenfassung


Zusammenfassung

[Ahx\(^{8-20}\)]NPY banden an Y\(_4\)-Rezeptoren von Ratten, nicht aber von Menschen oder Guinea Pigs, nach einem Modell mit zwei Bindungsstellen. Diese Resultate legen nahe, dass verschiedene volle Agonisten zwischen verschiedenen aktiven Konformationen von Guinea Pig Y\(_4\)-Rezeptoren unterscheiden können und dass Y\(_4\)-Rezeptoren in vivo funktionelle Unterschiede zwischen Menschen, Ratten und Guinea Pigs aufweisen (Kapitel 4).

On the Role of Neuropeptides in the Hypothalamic Regulation of Food Intake (Review)

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bInstitute of Biochemistry, University of Leipzig, Germany

Current Medicinal Chemistry, submitted
1.1 Abstract

Neuropeptides are molecules of outstanding importance in the regulation of most different physiological and biochemical processes. They act as signal transducing molecules that make them an ideal target for pharmacological treatment. Because of their possibility to act via different receptor subtypes within a whole receptor family, the peptides are able to selectively induce physiological processes. Therefore, peptide research provides important contributions to a detailed understanding of physiological processes. The hypothalamic regulation of food intake reveals a redundant system of neuropeptides, acting and interacting with each other in order to control energy homeostasis and body weight. Leptin and insulin are peripheral adiposity signals, inducing first order neuronal signalling in the arcuate nucleus of the hypothalamus. There, they inhibit anabolic peptides, whereas they stimulate catabolic peptides. Neurons from the arcuate nucleus project to candidate second-order neurons involved in hypothalamic response to leptin and insulin, located in the paraventricular nucleus and the lateral hypothalamic and perifornical area. However, anomalies in body weight, especially overweight and obesity, are not only genetically determined, but also by the environment. Cultural, socioeconomic and psychosocial factors, physical activity and other environmental factors are known for sustained influence of body weight. Medicinal chemistry importantly contributes to the molecular understanding of anomalies and diseases, which likely culminates in the development of appropriate medical treatments. Hereby, the development of receptor subtype selective agonists and antagonists is a key step. This is exemplified on a set of hypothalamic neuropeptides which are involved in the regulation of food intake.

Keywords: hypothalamus, neuropeptides, ligand-receptor-interaction, structure-affinity relationship, food intake, obesity
1.2 Peptides are molecules of growing importance

A large number of important physiological and biochemical processes that keep a body alive are mediated and regulated by peptides. Smaller and bigger peptides are involved in receptor mediated signal transduction in the central and peripheral nervous system, in the gastrointestinal tract, in immunological processes, blood pressure and many more. The raising knowledge on peptides and their functions led to an increasing pharmacological and medicinal interest in peptides as an ideal target for pharmacological treatment.

Peptides have the ability to adopt different three-dimensional structures that allow the interaction with different targets. In addition, they even can change their conformation according to the surrounding, for example during the process of membrane approaching or receptor binding. Accordingly, peptides activate or inhibit different receptor subtypes within a receptor family to initiate specific effects. Proteinogenic receptors are not the only structures that can be selectively recognized by peptides. Also DNA- or RNA-structures can be bound, initiating or inhibiting discrete functions, as it is shown for example most recently for aptamers that can antagonize peptide binding to a specific receptor.

Peptides are important molecules in the regulation of many different physiological processes. Especially neuropeptides evolve a wide spectrum of actions in most different fields of function. In addition to antisera in immunohistochemical studies, radioactive- or fluorescence-labeled peptides are important tools to screen the brain or other tissues on the search for physiologically and pharmacologically important structures – mostly distinct receptors – as it has been shown for example in the wide field of neuropeptide Y research. Labelled peptides are also convenient tools to follow their distribution in the body, for example the crossing of the blood-brain barrier, as it has been performed for the recently discovered neuropeptides orexin A and B.

Neuropeptide Y and the recently identified orexins are two of many other neuropeptides, that are involved in the regulation of food intake and energy homeostasis. Anomalies of this regulation might cause obesity, a disease that is beginning to replace undernutrition and infectious diseases as the most significant
1.3 Neuropeptides: key position in the regulation of food intake

1.3.1 Hypothalamic regulation of food intake

1.3.1.1 First order neuronal signalling

The hypothalamic area is known as an important center for the regulation of food intake and energy homeostasis within the central nervous system. Brain lesion and stimulating experiments in the first half of the 20th century showed the importance of this region on eating behaviour. These studies identified a ‘satiety center’ in the ventromedial hypothalamic nucleus (VMN), while the lateral hypothalamic area (LHA) was termed the ‘hunger center’ 9,10. Electrical stimulation of the VMN suppressed food intake while bilateral lesions induced obesity 11. The opposite set of responses, increase in food intake after stimulation and a lean phenotype after brain lesions, was observed in the LHA 12. Big effort in research in the field of central signal transduction have replaced this view of distinct centers in the brain that are responsible for specific actions by a view of discrete neuronal pathways that generate integrated responses to regulate and control body fat, energy expenditure and food intake 13. It is, however, important to emphasize that although knowledge has been increased over the past years,
the initial observations made in the 40s and 50s of the 20th century are still strikingly valid. Both hypothalamic areas, the VMN and the LHA, have recently been shown to contain neurons that express on the one hand neuropeptides, that play important role in the regulation of energy homeostasis and food intake. On the other hand, hypothalamic neurons express receptor proteins, that are targets for central as well as peripheral signalling molecules that regulate food intake. The most important known peripheral hormonal signals, which influence central regulation of food intake, are the peptide-hormones insulin and leptin (Table 1).

Insulin was the first hormonal signal shown to play a role in the control of body weight by the central nervous system (CNS). It is secreted by the pancreas, enters the brain from circulation by a putative insulin transporter and acts there to reduce energy intake 14,15. Leptin, a hormone secreted by adipocytes, was found as autosomal recessive mutation of the obese (ob) gene in ob/ob mice, which develop profound hyperphagia and obesity 16,17. Ob/ob mice weigh about three times more than normal mice and have a five fold increase in body fat. The ob gene encodes a protein of 167 amino acids, designated as leptin after the Greek word leptos = thin. It has been reported that endogenous leptin in human serum contains a 146 amino acid sequence (16 kDa). 18. Besides obesity, ob/ob mice show a large number of further defects, like decreased body temperature, decreased energy expenditure, decreased immune function, diabetes and infertility, which indicate that leptin has a wide range of effects in the organism 17. Exogenous administration of recombinant leptin corrects all of the above-mentioned defects in ob/ob mice (reviewed in 19). The observation, that low doses of centrally, but not peripherally, administered leptin reduces food intake and the fact that mice with hypothalamic lesions are leptin resistant, further supported the hypothesis, that the hypothalamus is an important site of leptin action 20-24. Some obese humans were found to express higher levels of leptin mRNA and leptin in plasma, while conversely, leptin levels fall during starvation 25-27.

Both hormones, insulin and leptin, circulate in the blood at levels proportional to the body fat content and enter the CNS in proportion to their plasma level 14,28-30. Deficiency of any of the two peptides induces food intake, whereas central administration into the brain does the opposite 15,17,20,31,32. Receptors of insulin
1.3 Neuropeptides: key position in the regulation of food intake

**Table 1.** Amino acid sequences of insulin and leptin. Cysteine residues that are involved in disulfid bridges are highlighted in grey and disulfid connections are marked. Leptin is shown as unprocessed precursor, the cleavable N-terminal signal site is underlined.

<table>
<thead>
<tr>
<th></th>
<th>Insulin</th>
<th>Leptin</th>
</tr>
</thead>
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<tr>
<td><strong>Length</strong></td>
<td>A-chain: 21 AA; B-chain: 30 AA</td>
<td>167 AA (unprocessed precursor)</td>
</tr>
<tr>
<td><strong>Molecular weight</strong></td>
<td>5786 Da</td>
<td>18640 Da (unprocessed precursor)</td>
</tr>
<tr>
<td><strong>Amino acid sequence</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-chain</td>
<td>GIVECCOTSICSLYQLENYN</td>
<td>MHWGTLCGLWLPYFLFYQAVPICKVQDDT</td>
</tr>
<tr>
<td>B-chain</td>
<td>FVNQHLGSHLVEALYLDGERFFYPKT</td>
<td>TLIKTIVTRINDISHTOSVSSKOKVTGLDFIPLHPIL</td>
</tr>
</tbody>
</table>

...and leptin are expressed in brain regions that are involved in energy regulation 33-35. The insulin receptor belongs to a class of transmembrane proteins, called receptor tyrosine kinases (RTKs). It is a $\alpha_2\beta_2$ heterotetramer consisting of two identical $\alpha$- and $\beta$-subunits that are connected by disulfide bridges. Insulin is bound extracellularly on both of the $\alpha$-subunits, initiating dimerization of the receptor and intracellular autophosphorylation on tyrosine residues of the $\beta$-subunits. The resulting phosphotyrosines bind and activate (phosphorylate) themselves and subsequently intracellular enzymes, that transmit the varies actions of insulin. The leptin receptor activates the JAK-STAT signalling pathway. In contrary to the insulin receptor, the tyrosine kinase activity of the leptin receptor is not part of the receptor protein itself, but of a bound cytoplasmatic tyrosine kinase of the janus kinase family (JAK). Activation of the leptin receptor results in intracellular binding of JAK2. Ligand-mediated receptor dimerization initiates kinase activity of the receptor dimer and subsequent activation of the bound JAKs by tyrosine phosphorylation. The kinase activity of the phosphorylated JAKs culminate in activation of signal transducers and activators of transcription (STAT). It has been shown, that leptin acts in vivo specifically via STAT3 proteins. They form dimers after phosphorylation, translocate into the nucleus and cause specific activation of gene transcription 19.
Comparing the role of insulin and leptin with respect to the regulation of energy homeostasis in the CNS, leptin seems to play a more vigorous role. For example, insulin deficiency can not induce obesity, whereas leptin deficiency causes severe obesity and hyperphagia. However, the function of the two hormones can not be clearly separated from each other, because insulin-stimulated glucose utilization in adipocytes is a key factor that links leptin secretion to body fat mass. Additionally, gain of weight cannot occur in case of insulin deficiency: even if food is consumed in large amounts plasma level of leptin remains low in rats and humans.37-39

Insulin and leptin initiate a series of catabolic or anabolic neuropeptide-containing pathways in the hypothalamic region of the CNS (Table 2). First order neuronal signalling is found in the arcuate nucleus (ARC), an 'arc-like' collection of neuronal cell bodies adjacent to the floor of the third ventricle, that's approximately one-half of the length of the hypothalamus. There is morphological evidence obtained by electron microscopy, which indicates that the ventral, but not the dorsal, part of the ARC lacks a blood-brain barrier (BBB). Leptin might be therefore considered to be transported to the ventromedial ARC via the general circulation, acting on leptin receptors devoid of a BBB. In contrast, it has been suggested that a truncated version of the leptin receptor may participate in the transport of leptin across the BBB.41,42 Further evidence for the existence of a specific uptake mechanism of leptin into the brain comes from New Zealand obese (NZO) mice that are resistant to peripheral administration of leptin but respond normally to centrally administered leptin, which suggests that the obesity derives from a defect of the leptin transport into the CNS.43 Food intake regulating action of leptin in the arcuate nucleus mainly involves four neuropeptides as known so far. They are co-localized in neurons by pairs, namely the two anabolic peptides neuropeptide Y (NPY) and Agouti related peptide (AGRP, also known as ART: Agouti related transcript)) as well as the two catabolic peptides pro-opiomelanocortin (POMC) and cocaine- and amphetamine-related transcript (CART).

An increased concentration of leptin / insulin leads to a diminished expression and subsequently an inhibition of the release of NPY and AGRP (Fig. (1)).
Table 2. Selection of neuropeptides that are involved in the hypothalamic regulation of food intake. Arrows indicate the direction of the effect exerted by one or both of the adiposity signals, insulin and leptin.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Size (amino acids)</th>
<th>Regulation by adiposity signals</th>
<th>Molecule</th>
<th>Size (amino acids)</th>
<th>Regulation by adiposity signals</th>
</tr>
</thead>
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<tr>
<td>catabolic</td>
<td></td>
<td></td>
<td>anabolic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-MSH</td>
<td>13</td>
<td>↑</td>
<td>NPY</td>
<td>36</td>
<td>↓</td>
</tr>
<tr>
<td>CART</td>
<td>89</td>
<td>↑</td>
<td>AGRP</td>
<td>112</td>
<td>↓</td>
</tr>
<tr>
<td>CRH</td>
<td>41</td>
<td>↑</td>
<td>MCH</td>
<td>19</td>
<td>↓</td>
</tr>
<tr>
<td>TRH</td>
<td>3</td>
<td>↑</td>
<td>Orexin A / B</td>
<td>33 / 28</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Galanin</td>
<td>30</td>
<td>↓</td>
</tr>
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</table>

NPY is one of the strongest stimulators of food intake and injections of NPY into the cerebral ventricles or directly into the hypothalamus of rats potently stimulate food intake and decrease energy expenditure. After continuous or repeated central administration of NPY, the treated animals become obese. Interestingly, the first transmitter candidate to be demonstrated in leptin receptor-expressing neurons was NPY. Leptin inhibits gene expression of NPY in the arcuate nucleus and genetic knockout of NPY, i.e. mice that lack NPY but are otherwise genetically normal, reduces hyperphagia and obesity in ob/ob mice. This indicates that the full response to leptin deficiency requires NPY signalling. Additionally, feeding response following NPY administration is inhibited by leptin, which suggests that leptin determines the sensitivity of the feeding response to exogenous NPY. In contrast, however, NPY knockout mice have more or less normal feeding responses. This raises questions about the need for NPY when leptin levels are normal. Obviously, such findings support the theory of the regulation of food intake as a redundant system, where distinct deficits can be replaced by other mechanisms. A related phenomenon is found in the NPY system itself. NPY involves not only one distinct receptor to enhance feeding behaviour, but an interplay of the Y1- and the Y5-receptor is suggested.

AGRP, is a second anabolic neuropeptide. It is co-localized in neurons with NPY, acts on the melanocortin (MC) signalling pathway, by displaying an
antagonistic effect on MC3 and MC4 receptors. The cloning of the AGRP gene was done shortly after the identification of the agouti gene. The Agouti gene leads to the expression of a protein ('agouti') that acts on the one hand as an antagonist on cutaneous MC1 receptors, lightening the coat colour to yellow. On the other hand, agouti antagonizes MC4 receptors, causing an obese phenotype as it is seen in the agouti (A^Y/a) mice, an autosomal dominant model of genetic obesity, which is characterized by a yellow coat colour and marked obesity. Intracerebroventricular (ICV) administration or transgenical expression of AGRP cause hyperphagia and the increase of food intake, even after a single ICV injection of AGRP, is sustained for up to a week. Although NPY is described as the most potent energy intake stimulating (orexigenic) molecule, its effects are short-lived in comparison to those of AGRP. AGRP must therefore be considered to be the most robust orexigenic molecule, if potency is measured as the cumulative increment of energy intake after a single ICV injection. Hypothalamic expression of AGRP is localized in the arcuate nucleus, like that of NPY. It is upregulated by leptin deficiency and by fasting, which indicates that melanocortin receptors play important role in body weight regulation.

Besides the anabolic leptin/insulin-first order signalling molecules NPY and AGRP, other hypothalamic first order signalling molecules are known, that however induce catabolic mechanisms. Best characterized are the α-melanocyte-stimulating hormone (α-MSH) and the cocaine- and amphetamine-regulated transcript (CART) (Fig. 1). Increased adiposity signals result in increased neuronal synthesis of these peptides in the brain. The most important, but also most complex is the melanocortin system with respect to energy homeostasis. Pro-opiomelanocortin (POMC) is the precursor molecule of the melanocortins, a family of peptides including ACTH, α-, β-, and γ-MSH. α-MSH binds to the so-called family of melanocortin receptors, named MC1 to MC5. The role of α-MSH in the regulation of body weight could be demonstrated by a synthetic agonist at the MC3- and MC4-receptor that suppressed food intake, whereas a synthetic antagonist had the opposite effect. Furthermore, mice lacking the MC4 receptor (MC4-knock-out mice) evolve a very obese phenotype, which indicates that MC4 receptor signalling reduces body weight and limits food intake. The MC4-receptor was found to be highly expressed in neurons of the paraventricular and
1.3 Neuropeptides: key position in the regulation of food intake

dorsomedial hypothalamic nuclei and in the lateral hypothalamic area 64. Fasted animals or ob/ob mice showed reduced POMC mRNA, that could be normalized by exogenous leptin administration 65. The opposite effect, increased POMC mRNA in the arcuate nucleus, could be observed after overfeeding 66. α-MSH and AGRP seem to be a kind of ying/yang-pair at the MC4 receptor, where α-MSH displays agonistic effects and AGRP its antagonistic counterpart. Soon after the important role of the MC4 receptor for regulation of food intake has been discovered, a dominant form of human obesity could be related to MC4 receptor mutations 67,68.

The second catabolic neuropeptide in the ARC, CART (cocaine- and amphetamine-related peptide) was identified as a mediator of leptin-activity in 1995 69. During starvation, or in ob/ob mice, CART mRNA is low in the arcuate nucleus, which can be reversed by exogenous leptin administration. In addition, leptin administration elevates CART mRNA in normal mice 61. Decreased food intake could be observed after ICV injection of CART peptides and, conversely, antibodies that block CART peptide action induce food intake 61,70.

Regarding the distribution of the mentioned neuropeptides in the hypothalamus, it could be shown that the anabolic peptides NPY and AGRP are co-localised in the arcuate nucleus neurons 59,60, while the catabolic POMC and CART are co-localised in a distinct, but adjacent subset of ARC neurons 71. This demonstrates that a single neuronal cell type can contain multiple effector molecules and indicates that the circuits originating in this brain area have highly specialized roles in energy homeostasis. The importance of the ARC brain region in the regulation of food intake could further be proven by inefficacy of leptin in reduction of food intake after destruction of the ARC brain region 72,73. A co-expression of leptin receptors has been found in both NPY/AGRP and POMC/CART neurons, indicating that both neurons are regulated by leptin, but in an opposing manner. The NPY/AGRP neurons are activated by low and inhibited by high leptin levels 23,48,59,60, and, conversely, low leptin levels inhibit POMC and CART expression in the ARC 61,65,74. Although less well studied, insulin seems to act in the same way as leptin and insulin receptors are found in high concentration throughout the arcuate nucleus 32,33,75.
Fig. (1). Hypothalamic regulation of food intake. The peripheral adiposity signals leptin and insulin induce first-order neuronal signals in the arcuate nucleus of the hypothalamus, acting on NPY/AGRP- and POMC/CART-containing neurons. Food intake is induced by NPY/AGRP neurons, while it is inhibited by POMC (α-MSH) and CART. Neurons of the ARC project to candidate second-order neurons involved in hypothalamic response to leptin and insulin. These include the PVN, that contains the catabolic neuropeptides TRH, OXY and CRH, and the LHA/PFA, where anabolic neuropeptides like MCH and orexins can be found.

ARC = arcuate nucleus, LHA = lateral hypothalamic area, PFA = perifornical area, PVN = paraventricular nucleus, NPY = neuropeptide Y, AGRP = Agouti related protein, POMC = pro-opiomelanocortin, α-MSH = α-melanocyte-stimulating hormone, CART = cocaine- and amphetamine-regulated transcript, TRH = thyrotropin-releasing hormone, CRH = corticotropin-releasing hormone, OXY = oxytocin, MCH = melanin-concentrating hormone
1.3.1.2 Second-order neuronal signalling

Nerve fibres from the arcuate nucleus project into other hypothalamic regions and can induce a second-order neuronal signal. The identification of such downstream neurons is just beginning, and energy homeostasis is probably regulated by integrated and redundant pathways, rather than by a discrete set of neurons connected in series to one another. However, the hypothesis of first-order signals in the ARC induced by leptin and insulin, followed by second-order signals in areas where ARC neurons project into, presents a possible model to understand central regulation of food intake and energy homeostasis 58.

Brain areas of the hypothalamus like the paraventricular nucleus (PVN), the perifornical area (PFA) and the lateral hypothalamus (LHA) are richly supplied by axons from the ARC 76,77. A number of neuropeptides that are synthesized in the PVN — known as the ‘satiety center’ 10 — reduce food intake after central administration, like corticotropin-releasing hormone (CRH) 78, thyrotropin-releasing hormone (TRH) 79 and oxytocin (OXY) 80. These catabolic downstream located effectors should be stimulated by melanocortin and/or CART signals but inhibited by NPY/AGRP signalling which will have to be proven in the future. However, surprisingly leptin receptor immunoreactivity is also present in CRH neurons of the PVN, which suggests an additional direct modulation of CRH signalling by leptin 81.

The LHA/PFA is known as the ‘hunger center’ 9,10. Its involvement in anabolic signalling is supported by studies on melanin-concentrating hormone (MCH), an orexigenic peptide located in this area. Energy restriction and leptin deficiency induce MCH synthesis 82, that is normalized after leptin administration. MCH knockout mice are excessively lean due to reduced food intake 83. Like CRH neurons, MCH neurons contain leptin receptors, which suggests the possibility of direct interaction of leptin on lateral hypothalamic MCH neurons. Similar to NPY receptors, another potent stimulator of food intake, MCH receptors belong to the family of G-protein coupled receptors. They belong to the Gi subfamily, that inhibits formation of cyclic AMP after activation by a ligand and consequently reduces signalling by protein kinase A 84,85. The opposite effect can be observed
at catabolic inhibitors of food intake, for example MC4 or CRH receptors, which are coupled to G\textsubscript{s} and consequently increase cAMP and PKA signalling.

Two recently discovered neuropeptides that are expressed exclusively in the LHA, zona incerta and PFA, enlarge the group of food intake regulating neuropeptides. They were termed orexin A and B \textsuperscript{86} or hypocretin 1 and 2 \textsuperscript{87}, respectively, by two groups that simultaneously discovered them. Orexin-containing neurons have extensive projections throughout the whole central nervous system \textsuperscript{88,89}. After central administration, these peptides increase food intake and display other functions like induction of generalized behavioural arousal or influence of the sleep/wake cycle \textsuperscript{86,90,91}. Both peptides derive from the same 131 amino acid precursor, prepro-orexin, whose mRNA is produced in defined regions of the lateral and perifornical hypothalamus (LH and PFH) and upregulated upon fasting. Orexin containing neurons have been shown to have leptin receptor immunoreactivity, which suggests a direct effect of leptin on the expression of orexin mRNA \textsuperscript{92}. Orexin A and B are endogenous ligands of two closely related heptahelical G-protein-coupled receptors termed OX\textsubscript{1} and OX\textsubscript{2}. Orexin A shows higher affinity at OX\textsubscript{1} receptors, whereas the affinity of the two peptides at OX\textsubscript{2} receptors is in the same range. Unlike NPY, the orexin receptors are coupled to G\textsubscript{q}-proteins, and transmit their effect by modulation of the intracellular Ca\textsuperscript{2+}-level. Up to now, no structure-activity relationships of any of the two orexin peptides have been reported, nor any potent subtype selective antagonist or agonist is known to characterise the physiological and pharmacological role of the different receptors. Their location in the LHA/PFA suggests them to be molecules of the second-order neuronal signalling, downstream of NPY. In contrast, recent studies showed the opposite: a synthetic NPY Y1 antagonist could block orexin A induced feeding behaviour in rats \textsuperscript{93}. A cross-communication between orexin- and NPY-containing neurons is furthermore indicated because orexin-containing fibers and nerve terminals are associated with NPY neuronal cell bodies and vice versa \textsuperscript{94,95}.

The unidirectional model of first- and second-order signalling neurons is enlarged by the fact, that many neurons of the PVN, PFA and LHA project back to the arcuate nucleus, which suggests a bidirectional neuronal traffic flow. Accordingly, second order neurons can modify the arriving signals and are not just
1.3 Neuropeptides: key position in the regulation of food intake

Recipients of information from the arcuate nucleus. Additionally, leptin receptors have been described on PVA and LHA neurons as well, which makes them to direct targets for adiposity signals from the periphery. However, the density of leptin receptors in the hypothalamus is significantly higher in the ARC than in all other hypothalamic sites.

One additional target of leptin in the hypothalamus is the anabolic neuropeptide galanin. Galanin is a 30 residue gut-brain peptide that is involved in a wide range of actions, including modulatory action on gut motility and central induction of food intake. It influences several memory tasks and is overexpressed in the basal forebrain in Alzheimer's disease. Galanin and galanin receptors are widely distributed within the central nervous system. Historically, much research has been focused on hypothalamic galanin systems, including the preoptic area, the PVN, the supraoptic nucleus (SON) and median eminence. Two distinct galanin receptors have been cloned and characterized, termed GalR1 and GalR2. GalR1 mRNA was demonstrated in the SON and magnocellular (mPVN) and parvocellular (pPVN) region of the PVN by in situ hybridization histochemical studies of rat brain, whereas GalR2 mRNA was found only in the pPVN and its existence in the SON is discussed controversially. Intracerebroventricular injection of galanin induces food intake and administration of leptin decreases the hypothalamic galanin gene expression in rats. The anabolic property of galanin and its expression in the PVN contradict somehow the thesis of a 'satiety center' in the PVN, which primarily contains catabolic signal molecules. However, it supports the hypothesis of an interconnected network composed of a series of signal transducting molecules that are involved in the regulation of food intake and energy homeostasis. Further evidence for this is given by direct contacts and interplay among galanin, neuropeptide Y and β-endorphin, that all are involved in the regulation of food intake.

The hypothalamic regulation of food intake and energy homeostasis is only part of a complex system that includes signals from the gastrointestinal tract, the liver, olfactory signals or signals from the environment, to mention only a few. However, a more complete understanding of the signal transduction in the hypothalamic area gives raise to the hope of finding pharmacological treatments to influence the
central regulation of food intake and thereby reduce the enormous cost to human health which arise from obesity and food intake.

1.3.2 Obesity, a question of genes and environment

What we eat and how much we eat may vary considerably from one meal to the next and from one day to the other. Energy intake is affected by factors like time of day, physical activity, emotions, social factors, convenience and cost, which are variables that are not biologically determined. However, regarding a longer period of time, energy intake and energy expenditure are in a quite precise equilibrium for most of us. This suggests a genetically determined regulatory process—called energy homeostasis—that matches energy intake and energy expenditure and promotes a stable body weight. Genetic mutations in mice and humans that result in an obese phenotype, support the theory of genetical regulation of food intake (reviewed in). The identification of the genes that mainly regulate energy homeostasis turned out to be very difficult. This might be due to a polygenic predisposition to obesity that involves many genes among each only has a small effect. Thus, it is not surprising that obesity mostly is only one amongst several anomalies in animals and humans with genetical variations. For syndroms like Prader-Willi, Cohen, Alstrom, Bardet-Biedl, and Borjeson-Forssman-Lehmann, the causative genes have not yet been isolated, although one or more map positions have been determined. Efforts to clarify the relationship between geno- and phenotype led for example to the so-called ‘human obesity gene map’. This contains entries for more than 40 genes and 15 chromosomal regions, which are indicated to have a relationship to adiposity or a related phenotype. However, the theory of obesity as genetic variation and high body weight as a genetically determined mismatch in energy homeostasis provides a starting point for efforts to identify the genes involved.

Regarding the dramatic raising of obesity especially in newly westernized (so-called ‘Coca-Cola-nized’) societies, the explanation of obesity as exclusively genetically determined illness seems to be insufficient. The most recent health survey in England and Wales has confirmed an increase in obesity from 6% in adult men and 8% in adult women in 1980 to 17% of men and 20% of women in
1997. Recent data from the United States show that about 20% of US men and 25% of US women are obese, as proven by the third National Health and Nutrition Examination Survey (1988-1994). Besides Europe and America, a rise of obese people is also seen in all populations in Southeast Asia. In Japan and China a pronounced increase of people with overweight or obesity has been observed during the past two decades. In Malaysia obesity increased that much, that it is now more prevalent than undernutrition in both urban and rural communities. But the most alarming situation is found in the Pacific region. The prevalence of obesity in urban Samoa is valued to be greater than 60% of adult men and 75% of adult women. Also in the Middle East, obesity is regarded to be a severe problem. In the United Arab Emirates obesity is recognized as a major public-health problem that may be important in the increasing occurrence of other chronic diseases.

The influence of cultural and environmental factors on body weight is frequently witnessed in migrant studies by a marked change of the Body Mass Index (BMI, defined as weight in kilograms divided by square of the height in metres, Table 3). For example, Pima Indians living in the United States are on average 25 kg heavier than Pima Indians living in Mexico. The situation for Afrikans is homologous. Nigerian men and women living in the United States have an average BMI of 27.1 and 30.8 respectively, whereas the BMI for Nigerians in Africa is only 21.7 and 22.6.

<table>
<thead>
<tr>
<th>BMI [kg/m²]</th>
<th>Popular description</th>
<th>WHO classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;18.5</td>
<td>Thin</td>
<td>Underweight</td>
</tr>
<tr>
<td>18.5 – 24.9</td>
<td>Normal</td>
<td>-</td>
</tr>
<tr>
<td>25.0 – 29.9</td>
<td>Overweight</td>
<td>Grade 1 overweight</td>
</tr>
<tr>
<td>30.0 – 39.9</td>
<td>Obesity</td>
<td>Grade 2 overweight</td>
</tr>
<tr>
<td>&gt; 40.0</td>
<td>Morbid Obesity</td>
<td>Grade 3 overweight</td>
</tr>
</tbody>
</table>

Table 3. Classification of overweight by a WHO expert committee. Body Mass Index (BMI) is the weight in kilograms divided by the square of the height in meters.
Besides cultural influences, energy expenditure is a further important environmental factor in the regulation of energy homeostasis. Mainly physical activity represents 20-50% of total energy expenditure. A direct relationship between obesity and reduced physical activity is found in developed countries. Children in the United States, for example, have a 5.3 times greater risk for obesity if they watch television for more than 5 hours each day, compared with children that watch less than two hours, even after correcting for a wide range of socioeconomic variables. In England and Wales, people of the low social classes IV and V spend significantly more time watching television and are more likely to define themselves as inactive compared with those in the high social class I. This is accompanied by a strong gradient in the prevalence of obesity related to social class, especially in women. 10.7% obese women in the high social class I are opposed by 25% in the low social class V.

Regarding all these facts, Kopelman concludes that obesity is not a single disorder but a heterogeneous group of conditions with multiple causes. Body weight is determined by an interaction between genetic, environmental and psychosocial factors acting through the physiological mediators of energy intake and expenditure. Although genetic differences are of undoubted importance, the marked rise in the prevalence of obesity is best explained by behavioural and environmental changes that have resulted from technological advances.

1.4 From the peptide to the target in medicinal chemistry

A big number of physiological and biochemical processes are mediated by peptides that display hormonal or neurotransmitter function. Even in our days new compounds are found frequently, like for example the neuropeptides orexin A and B (also known as hypocretin 1 and 2) that were discovered in 1998 on the search for endogenous ligands of orphan G-protein coupled receptors. Each recovery of a novel ligand starts a process to find, localize and define all possible targets, usually defined proteinogenic receptors that might split up into a number of subtypes with different pharmacological profiles. It is one of the main goals of medicinal chemistry, to contribute to a detailed understanding of the biochemical
processes that regulate and modify physiological processes in order to develop suitable medicinal therapies in the case of malfunction of cellular systems that cause illness. The broad research field of neuropeptide Y (NPY) is an good example for “from the peptide to the target”, or “how to get knowledge about distribution and function of a newly identified peptide”. NPY was isolated from pig brain in 1982. Already in 1986 Wahlestedt et al. predicted the occurrence of two receptor subtypes based on the requirement of the entire neuropeptide Y molecule for binding to the Y1-receptor, while C-terminal segments (13-36 and 18-36) of NPY bind to the Y2-receptors only slightly less (IC$_{50}$ 5-10-fold). Today six different NPY receptor subtypes are cloned (Y$_1$, Y$_2$, Y$_4$, Y$_5$, y$_6$, y$_7$) and further subtypes postulated, all of them correlated to a distinct pharmacological profile.

How to get these information? Receptor orientated research gives direct insight into distribution of an investigated receptor by immunohistochemistry using fluorescence-, confocal- and electron-microscopy. Selective anti-receptor antibodies can also be used in competition binding studies with the native ligand to give information about the binding site. Site-directed mutagenesis of a receptor provides knowledge on the positions that are important for ligand binding and/or for receptor activation. In case different subtypes of an investigated receptor have been cloned, modified ligands are frequently used to define the pharmacological properties. Frequently, however it is just the other way round. Different ligand analogs show distinct activities and lead to the suggestion of receptor subtypes based on the different pharmacological profile. Accordingly, modified peptide ligands are important tools to characterize signal transduction pathways.

Nowadays, two approaches are used to obtain compounds with the desired properties. The so-called combinatorial and the rational approach. In the rational approach, first the minimal sequence has to be identified. Native ligands are shortened by N- and/or C-terminal truncation or by deletion of amino acids along the sequence, so-called central truncation. The stepwise shortened ligands get tested regarding to binding and functional properties. Peptide ligands that are able to activate a receptor usually have to be longer than ligands with pure antagonistic properties, because a sufficient number of contact points between the ligand and
the receptor have to be ensured in order to cause a conformational change of the receptor that culminates in signal transduction. Sometimes it is already sufficient to exchange a peptide amide with the corresponding peptide acid to lose agonistic properties completely. After having detected the critical structural requirements for receptor binding and receptor activation, molecules with further modifications are synthesized to get more detailed information about ligand-receptor interaction, about putative receptor subtypes or to reach higher binding than the native ligand or get receptor subtype selectivity. Such modifications might be based on molecular modelling studies, that start from structural information about the native ligand. Computer based variations of the molecule, followed by energy minimization, give theoretical hints for the synthesis of new ligands. In order to understand the importance of each part of a peptide, single exchanges of each residue with L-alanine is performed. Such alanin-scans give information about the importance of each position with respect to binding, i.e. of the chemical properties of each side-chain. Information about the role of the orientation of the side-chains can be obtained by scans with D-isomers of the corresponding amino acids. A frequently applied way to reach receptor subtype selective ligands is based on the reduction of conformational flexibility of the native peptide ligand. Receptor selectivity due to increased rigidity will be obtained if the stabilized or induced conformation corresponds to that adopted from the native ligand during interaction with one specific receptor. If not, the reduced flexibility leads to reduced affinity at the receptor subtypes. Conformationally constrained peptides are performed by the introduction of special rigid amino acids or by backbone modification. Cyclisation of a peptide chain is another frequently used method to reduce the conformational flexibility of a peptide. This is performed for example by disulfide bridges, connecting the side chains of two cysteine residues, or by lactam bridges which are cyclic amids that connect an amino- and an acidic side chain. The combinatorial approach starts from a peptide library which consists of a high number of peptides that exhibit different amino acid sequences. Hereby they can correspond partly to the native peptide that should be mimicked, but this is no precondition. Peptide libraries can be obtained for example by solid phase peptide synthesis. The peptides, bound to a polystyrol-resin by a linker, are elongated amino acid by amino acid. The resin particles are mixed and newly apportioned
after each coupling step of different amino acids in different vessels. If binding
Tests of a peptide library result in a hit, the amino acid sequence (primary
structure) of the at first unknown peptide is subsequently analyzed.

What is the major aim of the study? The availability of highly receptor subtype
selective agonists and antagonists is a main target in structure-affinity and
structure-activity relationship studies. This allows the characterization of receptor
subtype specific ligand-receptor interaction and possibly the postulation of further
receptor subtypes. Additionally, ligands that bind selectively to distinct receptor
subtypes would be wonderful tools to localize the distribution of each receptor
subtype with radioactive analogs and to characterize their pharmacological and
physiological role in vitro and in vivo. A further aim of structure-affinity and
structure-activity relationship studies is to identify the bioactive conformation of a
ligand at a distinct receptor subtype, in order to develop selective and potent non-
peptide drugs. Because endogenous peptides themselves typically have some
limitations regarding bioavailability and oral activity, mimetics are used to
overcome the limits of the native peptides. Here it turned out that it is more difficult
to obtain selective non-peptide agonists for peptidic G-protein coupled receptors
than selective antagonists \(^{129}\). This might be due to a lack of contact points
between the ligand and the receptor in the case of the small non-peptide ligands
and the subsequent inability to induce the receptor to change from the inactive to
the active conformation. Peptide mimicry by design was focused on imitating
secondary structural motifs, particularly \(\beta\)-turns, but this approach has yielded few
pharmaceutical products. Today, the process of identifying and optimizing peptide
mimics is driven mainly by screening to obtain hits, followed by optimization, which
might include design based on arranging pharmacophores appropriately in three
dimensions. A consequence of this is that one of the more difficult problems in
drug discovery, namely the identification of non-peptide agonists at peptide
receptors, is beginning to be solved \(^{130}\).
1.5 Structure-activity studies on selected neuropeptides with respect to their role in obesity

Structure-activity studies are the basis for a detailed understanding of the interaction of a ligand at a distinct receptor or receptor subtype. A series of selected hypothalamic neuropeptides will be discussed with respect to the structure-affinity and structure-activity relationship and their role in obesity. Native amino acid sequences are listed in Table 4.

Table 4. Amino acid sequences of selected hypothalamic neuropeptides with influence on the regulation of energy homeostasis and food intake. Ac = N-terminal acetylation, U = pyroglutamic acid

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Size [no of amino acids]</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>146</td>
<td>VPIQKVQDDTKTLIKTIVTRINDISHTQSVMKSQKVTGGLRGHLIPNLNLTSMDQTLAVYQIQLTSMPSRNVIQISNDLENLRDDHLAFSKSCHLPWASGLETLDLSGLQVLEASGYSTEVVALSRLQGLQDMLWQLDLSPGC-COOH</td>
</tr>
<tr>
<td>α-MSH</td>
<td>13</td>
<td>Ac-SYSMEHFRWGKPV-NH2</td>
</tr>
<tr>
<td>CART</td>
<td>89</td>
<td>QEDAELOPRALDIYSADDASHEKEKOLEALQEVLLKLSKRPVTEYKEKYGQVPMCDAGEQCARVRKARGIKLCQPGTSCNSFLKCL-COOH</td>
</tr>
<tr>
<td>pNPY</td>
<td>36</td>
<td>YPSKPDIPNGEDAPAEFLYSAVDDASHEKELIEALQEVLLKLSKRPVTEYKEKYGQVPMCDAGEQ</td>
</tr>
<tr>
<td>AGRP</td>
<td>112</td>
<td>AQMGLAPMEGIRRPDQALLPELPGLGLRALKTTAEQAEDELLLQLAAGALAEVLDLQDREP</td>
</tr>
<tr>
<td>Orexin A</td>
<td>33</td>
<td>UPLPDCCRQKTSCRLYELLHGAGNHAGILTL-NH2</td>
</tr>
<tr>
<td>Orexin B</td>
<td>28</td>
<td>RSGPPGLQGRGQLLQASONHAAGILTMM-NH2</td>
</tr>
<tr>
<td>MCH</td>
<td>19</td>
<td>DFDMLPQMLGRVYRPCWQV-COOH</td>
</tr>
<tr>
<td>Galanin</td>
<td>30</td>
<td>GWTLNSAGYLLGMHAGNHRSFSDKNGLTS-COOH</td>
</tr>
</tbody>
</table>
1.5.1 Leptin

Leptin is a 146 amino acid signalling peptide that is processed of a 167 amino acid precursor by N-terminal truncation. The two C-terminal cysteins in positions 117 and 167 are connected to a disulfide bond and form a big C-terminal cycle. Leptin is produced in adipocytes and secreted to the systemic blood circulation. Its concentration in the blood is a value for the amount of body fat. Leptin influences energy homeostasis and reduces food intake by acting on specific receptors in the hypothalamus. Highest receptor density is found in the arcuate nucleus, but also other hypothalamic regions like the paraventricular nucleus (PVN), the lateral hypothalamus and perifornical area are found to express leptin receptors. The leptin receptor and various splice variants are cloned and found to have sequence similarity to the class I cytokine receptor family. Mouse leptin was found to form a four-helix bundle by NMR and to adopt a cytokine fold similar to the short-helix subfamily of cytokine folds. Structure-function studies of human leptin revealed that the C-terminal disulfide loop was not necessary to reduce food intake. Mutant-type leptin lacking the C-terminal disulfide bond reduced food intake as effective as the wild type leptin. Deletion of the 50 C-terminal amino acids resulted in a peptide that reduced food intake to a lesser extent, whereas a peptide with deletion of the N-terminal 93 amino acids lost any effect on food intake. This suggests that the N-terminal region of leptin is essential for receptor binding and biological activity. The C-terminal structure is suggested to be important for enhancing biological actions, whereas the C-terminal disulfide bond is not needed. In contrary to this, C57BL/6J ob/ob mice have a single base mutation of the ob gene in codon 105 which results in a truncated inactive form of leptin. This suggests that leptin activity may be localized, at least in part, to domains distal to amino acid residue 104. Accordingly, a synthetic peptide amide corresponding to amino acid residues 116-130 of mouse leptin, LEP-(116-130), was found to reduce body weight gain, food intake and blood glucose levels in ob/ob and db/db mice. Further investigations showed that the activity of LEP-(116-130) resides in a restricted sequence between amino acid residues 116-122. A synthetic peptide corresponding to this sequence (SCSLPYT) has been named OB3. A D-amino acid scan of OB3 showed a significant increase (2.6-fold) in the
weight-reducing effect of \([D-\text{Leu}^4]-\text{OB3}\) compared with OB3. Unlike native leptin, however, neither OB3 nor any of its D-amino acid-substituted analogs had any apparent effect on thermogenesis. Results like these indicate that synthetic peptide strategies may be useful in the development of potent stable pharmacophores with potential therapeutic significance in the treatment of human obesity and its related metabolic dysfunctions 136.

1.5.2 POMC and \(\alpha\)-MSH

The melanocortin system plays an important role in the field of regulation of food intake. Melanocortins are cleavage products of pro-opiomelanocotin (POMC), a 241 amino acid precursor, and bind to melanocortin (MC) receptors. Amongst the melanocortin receptors, named MC1 to MC5, the MC4 receptor is of outstanding importance concerning energy homeostasis. It belongs to the group of \(G_\alpha\) protein coupled receptors, that increase intracellular cAMP and PKA signalling after activation by an agonistic ligand. The N-terminally acetylated, thirteen amino acid peptide \(\alpha\)-MSH displays agonistic effect on the MC4 receptor and suppresses food intake, especially after injection into the paraventricular nucleus (PVN), a site where MC4-receptor gene expression is very high 137. It is a cleavage product of POMC, which firstly is processed into corticotropin (ACTH, POMC 112-150). This can be further cleaved to form \(\alpha\)-MSH (Ac-(112-124)-NH\(_2\)). Amino acids 6 to 9 of \(\alpha\)-MSH (HFRW), frequently referred to as the ‘active core’, are also present in other melanotropins (\(\beta\)-MSH, \(\gamma\)-MSH) and have been recognized to be necessary for efficient binding to and activation of the different melanocortin receptors 138,139. Phe and Trp are bulky aromatic amino acids in the active core and His and Arg are two basic hydrophilic amino acids that all are essential. This suggests that both, hydrophobic and hydrophilic interactions directly interact with the melanocortin receptors. A high number of analogs has been synthesized throughout the years. The core sequence of \(\alpha\)-MSH, which lacks the N- and C-terminal amino acids, is sufficient for activation of the melanocortin receptors and favoured the synthesis of cyclic MSH-analogs. Two of them shall be further discussed, because of their relevance in many pharmacological tests (Fig. (2)) 137,140 in the field of regulation of food intake. Both peptides are cyclic lactam
derivatives of \( \alpha \)-MSH: MTII \(^{141} \) (Ac-Nle\(^4\)-cyclo(5\( \beta \)-10\( \varepsilon \))(Asp\(^5\)-His\(^6\)-D-Phe\(^7\)-Arg\(^8\)-Trp\(^9\)-Lys\(^{10}\))-amide) is a potent but not selective agonist at the MC3-, MC4-, and MC5-receptors. In contrary, the almost similar derivative SHU9119 \(^{142} \) (Ac-Nle\(^4\)-cyclo(5\( \beta \)-10\( \varepsilon \))(Asp\(^5\)-His\(^6\)-D-Nal(2\(^\prime\))-Arg\(^8\)-Trp\(^9\)-Lys\(^{10}\))-amide) displays potent antagonistic activity at the human MC3- and MC4-receptor, but remaining agonistic activity at the MC5-receptor. Accordingly position 7 (Phe) in \( \alpha \)-MSH plays an important role in receptor activation. Whereas D-Phe in combination with cyclisation leads to a partially selective agonist, D-Nal\(^2\) at position 7 leads to antagonistic properties. Structure-function studies on MTII indicated that the basic, hydrophilic residues His\(^6\) and Arg\(^8\) are less essential for the activation of the MC receptors 3, 4 and 5 whereas the hydrophobic aromatic residues Phe\(^7\) and Trp\(^9\) seem to play a significant role in receptor recognition \(^{143,144} \).

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**Fig. (2).** Amino acid sequence of \( \alpha \)-MSH and its analogs MTII and SHU9119. MTII is a potent but not selective agonist at the MC3-, MC4- and MC5-receptor, whereas SHU9119 displays antagonistic activity at the human MC3- and MC4-receptor and agonistic activity at the MC5-receptor. The so-called "active core" of \( \alpha \)-MSH is highlighted in grey.
1.5.3 CART

In May 1998 the cocaine- and amphetamine regulated transcript peptide (CART) was added to the list of hypothalamic neuropeptides with effect on feeding behaviour. Besides its potent appetite suppressing activity, it modulates locomotor activity and stress-reaction, is involved in sensory processes in rats and may play a role in effects of psychostimulants. Clues to the existence of CART peptides first showed up in the brains of rats given cocaine and amphetamine by identification of elevated CART mRNA. The meaning of the acronym “CART” varies in the literature. In this article it refers to the peptide, other authors refer it to the corresponding mRNA.

In rats, CART mRNA encodes a peptide of either 129 or 116 amino acid residues, whereas only the short form exists in humans. The N-terminal 27 amino acids are predicted as signal sequence, resulting in a human CART peptide of 89 residues. Spiess and co-workers isolated a peptide from ovine hypothalamic extracts that could be identical to CART(42-89), thus corresponding to processing at the Lys$^{40}$-Arg$^{41}$ sequence. This C-terminal part is thought to constitute a biologically active part of the molecule. It contains six cysteine residues, which in the recombinant peptide have been shown to be disulfide linked ($C^{65-73}$, $C^{61-81}$, $C^{75-88}$) to form a rather compact structure. In rats, central administration of CART(1-26) could not increase locomotor activity, whereas the endogenously occurring C-terminal part of the longer splice variant, CART(55-102), dosedependently increased locomotor activity. Because of the influence of CART on food intake, it was recently hypothesized that genetic variation in CART may contribute to human obesity. A series of polymorphisms were found. However, up to now no clear association could be found between genetic variation in the CART locus and development of an obese phenotype.

1.5.4 Neuropeptide Y

Neuropeptide Y (NPY) is one of the most abundant neuropeptides in the brain. It belongs to the pancreatic polypeptide hormone family (also called NPY family), which further includes peptide YY (PYY) and pancreatic polypeptide (PP). All of these peptides are C-terminally amidated and consist of 36 amino
acids. The first isolation of NPY was performed from extracts of porcine brain in 1982. NPY is the most conserved peptide in the PP family, with at least 61% identity in all known species. Seven positions are constant among all different sequences of NPY, PYY and PP: Pro, Pro, Gly, Ala, Tyr, Arg and Arg. Another four positions are highly conserved: Pro, Tyr, Thr and Tyr. The three-dimensional solution structure of NPY has been investigated by 2D-NMR and circular dichroism spectroscopy (CD) and related to the structure of avian PP, that was determined by X-ray crystallography. Accordingly, aPP crystals consist of an extended type II polyproline helix (residues 1-8), followed by a turn (residues 9-13) and an amphipathic α-helix (residues 14-31). The resulting hairpin-like fold, also referred to as PP-fold, is assumed to be the structural feature common within the whole NPY family (Fig. 3)). A three-dimensional structure of human NPY in water at pH 3.2 was suggested by Darbon and co-workers to adopt a polyproline stretch (residues 1-10) followed by a tight hairpin structure (residues 11-14) and two short α-helices (residues 15-26 and 28-35). N- and C-terminal ends were kept close together by hydrophobic interaction. Slightly different from this model, Cowley and co-workers and Monks and co-workers found a dimeric NPY structure, that consists of an antiparallel, hydrophobic packing of two helical units (residues 11-36 or 13-36). In a recent study it has been shown that the monomer and the dimer of NPY both are simultaneously and in equilibrium present under NMR conditions.

NPY induces its effects by at least four different receptor subtypes. They are designated as Y, Y, Y, Y, Y and y-receptors and belong to the superfamily of heptahelical G-protein coupled receptors. The Y-receptor has been described only pharmacologically and its existence still is debatable. The y-receptor has been found in mice and rabbits, but not in primates, although its mRNA is present in various tissues. Because no physiologically relevant actions could be attributed to the y-receptor, it is designated by lower case y. NPY signalling inhibits the formation of cAMP by inhibition of the adenylyl cyclase through G-proteins of the G/G family. Additional effects like modulation of K+ and Ca2+ channels have been observed in neurons and in the vascular bed, respectively.
Fig. (3). (A) Model of the structure of the PP-family peptides. N-terminal polyproline helix and C-terminal α-helix are connected by a hairpin-like turn, called PP-fold. The seven constant positions are indicated and highlighted in grey, the four mostly constant positions in brackets and light grey. (B) Amino acid sequences of hNPY, pPYY and hPP. The constant positions among all investigated species are underlined. Constant positions among all peptides of the PP-family are highlighted in grey and light grey for mostly constant positions, respectively.

It is not yet fully understood, which receptor subtype is related to which physiological effect. In case of the regulation of energy homeostasis, an interaction of the Y₁- and Y₅-receptors is suggested that are both involved in the stimulation of feeding ⁵¹. Deficiencies in either the Y₁- or the Y₅-receptor change normal feeding response to fasting or NPY stimulation, which indicates that both receptors play an important role in NPY induced appetite control ¹⁶¹,¹⁶². The contribution of each amino acid of NPY to the ligand-receptor interaction was examined by a so-called L-Ala-scan. The most sensitive positions with respect to binding to the Y₁ receptor were found to be Pro², Pro⁵, Arg¹⁹, Tyr²⁰ and the whole C-terminal part 27 to 36, whereas the two Arg residues at positions 33 and 35 are found to be essential ¹⁶³. Pro in positions 2 and 5 and Tyr in positions 20 and 27 may be important in stabilizing the hairpin-like structure of NPY by hydrophobic
interaction. A D-amino acid scan of NPY showed a similar profile to the L-Ala-scan and suggests that the amino acids are equally sensitive to both: side chain orientation and its chemical properties \textsuperscript{164}. N-terminal as well as C-terminal truncation of NPY resulted in a loss of affinity at the Y\textsubscript{1}-receptor and indicate the importance of the peptide's terminals for ligand binding and receptor activation. Even the change of the peptide amide to the corresponding peptide acid resulted in a complete loss of affinity at the Y\textsubscript{1}-receptor. Despite that, GW1229 (also known as GR231118 or 1229U211), a dimer of an analog of the C-terminal nonapeptide of NPY was found to be a highly potent antagonist at the Y\textsubscript{1}-receptor and agonist at the Y\textsubscript{4}-receptor \textsuperscript{165}.

In contrary to the Y\textsubscript{1}-receptor, the Y\textsubscript{5}-receptor tolerates short N-terminal truncations like NPY\textsuperscript{2-36} and NPY\textsuperscript{3-36} \textsuperscript{124}. Regarding receptor selectivity at the Y\textsubscript{5}-receptor subtype, positions 31 and 32 seem to be of special importance. [A\textsuperscript{31}, Aib\textsuperscript{32}] NPY (Aib = \(\alpha\)-amino isobutyric acid) binds highly selective to the Y\textsubscript{5} receptor \textsuperscript{124}. This compound, as well as the more potent PP/NPY chimera [cPP\textsuperscript{1-7}, NPY\textsuperscript{19-23}, Ala\textsuperscript{31}, Aib\textsuperscript{32}, Gln\textsuperscript{34}]-hPP have been found to increase food intake in rat, the latter was even 3-fold as potent as NPY in binding to the Y\textsubscript{5}-receptor and in increasing food intake \textsuperscript{166}.

1.5.5 AGRP

Agouti related peptide (AGRP) is a rather long, naturally occurring antagonist of the brain melanocortin receptors MC3 and MC4 consisting of 112 amino acids. An N-terminal signal sequence of 20 amino acids is cleaved and therefore the numbering varies between either AGRP\textsuperscript{1-112} or, mostly applied, AGRP\textsuperscript{21-132}, which stands for the same peptide. AGRP is structurally related to agouti protein. Both of them contain 10 cysteine residues in the C-terminal domain that are arranged in five disulfide bonds. The exact mechanisms of AGRP and Agouti protein have been difficult to examine, partly because of difficulties in producing homogeneous forms of these molecules \textsuperscript{167}. N-terminal truncation of AGRP revealed that the cysteine-rich C-terminal part of AGRP is sufficient for receptor binding at the MC3, MC4 and MC5 receptor. AGRP\textsuperscript{87-132} displaced the binding of a \([\textsuperscript{125}I]-\alpha\)-MSH analog at MC-receptors and vice versa. Accordingly, it has been suggested that these
molecules bind in a competitive manner to melanocortin receptors. The antagonistic properties of the C-terminal fragments of AGRP at MC3 and MC4 receptors have been tested by intracerebroventricular (ICV) administration of AGRP83-132, that increased food intake over a 24-h period similar to the synthetic MC3 and MC4 receptor antagonist SHU9119 (see above). In addition, AGRP83-132 and SHU9119 both blocked the reduction of 1-h food intake after ICV administration of α-MSH. Cyclic AMP assays of AGRP83-132 showed an inhibitory effect similar to that reported for the full length peptide. Further investigations revealed the importance of the residues 110 to 117. One possible arrangement of the disulfide bonds could be an octapeptide loop formed by Cys110-Cys117. Four residues within this loop, namely Arg111-Phe112-Phe113-Asn114, bear striking resemblance to the most important amino acid sequence of α-MSH (His6-Phe7-Arg9-Trp9) including two aromatic and two basic amino acids. Cyclic synthetic analogs based on the sequence of this loop (AGRP110-117) were found to be functional antagonists of the human MC4 receptor, while they exhibit a lower affinity at the MC3 receptor. The RFF111-113-triplet appeared to be the most important portion of AGRP with respect to binding to MC3 and MC4 receptors. These findings suggest that the octapeptide loop of AGRP110-117 mimics the ‘active core’-conformation of α-MSH. A most recent work confirmed the antagonistic activity of the disulfide bridged Y-cyclo[CRFFNAFC]-Y and revealed an unexpected agonistic MC1-receptor activity of this cyclopeptide.

1.5.6 Orexin A and B

Orexin A and B (also known as hypocretin 1 and 2) are two newly discovered neuropeptides, that are involved into energy homeostasis and display a potent stimulation of food intake after ICV administration. Both peptides derive from prepro-orexin, a 131 amino acid acid precursor, produced in defined regions of the lateral and perifornical hypothalamus, whose mRNA is upregulated upon fasting. Orexin A is a 33 amino acid peptide amide containing two intramolecular disulfide bonds from Cys6 to Cys12 and Cys7 to Cys14, respectively. The linear peptide amide orexin B consists of 28 amino acids and shares 46% structure identity with orexin A, which is mainly comprised at the C-terminus. The three-
1.5 Structure-activity studies on selected neuropeptides with respect to their role in obesity

dimensional solution structure of orexin B, determined by 2D-NMR, has been published recently. Accordingly, orexin B consists of two alpha-helices, connected by a short linker sequence from position 20 to 23. The orexins bind to two closely related Gq-protein coupled receptors (64% amino acid identity), which are named OX1- and OX2-receptor. Whereas orexin A has a higher affinity to the OX1-receptor, equipotent affinity was found for orexin A and B to OX2.

Up to now, no structure-affinity studies of the orexins have been published, nor receptor subtype selective ligands are known that would allow pharmacological and physiological characterization of the two orexin receptors. According to preliminary data, the almost identical C-terminal segments of orexin A and B are sufficient for high affinity and signal transduction at the orexin receptors, while N-terminal segments result in a marked decrease or complete loss of binding. The binding of orexin A was found to be thermodynamically weak by rapid dissociation upon dilution, as it would be expected for an association mainly based on ionic interactions.

1.5.7 MCH

Melanin-concentrating hormone (MCH) is a cyclic, nineteen amino acid hypothalamic neuropeptide with an intramolecular bond of Cys7 to Cys16. It was first isolated and characterized from chum salmon pituitary as a hormon that is responsible for color changes in response to the environment. Its involvement in the regulation of food intake was supported by the observation of an increased feeding in rats after ICV injection of MCH. MCH mRNA was up-regulated during fasting and in leptin deficient obese ob/ob mice. The food intake restricting effect of melanocortins in the hypothalamus was antagonized by MCH and MCH knockout mice displayed reduced body weight and an increase in metabolic rate.

The disulfide bond Cys7-Cys16 was reported to be essential for MCH-like activity, since contractions of the ring led in almost all cases to inactive peptides. N-terminal truncation of MCH was tolerated and the minimal fragment that was found to possess equipotency to MCH was the sequence MCH(5-15). The C-terminal part of MCH was shown to be involved into ligand-receptor
interaction because analogs with exchanged Trp in the C-terminal sequence, lost full agonism and equipotency to MCH. A most recent work suggests that Arg$^{11}$ (in the middle of the Cys-loop) is required for the formation of the MCH peptide-receptor-complex, by forming a direct interaction with Asp$^{123}$ of the MCH receptor, as indicated by molecular modelling. Substitution of Arg$^{11}$ by Ala displayed a 3000-fold reduction in binding and a complete loss of measurable functional activity. [Lys$^{11}$]-MCH and [D-Arg$^{11}$]-MCH were found to maintain a reduced affinity for the MCH-receptor, with the lysine analog being a partial agonists (approximately 67%) and the D-arginine analog being a functional antagonist at MCH-receptors.

1.5.8 Galanin

Galanin is a linear 30 residue peptide that is widely distributed within the central nervous system and the periphery. Galanin was first isolated from porcine small intestine. The porcine galanin structure, beginning with an N-terminal glycine and ending with a C-terminal alanine gave the peptide its name. The human sequence differs slightly from the porcine sequence. However, galanin shows 90% homology throughout the species, but little similarity to other peptides. Galanin receptors are widely distributed throughout the body. Centrally, it coexists with classical neurotransmitters such as acetylcholine, norepinephrine, GABA and serotonin. Galanin causes a diverse set of physiological responses such as a decrease in the release of the neurotransmitters norepinephrine and acetylcholine. In the hypothalamic, galanin is found in the preoptic area, the PVN, the supraoptic nucleus (SON) and median eminence. There it induces feeding behavior, stimulates growth hormone release, and inhibits corticosterone release. Peripherally, galanin inhibits glucose induced insulin secretion and somatostatin release. Galanin also causes smooth muscle contraction. Receptors have been found throughout the gastrointestinal and genitourinary tracts.

Injection of galanin into the PVN of rats increased food intake. While NPY primarily stimulates the carbohydrate uptake, galanin favors the uptake of fat. For analysis of the critical amino acid sequence of galanin that is necessary to stimulate feeding behavior in rats, synthetic fragments of rat galanin
were administrated intraventricularly or into the PVN ⁹⁶. The entire galanin molecule rGAL(1-29) and the N-terminal galanin fragment rGAL(1-16) significantly increased feeding behavior. No significant effect was found for the fragments rGAL(1-9), rGAL(10-20), rGAL(12-29), rGAL(17-29) or rGAL(21-29), and for the analog [D-Trp⁵]-rGAL(1-16). Displacement experiments using rat hypothalamic membranes and ¹²⁵I-rGAL showed similar results. Therefore, the first 16 N-terminal amino acids are suggested to contain galanin agonist activity on increasing food consumption and the ability to bind to the galanin receptor in the rat hypothalamus ⁹⁶. Consistent with this hypothesis were the findings of Yanaihara and co-workers ¹⁸⁷. They tested 23 galanin related peptides and suggest that the fifteen N-terminal residues of galanin are crucial for its activity in glucose-stimulated insulin release, gastrin and somatostatin release, neurally evoked muscle contractions and C-fiber response in the isolated spinal cord of the new born rat. The galanin analogs [D-Thr⁶, D-Trp⁸,⁹]-galanin(1-15)ol and [D-Trp⁸,⁹]-galanin(1-15)ol were found to be potent antagonists for the inhibitory effect of galanin on the glucose-induced insulin release ¹⁸⁷.

1.6 Conclusion

In the past three decades, peptides reached more and more importance in the field of medicinal chemistry. Many peptides have been described to have important functions in signal transduction, displaying hormonal or neurotransmitter properties, and the lists are still growing. Big advances in synthetical and analytical methods allow the fast preparation and characterization of endogenously occurring peptides. Synthesis and examination of a big number of corresponding fragments and analogs give insight into pharmacological properties and ligand-receptor interaction of the native peptide ligands. As a consequence, genetic variation and polymorphism may be accounted for individual anomalies and illnesses.

The hypothalamic regulation of food intake represents a complex network of neuropeptides that act and interact within a redundant system. Leptin and insulin, two important peptidic peripheral adiposity signals, dose dependently modulate the expression and release of a series of neuropeptides in the hypothalamus,
mainly in the arcuate nucleus (ARC), but also in other hypothalamic sites. In the form of a first order neuronal signalling in the ARC, leptin and insulin stimulate the co-expressed catabolic peptides pro-opiomelanocortin (POMC) and cocaine- and amphetamine related transcript peptide (CART), whereas they inhibit the likewise co-expressed anabolic peptides neuropeptide Y (NPY) and Agouti-related protein (AGRP). Nerve fibres that contain these peptides project into other hypothalamic sites, where they initiate a second order neuronal signalling. Inhibitory effects on the lateral and perifornical hypothalamus (LHA/PFA), which comprise mainly anabolic peptides like MCH and the orexins A and B, are accompanied by stimulatory effects on the paraventricular nucleus (PVN), which comprises mainly catabolic neuropeptides like TRH, OXY or CRH.

One major aim of medicinal chemistry is to provide highly receptor subtype selective ligands in order to investigate the role of a single receptor subtype or to provide appropriate medical treatment. Because endogenous peptides themselves typically have some limitations regarding bioavailability and oral activity, non-peptide mimics are sought. The needful information to synthesize non-peptide ligands for peptide receptors might derive out of structure-affinity and structure-activity studies of the native peptide. Peptide fragments and analogs are screened for their activity and affinity to a distinct receptor and correlated with their three-dimensional structure. Synthetic peptide strategies turned out to be useful in the development of potent stable pharmacophores with potential therapeutic significance in the treatment of diseases like human obesity and its related malfunctions.
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CHAPTER 2

Novel Analogs of Neuropeptide Y with Preference to the Y1-Receptor

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European Journal of Biochemistry, submitted
2.1 Abstract

Neuropeptide Y (NPY) is one of the most abundant neuropeptides in the mammalian brain and evolves its action in human via at least three receptor subtypes, namely Y₁-, Y₂-, and Y₅-receptors. Whereas for the Y₂- and Y₅-receptors selective agonists and antagonists are known, the Y₁-receptor still lacks a highly selective agonist. This work presents the first NPY-based analogs with Y₁-receptor preference and agonistic properties. Furthermore, the importance of specific amino acids of NPY for binding to the Y-receptor subtypes is presented. Amongst the tested analogs, [F⁷, P³⁴] pNPY has been found to show most significant Y₁-receptor preference (>1:3000-fold), with subnanomolar affinity to the Y₁-receptor, and Kᵢ values of about 30 nM at the Y₂- and Y₅-subtype, respectively. Variations of position 6, especially [R⁶, P³⁴] pNPY and variations within positions 20-23 of NPY were found to result in further analogs with significant Y₁-receptor preference (1:400 - 1:2000). In contrast, cyclo S-S [Cys²⁰,²⁴] pNPY was found to be a highly selective ligand at the Y₂-receptor, binding only 3-fold less efficient than NPY. Analogs containing variations of positions 31 and 32 showed highly reduced affinity to the Y₁-receptor, while binding to the Y₅-receptor was less affected. Inhibition of cAMP-accumulation of selected peptides with replacements within position 20-23 of NPY showed preserved agonistic properties. The tested NPY analogs give insights into ligand-receptor interaction of NPY at the Y₁-, Y₂- and Y₅-receptor and contribute to our understanding of subtype selectivity. Furthermore, the Y₁-receptor preferring peptides are novel tools, that will provide insight in the physiological role of the Y₁-receptor.

Keywords: NPY, neuropeptides, structure-affinity relationship, food intake
2.2 Introduction

Obesity, food intake, energy homeostasis are keywords of still growing importance, since obesity is beginning to replace undernutrition and infectious diseases as the most significant contributor to ill health. Neuropeptide Y (NPY) is one of the most important effector molecules of leptin, the food intake modulating molecule plainly. Involving different receptor subtypes, NPY elicits a number of physiological effects. Profound effects on stimulation of food intake, secretion of luteinizing hormone, growth hormone and insulin release suggest an important role of NPY in the pathophysiology of obesity and diabetes. A wide range of other effects of NPY have been reported, like potent vasoconstriction, facilitation of learning and memory, modulation of locomotor behaviours, induction of hypothermia, inhibition of sexual behaviour, shifts in circadian rhythms, modulation of cardiorespiratory parameters, anxiolytic potency and inhibition of alcohol consumption and resistance.

NPY, a 36 amino acid peptide amide, belongs to the family of pancreatic polypeptides, that includes also pancreatic polypeptide (PP) and peptide YY (PYY). In 1982 it was isolated for the first time from pig brain.

NPY is widely distributed within the central nervous system and in the periphery. Its wide range of action is exerted via several receptor subtypes, named Y₁, Y₂, Y₅ and Y₆ receptor subtypes, whereas pancreatic polypeptide (PP) binds to Y₄/PP₁. A putative Y₃-receptor has been only described pharmacologically and no specific agonists or antagonists are known. All receptors belong to the superfamily of G-protein coupled receptors. The relation of a certain receptor subtype to a distinct physiological effect is not yet fully understood. Recent studies even suggest a team play of different receptor subtypes in a redundant system, as it is shown for the Y₁ and Y₅-receptor in the regulation of food intake. The findings, that deficiencies in either the Y₁ or the Y₅-receptors impaire the normal feeding response to fasting or NPY stimulation, strongly indicate that both Y receptors are involved in appetite regulation by NPY. Selective receptor agonists or antagonists are useful tools to study the functions of a single receptor subtype. In order to clarify the contribution of the Y₁- and the Y₅-receptor to the regulation of food intake and energy homeostasis within their interplay, it is
essential to be able to activate or inhibit each receptor selectively. Exchange of single or multiple amino acids, introduction of conformationally constraint amino acids or C-terminal, N-terminal or central truncations of native peptides are the mostly adopted ways to find receptor-selective peptide analogs. Efforts to obtain potent and selective nonpeptide agonists for G-protein coupled receptors have been more difficult than to obtain potent and selective antagonists, may be due to a lack in numbers of contact points that inhibits the conformational change of the receptor from the inactive to the active form 31.

For the Y5-receptor, selective agonists and antagonist have been reported. The relatively selective Y5-receptor agonist [D-Trp32] NPY was found to produce a modest feeding response only at high doses 32. [Ala31, Aib32] pNPY, a recently developed Y5-receptor selective agonist, increased affinity at Y5-receptors compared to NPY and was even 3-fold more potent in stimulation of food intake than NPY itself 33. The nonpeptide Y5-receptor antagonist CGP71683A is characterized by nanomolar affinity to the Y5-subtype and only micromolar affinity to the other receptors. Data have shown inhibitory effects on NPY induced food intake either in lean or obese rats, supporting the hypothesis that the Y5-receptor is involved in the regulation of feeding behaviour 34.

For the Y1-receptor, several potent antagonists are known, e. g. BIBP3226 35, LY357897 36 or BIBO 3304, a nonpeptide Y1-antagonist that is even capable of inhibiting NPY-stimulated food consumption in rats after ICV administration 37. GW1229 (also known as 1229U91 or GR231118), a symmetrical dimeric peptide, has very high affinity for the Y1-receptor, but binds also to the Y4-subtype with agonistic properties 38,39. But up to the present, no selective agonists for the Y1-receptor have been published. Interesting compounds with respect to the binding profile were obtained by chimeric combination of NPY and PP 40. NPY and hPP (see Table 1) reveal completely different affinity at the Y-receptors. NPY shows high affinity to the Y1-, Y2- and Y5-receptor and only moderate affinity to the Y4-receptor, whereas hPP binds to the Y4-receptor, has moderate affinity to the Y5-receptor and binds only weakly to the Y1- and Y2-receptors.
Table 1. Amino acid sequences of pNPY, hPP and cPP. Constant positions among the different species are underlined.

<table>
<thead>
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<th>Peptide</th>
<th>Amino acid sequence</th>
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<tbody>
<tr>
<td>pNPY</td>
<td>YPSKPDNPEDAPAEDLARYSYSLRHYINLITRQRY-NH₂</td>
</tr>
<tr>
<td>cPP</td>
<td>GPSQPTYPGDAPVEDLIRFYNDLQQYLNVVTRHRY-NH₂</td>
</tr>
<tr>
<td>hPP</td>
<td>APLEPYPGDNATPEQMAQYAADRLRRYINMLTRPRY-NH₂</td>
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</table>

The aim of this work was to further characterize the binding mode of NPY to the Y₁-receptor and to increase Y₁-receptor selectivity comparing to NPY. Analogs of NPY containing single or multiple amino acid exchanges and chimera of NPY with cPP or hPP, respectively, were synthesized. The binding of all analogs to Y₁-, Y₂- and Y₅-receptors was tested on appropriate cell lines and correlated with the conformational properties according to circular dichroism spectroscopy.

2.3 Experimental Procedures

2.3.1 Materials

The N⁰-Fmoc-protected amino acids were purchased from Alexix (Läufelfingen, Switzerland), Novabiochem (Läufelfingen, Switzerland) and Bachem (Bubendorf, Switzerland). The side-chain protecting groups were: tert-butyl for Asp, Glu, Ser, Thr and Tyr, trityl for Asn, Cys, Gln and His, Boc for Lys and Trp, 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg. The 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink amide) resin was obtained from Novabiochem. Acetic acid (p.a.), N-hydroxybenzotriazole (HOBt), TFA, thioanisole, p-thiocresol, 1,2-ethanedithiol, piperidine, 1-methyl-2-pyrrolidinone, tert-butanol, DMF (puriss.), sodium hydrogenphosphate, potassium dihydrogenphosphate and Pefabloc SC were obtained from Fluka (Buchs, Switzerland). N,N'-diisopropylcarbodiimide (DIC) was purchased from Aldrich (Buchs, Switzerland). DMF (pure), chloroforme, dichlormethane, methanol and diethylether were purchased from Scharlau (La Jota, Barcelona, Spain). Acetonitrile was obtained from Romil (Cambridge, England). 50% Dulbecco’s modified Eagle medium/50% Ham’s F12, minimum essential medium (MEM) with Earl’s salts, sodium pyruvate and phosphate buffered saline (PBS) were
2.3.2 Peptide synthesis

The peptides were synthesised by automated multiple solid phase peptide synthesis on a peptide synthesiser (Syro, MultiSynTech, Bochum, Germany) using the Fmoc-strategy on a Rink Amide resin (30 mg, resin loading 0.6 mmol/g). N-Fmoc amino acids were attached by a double coupling procedure and a ten-fold excess of amino acid, HOBt, DIC in DMF (2 x 40 min). Fmoc-deprotection was accomplished with 40% piperidine in DMF for 3 min, 20% piperidine for 7 min and 40% piperidine for 5 min, finally. The peptides were cleaved from the resins using a mixture of TFA/thioanisole/p-thiocresol (90:5:5 v/v). Trp and Cys containing peptides were cleaved using a cleavage cocktail of TFA/thioanisole/1,2-ethanediithiol (90:7:3 v/v). The fully deprotected peptides were precipitated from ice-cold diethylether, collected by centrifugation, washed with diethylether four times and lyophilised from water/tert.-butanol (3:1 w/w). Disulfide-cyclisation of [Cys20,24] NPY was achieved by iodine oxidation of the peptide on the resin. 15 equivalents (34.2 mg) of iodine dissolved in 1 ml DMF were added to the pre-swollen resin (15 mg). After one hour, the resin was washed with DMF, methanol and diethyl ether (4 ml each) and the peptide was cleaved from the resin with a mixture of TFA/thioanisole/water (90:5:5). The peptide was purified by preparative HPLC on a C-18 column (Waters, 5 μm, 25 x 300 mm) with a linear gradient of 20 to 40% A in B over 30 min (A = 0.08% TFA in acetonitrile, B = 0.1% TFA in water) at a flow rate of 15 ml/min, the relevant fractions were collected and lyophilized.

Characterisation of the peptides was achieved by electrospray ionisation mass spectrometry (SSQ 710, Finnigan MAT, Bremen, Germany) and by analytical reversed-phase HPLC on a LiChrospher RP-18-coloumn (5 μm,3x 125 mm, Merck, Darmstadt, Germany). Gradient: 25 to 75% A in 30 min. Flow rate 0.6 ml/min.

Amino acid sequences and analytical data of the peptides are listed in Table 2.
2.3.3 Cell culture

SK-N-MC cells (neuroblastoma, hY₁) were cultivated in MEM with Earl's salts supplemented with 10% (v/v) fetal calf serum, 4 mM L-glutamine, 0.2 mM non-essential amino acids and 1 mM sodium pyruvate. SMS-KAN cells (neuroblastoma, hY₂) were grown in 50% Dulbecco's modified Eagle medium/50% nutrient mix Ham's F12 with 15% fetal calf serum, 4 mM L-glutamine and 0.2 mM non-essential amino acids. BHK cells transfected with rY5-receptors were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum, and 0.05% geneticin. Cells were grown to confluency at 37 °C and 5% CO₂.

The hY₄ receptor was cloned by screening of genomic DNA libraries, using rY₄ as probe, which had been cloned by PCR with degenerate primers. Cloning and generation of stable cell lines for the Y₄ receptors have been reported.

2.3.4 Binding assays

For binding assays at Y₁, Y₂ and Y₅-receptors, cells were resuspended in incubation buffer MEM with Earl's salts containing 0.1% bacitracin, 50 mM pefabloc SC and 1% bovine serum albumin. 200 µl of the suspension, containing ca. 2.5 million cells per ml, were incubated with 25 µl of a 10 nM solution of ³H-propionyl-NPY and 25 µl of NPY or analog in a concentration range of 10 µM to 10 pM. Non specific binding was defined in the presence of 1 µM cold NPY. After 2 hours at room temperature, the incubation was terminated by centrifugation at 1600 g and 4 °C for 5 minutes. The pellets were washed once with 400 µl PBS, centrifugated again and the washed pellets resuspended in 100 µl PBS. The cell suspension was mixed with 3 ml scintillation cocktail and radioactivity was measured by a β⁻ counter.

At the hY₄-receptor, competition assays were performed on cell homogenates as described in ⁴³ using ¹²⁵I-hPP (2000 Ci/mmol, Eurodiagnostica, Malmö, Sweden) as radioligand. 100 nM bPP was used to define non-specific binding. The reactions were incubated for eight hours at RT and terminated by rapid filtration through a GF/C filter using a Tomtec Mach III harvester. Radioactivity remaining on the filter was determined using a β⁻ counter.

Kᵢ values of the binding curves were calculated by non-linear regression on a one-site composition based model using Prism 3.0 (see table 3 and 5).
2.3.5 Circular dichroism spectroscopy

The CD spectra were recorded on a JASCO J720 spectropolarimeter at 20 °C over a range of 185 to 250 nm in a N2 atmosphere. The peptides were solved in phosphate buffer (20 mM, pH 7.0) in concentrations of 30-40 μM. The peptide solutions were measured three times in a thermostatable sample cell with a path of 0.02 cm. Instrumental parameters were chosen as follows: response time of 2 s, sensitivity of 10 mdeg, band width of 2 nm, step resolution of 0.2 nm and scan speed of 20 nm/min. All CD spectra were corrected by subtraction of the CD spectrum of the solvent to eliminate the interference from cell, solvent and optical equipment. A low-path Fourier-transform filter was used to reduce high-frequency noise. The ellipticity was expressed as mean-residue molar ellipticity [θ]R in deg cm² dmol⁻¹. The secondary structure components were calculated by the Secondary Structure Estimation Program (JASCO), based on the reference spectra of Yang 44.

2.3.6 cAMP Enzyme-immunoassay

SK-N-MC cells expressing hY₁ receptors grown to confluency were resuspended in cAMP-buffer (145 mM NaCl, 1 mM MgSO₄, 5 mM KCl, 10 mM HEPES, 0.5% BSA, 10 mM glucose, 0.1 mM 3-isobutyl-1-methylxanthine, pH 7.4). 1 Mio cells were incubated with 10 μl of a 1.5 mM solution of forskolin and different concentrations of peptides for 1 hour at 37 °C. Incubation was stopped by addition of 100 μl of a 1M solution of HCl. Cell lysis was done by freezing, followed by centrifugation and the supernatant was diluted 1:250.

The intracellular amount of cAMP was determined by using a cAMP enzyme-immunoassay (Biotrak). Reactions were performed according to the protocol of the manufacturer, and optical density was determined at 450 nm.
Table 2. Sequences of the synthesized peptides and analytical data as described in experimental procedures. Special amino acids: Aib = \(\alpha\)-amino-isobutyric acid. Deviations from pNPY are marked in grey.

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<th>Sequence</th>
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</table>
2.4 Results

2.4.1 Peptide synthesis and testing

The peptides were designed as NPY analogs with single or multiple amino acid exchanges or chimeras of NPY and cPP or hPP, respectively (Table 2). Almost all peptides were synthesized containing proline in position 34, in order to reduce Y2-receptor binding. A first set of peptides (2 to 6) comprised NPY/PP-chimeras. A second set of NPY analogs (7 to 19) included replacements in positions 6 to 8. A third set of peptides (20 to 23) showed exchanges in the hairpin-like structure of NPY, namely positions 11, 13 and 14. Prolongations of the polyproline helix and introductions of the turn inducing amino acid Aib (α-amino-isobutyric acid) were performed. A final fourth set of analogs (24 to 30), included peptides with single or double amino acid exchanges in the C-terminal α-helix, in order to get more detailed information of the importance and variability of these positions. Unique amongst these was peptide no 25, cyclo S-S [C20,24] pNPY. Binding experiments were performed on SK-N-MC cells expressing the hY1-receptor, on SMS-KAN cells expressing the hY2-receptor and on BHK-cells, stably transfected with the rY5-receptor. Furthermore, NPY/PP chimeric peptides were additionally tested at the hY4-receptor (Table 3, Table 4). Aqueous solutions of the analogs were investigated by circular dichroism spectroscopy and spectra were subsequently analyzed according to the method of Yang 44 (Table 3).

2.4.2 NPY/PP-chimeric peptides

In a first set of analogs, chimeras of NPY and cPP or hPP, respectively, were performed by introduction of relevant sequences of the pancreatic polypeptides (positions 1-7 and 20-23) into [P34] pNPY. This resulted in an expected complete loss of affinity at the Y2-receptor. Exchange of the residues 20-23 of [P34] pNPY (YYSA) by the corresponding cPP segment (FYDN) (2) resulted in a 9-fold loss of affinity at the Y1-receptor (K, = 0.107 nM) and a 26-fold loss at the Y5-receptor (Ki = 42 nM). This modification caused in CD-spectroscopy a marked shift of the cut of the zero-
line to shorter wavelengths and a slight increase of the negative Cotton-effect at 209 nm. However, mathematical analysis of the curve revealed an unchanged amount of helicity compared to pNPY, whereas β-sheet structure was reduced in favour of turn and random structure (Figure 1A, Table 3).

Replacing positions 1-7 of [P34] pNPY by the corresponding cPP segment (3) reduced binding to the Y1-receptor \( (K_i = 0.015 \text{ nM}) \) only slightly compared to NPY, whereas affinity to the Y5-receptor \( (K_i = 19 \text{ nM}) \) was significantly reduced, showing about 1280-fold better binding to Y1 than to Y5. Combining these two modifications in \([cPP^{1-7}, P^{34}]\) pNPY (4) resulted in a 41-fold decrease of affinity at the Y1- and 35-fold decrease at the Y5-receptor. Interestingly, compounds 3 and 4 into [P34] pNPY did not severely change the CD spectrum of pNPY.

Exchanging only positions 22-23 of NPY by the corresponding cPP-segment, resulted in a 10-fold decrease at the Y1- \( (K_i = 0.13 \text{ nM}) \) and a 22-fold decrease at the Y5-receptor \( (K_i = 36 \text{ nM}) \) (5). Introducing the corresponding hPP-segment at positions 21-23 of [P34] pNPY (AAD instead of YSA) reduced binding to the Y5-receptor to 54 nM, which corresponds to a 33-fold loss of affinity, whereas affinity at the Y1-receptor was reduced only about 20-fold \( (K_i = 0.29 \text{ nM}) \) (6). Surprisingly, compounds 5 and 6, enlarged the amount of helicity dramatically to about 55%, remaining an almost isodichrotic cut of the zero-line.

Affinity of the NPY/PP-chimera to the Y4-receptor was not increased compared to NPY (Table 4). NPY/PP-chimera were found to influence receptor subtype selectivity and to likely result in receptor selective ligands, as shown for \([cPP^{1-7}, P^{34}]\) pNPY (3). This is suggested to be driven rather by chemical than by conformational properties.

### 2.4.3 Analogs with replacements at position 6-8 of NPY

A second set of analogs covered replacements of the residues 6-8 of [P34] pNPY. Exchanging Asp\(^6\) by Phe, Arg, Cys and Asn (7-10) did not dramatically change affinity to the Y1- but markedly reduced affinity to the Y5-receptor (about 7-fold). Interestingly, the complete loss of affinity to the Y2-receptor due to Pro\(^{34}\) could be reduced by Phe\(^6\) and Arg\(^6\) to 40 nM and remarkable 17.2 nM, respectively. Amongst the exchanges at position 6, \([Arg^6, P^{34}]\) pNPY displayed the most significant difference between Y1 and Y5, with a relationship of 1 : 1754, leading to a compound with >1000-fold Y1-receptor preference.
Replacing Asn$^7$ of [P$^{34}$] pNPY with Phe, Arg, Cys and Asp showed a comparable picture (peptides 11 to 14). Again Phe and Arg partially recovered binding to the Y$_2$-receptor. Affinity to the Y$_1$-receptor was not decreased by Phe$^7$, slightly decreased by Arg$^7$ (6-fold, K$_i =$ 0.07 nM) and markedly decreased by Cys$^7$ and Asp$^7$ (35- and 53-fold, K$_i =$ 0.42 nM and 0.65 nM). At the Y$_5$-receptor, modifications in position 7 resulted in reduction of affinity vice versa to the Y$_1$-receptor. Phe$^7$ displayed the highest K$_i$ value (34 nM), whereas Asp$^7$ bound best (15 nM). This lead to the surprising relationship of 1 : 3783 (Y$_1$ : Y$_5$) for compound (11), [Phe$^7$, P$^{34}$] pNPY.

An interesting set of peptides was synthesized by the exchange of Pro$^8$ of [P$^{34}$] pNPY with Phe, Arg, Cys, Asp and Asn, respectively (15-19). Binding affinities to all tested receptors were significantly decreased, or lost in case of the Y$_2$-receptor. Selectivity to Y$_1$ was not increased, it was even slightly diminished compared to NPY. This suggests the importance of the Pro$^8$ residue and an intact N-terminal polyproline-helix.

Conformational analysis revealed only minor changes after modifications in positions 6 to 8 (7-19). Helicity varied in the range of 50%, slightly increased compared to NPY and β-sheet properties were reduced. Accordingly, no structural changes, but specific amino acids at positions 6 and 7 lead to Y$_1$-receptor preferring analogs.

### 2.4.4 Analogs with replacements in the turn-segment of NPY

A third set of peptides included variations in the turn of pNPY, namely positions 11, 13 and 14 (20-23). Pro$^{11}$ and Aib$^{13}$ (α-amino isobutyric acid) reduced affinity to the Y$_1$-receptor to 0.09 nM (7.5-fold reduction) and 0.33 nM (27-fold reduction), respectively. In combination, these two modifications reduced affinity to the Y$_1$-receptor 162-fold (K$_i =$ 1.98 nM). Comparable are the results at the Y$_5$-receptor, where the single mutations reduced binding about 10-fold, while the combination reduced binding 31-fold. An interesting binding profile was yielded by [P$^{11}$, T$^{13}$, P$^{14}$, P$^{34}$] pNPY. Binding affinities at the Y$_1$- and Y$_5$-receptors were only moderately (5-fold) decreased comparing to pNPY. Surprisingly, this variation in the loop of NPY could partially recover binding to the Y$_2$-receptor (K$_i =$ 19.5 nM), despite proline in position 34. Selectivity for the Y$_1$-receptor could not be improved by this type of analoga. The selectivity Y$_1$ : Y$_5$ ranged from 1:25 to 1:206, which is comparable to NPY (1:134). Conformational changes after modifications in the turn-region were
heterogenous. Aib$^{13}$ (20) reduced helicity from 35% down to 27% in favour of β-sheet. Pro$^{11}$ (21) increased helicity to 47% to the loss of β-sheet, as can be deduced from the remarkable increase of the Cotton-effect especially at 209 nm. Obviously, neither structure nor specific residues significantly contribute to the binding mode.

2.4.5 Exchanges within the α-helix

A fourth set of peptides consisted of compounds with single or multiple amino acid exchanges in the α-helical part and the C-terminus of NPY (24 to 30). [A$^{21,25}$, P$^{34}$] pNPY reduced affinity to the Y$_1$-receptor dramatically ($K_i = 8.7$ nM, about 700-fold reduction comparing to pNPY), whereas binding to the Y$_5$-receptor was less reduced ($K_i = 112$ nM, 68-fold reduction). A very interesting binding profile was obtained with compound 25, cyclo S-S [C$^{20,24}$] pNPY. Complete loss of binding at the Y$_1$- and Y$_5$-receptors was contrasted by a remained binding at the Y$_2$-receptor ($K_i = 0.38$ nM), that was almost as high as pNPY itself. The introduction of Trp in position 24 moderately reduced binding to all tested receptor subtypes. In contrast, Trp in position 31 and the combination [Trp$^{24,31}$] pNPY reduced binding to the Y$_1$-receptor dramatically ($K_i$ about 40 nM, about 3000-fold reduction), whereas binding to the Y$_2$- and Y$_5$-receptor remained almost unchanged. Sensitivity of positions 31 and 32 concerning the binding to the Y$_1$-receptor was also confirmed by [C$^{31}$, P$^{34}$] pNPY and [F$^{32}$, P$^{34}$] pNPY (29, 30), that showed >1000-fold reduced binding to the Y$_1$-receptor compared to pNPY, complete loss of affinity at the Y$_2$-receptor but maintained binding at the Y$_5$-receptor with $K_i$ values of 32 nM and 18 nM, respectively.

Variations within the α-helical part of pNPY furthermore revealed an interesting set of CD spectra. [A$^{21,25}$, P$^{34}$] pNPY (24) and cyclo S-S [C$^{20,24}$] pNPY (25) surprisingly showed quite similar CD-spectra with isodichroic cut of the zero-line at markedly reduced 198 nm. In contrary, the three Trp containing analogs (26 to 28) remained an isodichroic cut of the zero-line with pNPY at 203 nm but showed a much higher helicity of up to 64%. Single amino acid exchanges in the C-terminal part of NPY showed very different influences to the CD spectra. [C$^{31}$, P$^{34}$] pNPY (29), hardly did not change the CD spectrum of pNPY, whereas [F$^{32}$, P$^{34}$] pNPY dramatically reduced helicity of pNPY down to 16%, mainly in favor of β-sheet, that was elevated to 55%. The modifications don't correspondingly influence binding and secondary structure,
which suggests the importance of direct ligand-receptor interaction with respect to binding.

### 2.4.6 Functional studies

A selection of five peptides (1, 2, 4-6) were tested in a cAMP assay on a hY₁-receptor expressing cell-line, in order to see, whether agonistic properties are still maintained. $K_i$ and $EC_{50}$ values are given in Table 4. The agonistic effect at the hY₁-receptor, which results in a reduced cAMP concentration, was only slightly reduced for all analogs. With respect to the binding, efficacy was increased about 5- to 10-fold compared to NPY. A 10-fold loss in affinity, but almost equal efficacy was found for $[cPP^{20-23}, P^{34}]$ pNPY (2).

**Table 4.** Peptide activity of pNPY, hPP and compounds 2, 4, 5, and 6 at the human Y₁ receptor on SK-N-MC cells in a cAMP enzyme-immunoassay and affinity at the Y₄-receptor.

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<td>hY₁ $K_i : EC_{50}$</td>
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<td>14,9</td>
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</tr>
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<td>$[hPP^{21-23}, P^{34}]$ pNPY</td>
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Table 3. Affinity of pNPY and NPY analogs at the hY1-, hY2- and rY5-receptor. Secondary structure components were calculated based on the reference spectra of Yang. Aib = α-amino isobutyric acid.

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<th>No.</th>
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<th>hY1 $K_i$ [nM]</th>
<th>hY2 $K_i$ [nM]</th>
<th>rY5 $K_i$ [nM]</th>
<th>$K_i$ (hY1) : $K_i$ (hY5)</th>
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<th>β-sheet</th>
<th>turn</th>
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<td>&gt;500</td>
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<td>16</td>
<td>55</td>
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Figure 1. CD spectra of pNPY and pNPY-analogs in aqueous solution (pH = 7)
2.5 Discussion

Neuropeptide Y (NPY) belongs, together with pancreatic polypeptide and peptide YY, to the pancreatic polypeptide hormone family (PP-family). It is one of the most conserved peptides during evolution known so far. NPY exerts its wide range of action in human via at least three different receptor subtypes, designated as Y\textsubscript{1}-, Y\textsubscript{2}-, and Y\textsubscript{5}-receptor. One of the most striking neurotransmitter-effects of NPY within the central nervous system is its involvement in the regulation of food intake and body weight. Central administration of NPY results in a significant raise of food intake and repeated injection is followed by the development of an obese phenotype. However, in this field of action of NPY (regulation of food intake), the involved receptor subtypes are still matter of dispute. Latest theories suggest an interplay of the Y\textsubscript{1}- and the Y\textsubscript{5}-receptor, because deficiencies in either the Y\textsubscript{1}- or the Y\textsubscript{5}-receptor impair the normal feeding response to fasting or NPY stimulation. Physiological prove for the involvement of the Y\textsubscript{1}- and / or the Y\textsubscript{5}-receptor subtype in the regulation of food intake would be most strikingly obtained by the application of a set of highly receptor subtype selective agonists and antagonists. At the Y\textsubscript{5}-receptor, highly selective agonists and antagonists have been reported. For the Y\textsubscript{1}-receptor, several potent antagonists are known. The lack of a selective Y\textsubscript{1}-receptor agonist might be due to a sensitive binding pocket, that requires intact N- and C-terminus of NPY, C-terminal amidation and tolerates only slight modifications throughout the peptide. Modification of the C-terminal amide of NPY to a carboxylic group led to a complete loss of affinity (> 10 000 nM) at the Y\textsubscript{1}-receptor. Single exchange of each residue of NPY by L-Ala revealed that the most sensitive positions are: Pro\textsubscript{2}, Pro\textsubscript{5}, Arg\textsuperscript{19}, Tyr\textsuperscript{20} and the C-terminal positions 27 to 36, especially the two Arg residues at positions 33 and 35. The importance of the Pro residues 2 and 5 and of the Tyr residues 20 and 27 is probably due to their role in stabilizing the hairpin-like structure of the hormone by means of a hydrophobic core. Kirby and co-workers performed a D-amino acid scan of NPY, that showed an affinity profile very similar to that of the L-Ala scan. This suggests, that important positions are sensitive to both: side chain orientation and side chain itself. Although the Ala-scan of NPY showed no
essential importance of the central positions of NPY for the binding to the $Y_1$-receptor 47, central truncation of NPY by connecting the N- and C-terminus by a spacer (e. g. 6-amino hexanoic acid) led in almost all cases to a significant reduction or complete loss of affinity 50. High $Y_1$-receptor affinity was only obtained, if the number of centrally truncated residues was limited to 8. The best centrally truncated $Y_1$-receptor ligand turned out to be cyclo(7/21)-des-AA$^{10-17}$[Cys$^7,21$]-NPY, which was almost as potent as NPY itself 51.

The lack of a highly selective $Y_1$-receptor agonist prompted us to synthesize a series of NPY analogs for structure-affinity studies, in order to improve $Y_1$-receptor preference. Almost all analogs were synthesized with proline in position 34, because this is known to lead to a loss of $Y_2$-receptor affinity, without severely effecting the binding to the $Y_1$- 52, or $Y_5$- 48 subtype. Recent synthesis of chimeras of NPY and hPP showed an important role of the NPY segments 1-7 or 19-23 or both of them for the binding to the $Y_1$-receptor 40. New chimeric analogs of NPY and cPP or hPP, respectively, within positons 20-23 resulted in agonistic analogs with slight $Y_1$-receptor preference, but no highly selective analog could be obtained. Only exchange of the N-terminal part of NPY by the corresponding cPP sequence, [cPP$^{1-7}$, P$^{34}$] pNPY, significantly increased $Y_1$-receptor preference, binding 1279-fold better to the $Y_1$- than to the $Y_5$-receptor. This might be due to the aromatic tyrosine residue in position 7, because highest $Y_1$-receptor preference was obtained with the analog [F$^7$, P$^{34}$] pNPY (11).

Modifications in positions 6 and 7 were interesting because of their moderate influence for binding of NPY to the $Y_1$-receptor, as shown by the Ala-scan 47. Amino acid exchanges at position 6 of NPY were found to be better tolerated at the $Y_1$- than at the $Y_5$-receptor. Especially the exchange of the acidic Asp$^6$ with the basic Arg residue in [R$^6$, P$^{34}$] pNPY (8) displayed an interesting binding profile with about 2-fold increased affinity at the $Y_1$- and 7-fold decreased affinity at the $Y_5$-receptor, corresponding a 1754-fold better binding to the $Y_1$- than to $Y_5$-receptor. Most significant $Y_1$-receptor preference was obtained with the analog [F$^7$, P$^{34}$] pNPY, which displayed a $K_i$ value of 0.009 nM at the $Y_1$-receptor versus 34 nM at the $Y_5$-receptor, corresponding about 3780-fold better binding to $Y_1$ than to $Y_5$.

This suggests the importance of an aromatic residue in position 7 for preference to the $Y_1$-receptor.
Exchange of the proline residue in position 8 ruins the N-terminal polyproline helix and exchanges within the turn-segment of NPY may severely affect the orientation of the C- with respect to the N-terminus. This might give interesting insights into the conformational role of the N-terminal part and the turn of NPY. As expected, variations of position 8 and within the turn-segment were found to decrease binding affinities to all tested Y-receptors, suggesting the importance of these regions for the building of the correct binding-conformation of NPY. However, general functional correlation between a distinct secondary structure of an analog and its affinity could not be found. Figure 2 shows graphics, which correlate $K_i$ values at the $Y_1$, $Y_2$- and $Y_5$-receptors with the amount of $\alpha$-helicity. Similar distributions were found for $\beta$-sheet-, turn- and random-structure. Obviously, modifications of NPY heterogeneously affect binding and peptide conformation. This suggests an important contribution of direct interactions of the amino acids with the receptor. However, previous results that high $\alpha$-helicity favors binding to the $Y_1$-receptor 48, could be confirmed, because no high affinity ligand at the $Y_1$-receptor displayed an $\alpha$-helical amount < 30%. Nevertheless, unfavorable direct ligand-receptor interaction significantly reduces binding despite suitable secondary structure, as shown for [W31] pNPY (27) and [W24,31] pNPY (28) that bind to the $Y_1$-receptor with $K_i$ values of only about 40 nM and have a very high helical content of about 60%.

![Figure 2](image)

**Figure 2.** Correlation of affinity to the $Y_1$, $Y_2$- and $Y_5$-receptors with amount of $\alpha$-helicity.

A set of analogs, which contained modifications within the $\alpha$-helix and C-terminal part of NPY, revealed further information and results: The cyclic disulfide analog cyclo S-S[C20, 24] pNPY is presented here as new highly $Y_2$-receptor selective analog of neuropeptide Y. Amino acid positions 31 and 32 of NPY were found to
be crucial for the binding to the Y₁-receptor, because all corresponding analogs dramatically lost affinity to the Y₁-receptor subtype, whereas binding to the Y₅-receptor was much less affected. This hypothesis is supported by the Y₅-receptor selective agonists [A³¹, Aib³²] pNPY and [D-Trp³²] NPY, which also show modifications of amino acid positions 31 and 32.

This work presents the first NPY analogs with preference to the Y₁-receptor, gives information about the importance of distinct amino acid positions for binding to the Y-receptor subtypes, provides information for the synthesis of nonpeptide ligands to the Y-receptors and is therefore a further step on the search for highly selective Y-receptor ligands.
2.6 References


CHAPTER 3

Novel Short and Selective Peptide Ligands for the NPY $Y_1$- and $Y_4$-Receptor

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Biological & Medicinal Chemistry, submitted
3.1 Abstract

GR231118, a homodimer based analog of NPY28-36, is a frequently used Y₁-receptor antagonist, which however was found to further show agonistic effects at the Y₄-receptor. In this paper we report on a novel set of analogs of the corresponding monomer, in order to improve Y₁-receptor or Y₄-receptor selectivity. Among the 36 newly synthesized peptides, analogs with up to 50-fold Y₁-receptor selectivity for [P³⁰,C³¹,F³²,L³⁴]NPY28-36 and > 2500-fold Y₄-receptor selectivity for [P³⁰,Y³²,D³⁴]NPY28-36 were identified. Computer based analysis of the sequences and corresponding binding data revealed a high sensitivity of the positions 32 and 34 onto amino acid exchanges and suggested their importance for ligand-receptor selectivity. Based on the synthesized compounds, a computer based prediction of novel analogs turned out to be a useful tool in the analysis of large sets of analogs. Accordingly, this work provides new insights into ligand-receptor interaction at the Y₁- and Y₄-receptor and provides data for the synthesis of highly selective Y₁-receptor analogs that lack Y₄-receptor agonism.

Key words: NPY, GR231118, neuropeptides, food intake
3.2 Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide amide, that belongs to the pancreatic polypeptide hormone family together with pancreatic polypeptide (PP) and peptide YY (PYY). It is widely distributed peripherally and centrally and was first isolated from pig brain in 1982. NPY elicits diverse physiological effects transmitted by different receptor subtypes. Stimulation of food intake, secretion of luteinizing hormone, growth hormone and insulin release suggest an important role of NPY in the pathophysiology of obesity and diabetes. Other effects of NPY have been reported, like potent vasoconstriction, memory retention, modulation of locomotor behaviours, induction of hypothermia, inhibition of sexual behaviour, shifts in circadian rhythms, modulation of cardiorespiratory parameters, anxiolytic potency and inhibition of alcohol consumption and resistance. NPY exerts its wide spectrum of action via several receptor subtypes. Five of them, all belong the superfamily of G-protein coupled receptors, have been cloned, pharmacologically characterized and named Y₁-19-21, Y₂-22-24, Y₄/PP₁-25,26, Y₅-27 and Y₆-receptor subtypes. For NPY, a number of receptor subtype selective ligands have been synthesized. However, each recovery of a new receptor subtype questions the subtype-selectivity of the so far used analogs. In fact, among Y-receptor agonists, [¹²⁵I]-[Leu³¹, Pro³⁴]-PYY was developed as Y₁-receptor agonist radioligand, which was later demonstrated to bind with high affinity to the Y₄- and Y₅- receptors. Similarly, [¹²⁵I]-PYY(3-36) was first proposed as a selective Y₂-receptor probe and later found to also bind to the Y₅ and possibly Y₆-subtypes. A number of antagonists have been synthesized, for example the non-peptide Y₁-receptor antagonist BIBP3226. BIBP3226 demonstrated no activity at the Y₂-, Y₄- and Y₅-subtype and was used as radioligand. However, audioryadigraphic studies have shown that the affinity of [³H]-BIBP3226 was not very good, which resulted in high non-specific labelling and low resolution compared to [¹²⁵I]-[Leu³¹, Pro³⁴]-PYY. Recently, BIBO3304 was reported by the same group of investigators to be a further Y₁-receptor antagonist, that has a 10-fold higher affinity for the Y₁-receptor than BIBP3226 with a similar selectivity profile. However, BIBO3304 is not yet...
available in radiolabeled form. In order to get a radiolabeled antagonist with very high affinity to the Y₁-subtype, Dumont et al. recently presented [¹²⁵⁺]-GR231118 as new high affinity radioligand to investigate Y-receptor subtypes. The homodimeric, peptidergic GR231118 (also known as GW1229 or 1229U91) is based on the sequence Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-CONH₂ (Dpr = Diamino propionic acid). First reported as a potent antagonist for the Y₁-receptor subtype, GR231118 was recently shown to be a potent agonist at the Y₄- and possibly Y₆-receptor subtypes.

The aim of this work was to give new insights into binding mode and ligand receptor interaction at the Y₁- and the Y₄-receptor by a series of new C-terminal nonapeptide analogs of NPY. This information provides data for the synthesis of highly selective Y₁-receptor antagonists that lack Y₄-receptor agonism. The peptides were synthesized by solid phase peptide synthesis. Their binding properties were tested on hY₁-, hY₂- and hY₄-receptors, stably expressed in BHK-cells, and their conformation investigated by circular dichroism spectroscopy (CD).
3.3 Results

3.3.1 Structure-affinity relationship

Affinity of the 36 synthesized analogs of neuropeptide Y 28-36 were investigated by competition binding assays on cell lines selectively expressing the Y₁- or Y₄-receptor (Table 1). Compounds (25 - 30) were additionally tested at the hY₂-receptor and an expected complete loss of binding was found. Binding affinities at the Y₁-receptor of compound (1) (I²⁸N²⁹P³⁰L³¹Y³²R³³L³⁴R³⁵Y³⁶-NH₂) and the dimeric compound (33) have already been published ⁴⁴ and were confirmed in the presented work. Amino acids in positions 28, 29, 31, 32 and 34 of the nonameric NPY28-36 analog (1) were substituted by amino acids with side chains of different size, rigidity, acidity, charge, hydrophobicity or geometry (D-amino acids).

An aromatic side chain was found to be crucial at position 32. All compounds with aliphatic side chain at position 32 (4, 5, 14-16, 21, 27) displayed significantly reduced binding mainly at the Y₁ but also at the Y₄-receptor. Contrarily, the Bpa³² containing analogs (2, 9-13), which have even two phenyl rings in the side chain of position 32, revealed very high binding affinities, especially at the Y₁-receptor. Bpa³² even partly reduced the reduction of binding after introduction of Phe or Trp in position 34 (6, 7 vs. 9, 10).

Acidic side chains (3-5, 29) were not tolerated at the Y₁-receptor throughout, followed by complete loss of affinity (Kᵢ > 1500 nM). Contrarily, the Y₄-receptor tolerated acidic side chains, except in position 32, where an aromatic structure is required. This can be used to increase Y₄-receptor selectivity, as most significantly shown for [P³⁰,Y³²,D³⁴]NPY28-36 (29) with Kᵢ values of 7703 nM and 2.8 nM at Y₁ and Y₄, respectively, corresponding > 2500-fold Y₄-receptor selectivity.

D-amino acids were found to heterogeneously influence receptor selectivity. The D-Phe³² analog (18) reduced Y₁-receptor affinity about 280-fold compared to the
L-Phe\textsuperscript{32} compound (17), whereas the slightly reduced Y\textsubscript{4}-receptor affinity was not affected. Contrarily, D-Trp\textsuperscript{32} (20) even slightly increased the Y\textsubscript{1}-receptor affinity of the L-Trp\textsuperscript{32} analog (19), but affinity to the Y\textsubscript{4}-receptor was reduced about 4-fold.

Dimerization of two peptide monomers by disulfide bridges significantly increased binding, displaying K\textsubscript{i} values in the subnanomolar range. Dimerization of [P\textsuperscript{30},C\textsuperscript{31},F\textsuperscript{32},L\textsuperscript{34}] NPY28-36 (30) increased binding at the Y\textsubscript{1}-receptor 5-fold (K\textsubscript{i} = 0.92 nM) (34) and dimerization of [P\textsuperscript{30},C\textsuperscript{31},W\textsuperscript{32},L\textsuperscript{34}] NPY28-36 (31) even increased it 10-fold at the Y\textsubscript{1}- (K\textsubscript{i} = 0.75 nM) and at the Y\textsubscript{4}- (K\textsubscript{i} = 1.2 nM) receptor (36). Disulfide bridging in position 31 was found to result in higher binding affinities at the Y\textsubscript{1}-subtype than disulfide bridging in position 29, as shown by compounds 32 and 33 with K\textsubscript{i} values of 1.0 nM and 0.28 nM, respectively. The Cys\textsuperscript{31} compound [P\textsuperscript{30},C\textsuperscript{31},F\textsuperscript{32},L\textsuperscript{34}] NPY28-36 (30) turned out to have interesting binding properties also in monomeric form. It displayed K\textsubscript{i} values of 4.8 nM and 232 nM at the Y\textsubscript{1}- and Y\textsubscript{4}-receptor, respectively, which was the most significant Y\textsubscript{1}-receptor preference (50-fold) of all synthesized analogs.

Amino acid position 34 was found to be very sensitive with respect to amino acid exchange. All substitutions of Leu\textsuperscript{34} (6-10, 25, 26, 28, 29) reduced binding affinities at both receptor subtypes. However, this reduction was found to be strongly dependent on the chemical properties of the newly introduced amino acid. The exchange of the aliphatic Leu\textsuperscript{34} by the aromatic Phe\textsuperscript{34} (6) reduced binding at the Y\textsubscript{1}-subtype only about 12-fold and at Y\textsubscript{4} about 56-fold, whereas the Trp\textsuperscript{34} analog (7) lost binding about 125-fold at the Y\textsubscript{1}- and completely at the Y\textsubscript{4}-receptor. This is suggested to be due to an unfavorable role of the indole structure of Trp\textsuperscript{34} because the CD spectra of these compounds in methanol (Figure 2) have almost the same shape. The Pro\textsuperscript{34} and the Asp\textsuperscript{34} analogs showed subtype selectivity between the Y\textsubscript{1}- and the Y\textsubscript{4}-receptor. The Asp\textsuperscript{34} analog (29) displayed significant (> 2500 : 1) preference to the Y\textsubscript{4}-subtype, whereas the Pro\textsuperscript{34} analog (25) bound 18-fold better to the Y\textsubscript{1}-receptor. This Y\textsubscript{1}-preference might be supported by a conformational change, which is induced by the Pro\textsuperscript{34} residue and results in a significant decrease of \(\alpha\)-helicity.
3.3.2 Circular Dichroism

The conformation of the peptides was investigated by CD spectroscopy in three different solvents: a) methanol, b) aqueous solution (pH = 7.0), c) aqueous solution (pH = 7.0) : TFE = 7 : 3. The results were expressed as mean-residue molar ellipticity $\Theta_R$. The spectra were analyzed in terms of amount of defined secondary structure according to the method of Yang. A pronounced surrounding-dependency was noticed and suggests the formation of discrete secondary structure of the peptide analogs. Whereas in aqueous solution (b) the peptides were shown to form mainly random structure (>50%), they were found to adopt high $\alpha$-helicity after addition of 30% TFE (c). The CD spectra in solvent (b) and (c) of compound (5) are shown in Figure 1. Most significant differences regarding the formation of secondary structure were achieved in methanol, which allowed suggestions about the tendency of a peptide to form a distinct conformation. The corresponding CD-spectra are shown in Figure 2 and the results of the conformational analysis in Table 1. The ability to adopt a distinct secondary structure depended on the place of amino acid substitution and on the chemical properties of the newly introduced residue.

In position 29-30 of INPIYRLRY-NH$_2$ (1), the turn-inducing motive Asn-Pro-Xxx is found. Exchange of Asn$^{29}$ by Phe$^{29}$ (13) results in an absolutely different CD spectrum with complete loss of $\alpha$-helicity, which is suggested to be a consequence of the loss of the Asn-turn. Interestingly, binding affinities to the $Y_1$- and $Y_4$-receptor were reduced only about 5-fold.

The mostly varied position 32, was found to influence secondary structure according to the chemical properties of the introduced amino acid. Whereas Glu$^{32}$ (5) and hPhe$^{32}$ (22) increased $\alpha$-helicity and reduced turn-structure, 2NaI$^{32}$ (24) and Aib$^{32}$ (27) reduced $\alpha$-helicity dramatically, the latter maybe due to its turn-inducing effect. Already small deviations of the amino acid in position 32 might result in a big change of the secondary structure. The replacement of L-Phe$^{32}$ (17) by D-Phe$^{32}$ (18) resulted in a complete loss of the positive Cotton effect at 197 nm and a marked decrease in $\alpha$-helicity of 28% to 16%. Even more drastic were the
conformational changes after exchange of 1NaI$^{32}$ (23) by 2NaI$^{32}$ (24). This small variation caused a tremendous reduction of $\alpha$-helicity of 22% to 6%, mainly in favor of $\beta$-structure. Interestingly, compounds (23) and (24) revealed almost the same binding profile at the Y$_1$- and Y$_4$-receptor, which suggests, that direct interactions between the ligand and the receptor importantly contribute to the binding.

Position 34 was found to be involved in the formation and stabilization of $\alpha$-helical structure, because all variations of position 34 reduced helicity compared to compound (1), except Aib$^{34}$ (28), which slightly increased it. Pro$^{34}$ (25) even reduced helicity to 2%, mainly in favor of $\beta$-structure.

The peptide dimer (36) interestingly did not adopt a stable secondary structure with a random part of even 69% in methanol. This might be explained by the flexible disulfide bond, which allows the rotation of one monomer with respect to the other and, consequently, a big number of possible positions and interactions of the two monomeric parts.

![CD spectra](image)

**Figure 1.** CD spectra of compound no 5, [P$^{30}$, E$^{32}$, L$^{34}$]NPY28-36, in aqueous solution (b) and in aqueous solution : TFE = 7 : 3 (c).
Table 1. Binding affinities of the peptides at the hY1-, rY4- and hY4-receptors and conformational analysis by CD spectrometry in methanol solutions based on the reference spectra of Yang et al. 45.

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Figure 2. Circular dichroism spectra of the monomeric compounds 1 to 31 and the dimeric compound no 36 dissolved in methanol in concentrations of 150 µM.
3.3.3 Mathematical analysis of binding data

The big number of structural variants and binding data asked for a mathematical approach to correlate the influence of amino acid sequence with the binding and to likely predict new sequence variants, which display special properties, like high affinity and receptor subtype selectivity. A computer program was developed, which correlated binding of each analog to its sequential features. Sequential features were given by the individual amino acid sequence of each peptide. In order to be able to cope with the mathematical expenditure, the peptides were divided into three classes, covering $K_i$ values $<10$ nM, $<100$ nM and $<10'000$ nM. A complementary library to the synthesized peptides was created, including all theoretically possible amino acid combinations except these synthesized peptides. ‘Theoretically possible’ hereby means, that if in one position only two different amino acids occur among the synthesized peptides, then the complementary library will also contain only these two amino acids at this position. A complementary library of 1792 amino acid sequences could be calculated and expected binding affinities were assigned to each sequence. Attribution of the expected binding to a sequence of the complementary library was based either on pattern recognition by following minimal sets which cover the computational space or on probabilistic theory. Predicted binding affinities based on pattern recognition were more likely to be correct than those based on probabilistic theory, but only few sequences met the criteria for pattern recognition.

The level of correctness of the predicted binding affinities was verified by cross-validation of the synthesized and tested sequences. One of the tested sequences was left out at a time and the affinity, calculated by the trained algorithm with remaining data for this sequence, was compared with the previously measured affinity. A correct prediction was achieved in 60% at the $Y_1$-receptor and in 73% at the $Y_4$-receptor. The influence of a distinct amino acid position onto the correctness of the decision was expressed in a so-called ‘score-value’. This score-value allowed predictions onto the sensitivity of a position with regard to amino acid exchange, which may correlate with the importance of this amino acid for ligand-receptor interaction or building of a favorable secondary structure of the
peptide. The bigger the score-value, the more sensitive is the position with regard to amino acid exchange. Score-values are listed in Table 2. According to this, positions 32 and 34 were found to be the most sensitive positions for ligand-receptor interaction, whereas the positions 28, 29 and 31 are suggested to play a minor role.

The complementary library consisted of 1792 new analogs with a mathematically predicted affinity. Most interesting were sequences with high affinity, predicted based on pattern recognition. These variants should be synthesized first, if high affinity ligands should be obtained. A second criterion would be Y₁-receptor subtype selectivity. Table 3 shows a list of selected peptides including all high affinity analogs (Kᵢ = 10 nM), which were predicted based on pattern recognition and a series of peptides with high predicted Y₁-subtype selectivity.

Table 2. Score values of the prediction of binding data of a complementary library. Score-values describe the influence of a distinct amino acid position onto the correctness of the predicted affinity. The bigger the score-value, the more sensitive is a position with regard to amino acid exchange. Initial sequence and sequence position within NPY are listed in the top row. Positions, that were not varied are marked with an asterisk *

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Table 3. Selection of combinationally arranged amino acid sequences and predicted affinities (K_i pred.) at the Y_1- and Y_4-receptor, calculated based on pattern recognition (p) or probabilistic theory (t). Initial sequence and sequence position within NPY are listed in the top row. Positions, that were not varied are marked with an asterisk *.

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3.4 Discussion

A series of monomeric and dimeric peptide analogs of the C-terminal nonapeptide of NPY were synthesized to achieve new insights into ligand-receptor interaction at the NPY receptor subtypes \( Y_1 \) and \( Y_4 \). Previous works by Daniels et al. showed a tremendous raise in binding of the C-terminal decapeptide of NPY (NPY27-36) to the rat brain \( Y_2 \)-receptor after exchange of Thr\(^{32}\) by Tyr and Gln\(^{34}\) by Leu, respectively. The agonistic effect of this decapeptide was turned into an antagonistic effect by exchange of Leu\(^{30}\) by Pro. This might be due to a stabilisation of an Asn-turn, because the sequence Asn-Pro-Xxx is especially prone to the formation of an Asn-turn. Deletion of the N-terminal Tyr led to a nonapeptide (INPIYRLRY-NH\(_2\)) with antagonistic activity and an affinity (IC\(_{50}\)) of 9 nM at \( Y_1 \)- and 170 nM at \( Y_2 \)-receptors. The binding of corresponding nonamers has been reported to be raised strongly after dimerization, as it is clearly shown by the well studied substance GR231118 (also known as GW1229 or 1229U91), a potent antagonist at the \( Y_1 \)- and agonist at the \( Y_4 \)- and possibly \( Y_6 \)-receptor subtype.

In order to increase receptor subtype selectivity and affinity, single or multiple amino acids of INPIYRLRY-NH\(_2\) (1), corresponding \([P^{30}, Y^{32}, L^{34}]\)NPY28-36, were exchanged. Pro\(^{30}\) in position 3 of the nonamer was conserved to preserve the antagonistic effect of the peptide. Arg\(^{33}\) and Arg\(^{35}\) in positions 6 and 8 as well as the C-terminal Tyr\(^{36}\) in position 9 were also kept unchanged, because they are of major importance for the binding as shown by a complete Ala-scan of the full-length NPY and by variation of the Arg residues in an analog of the C-terminal pentapeptide of NPY. All the other positions of the nonapeptide INPIYRLRY-NH\(_2\), were varied, especially Tyr\(^{32}\) and Leu\(^{34}\) in positions 5 and 7 respectively. The newly introduced amino acids were chosen according to the properties of their side chains. Variation of acidity, size, rigidity, hydrophobicity and geometry (D-amino acids) were intended to give new perceptions of ligand-receptor interaction and to increase receptor subtype selectivity with regard to the \( Y_1 \)- and the \( Y_4 \)-receptor. Additionally, new cystine dimers were synthesized in order to raise the affinity.
The presence of an aromatic system in the side-chain of position 32 and the absence of an acidic residue throughout the peptide were found to be two general structural requirements for high Y₁-receptor affinity, which could be significantly (10-fold) increased by dimerization. The distance of the aromatic system in position 32 from the peptide chain was found to be important, because homophe₃² analog (22) lost about 67-fold in binding at the Y₁-receptor compared to the Phe₃² analog (17). However, increase of the aromatic systems in this peptide segment by introduction of Phe (11) or Trp (12) in position 31 reduced binding at the Y₁- as well as at the Y₄-receptor. Introduction of amino acids with acidic side chains (3-5, 29), was found to culminate in a complete loss of affinity at the Y₁-receptor (Kᵢ > 1500 nM), whereas the Y₄-receptor was found to be much less sensitive to these acidic side chains (Kᵢ between 2.8 nM and 82 nM). The absence of significant conformational changes after introduction of these acidic amino acids suggests their unfavourable role in direct ligand-receptor interaction.

The development of a highly Y₄-receptor selective ligand based thereafter on the existence of an acidic residue. Mostly pronounced Y₄-receptor preference was found for [P₃₀, Y₃², D₃⁴] NPY28-36 (29), with a ratio of 2750 : 1 (Y₄ : Y₁). Preference to the Y₁-receptor could not be correlated to a sequential or structural motive. Significant (>10-fold) preference to the Y₁-receptor was found for compounds (7, 9, 17, 20, 25 and 30). Highest preference at this interestingly exerted the Cys₃¹ containing compound 30, with (Y₁ : Y₄

Studies by CD spectrometry revealed a variety of information about the tendency of each peptide analog to adopt a distinct secondary structure. However, a generally applicable relationship between secondary structure and affinity could not be found, as shown in Figure 3, which correlates amount of α-helicity with the affinity. Similar distributions were found for β-sheet, turn and random structure. This suggests, that receptor affinity and receptor subtype selectivity is mainly driven by direct ligand-receptor interaction. The secondary structure of these relatively short monomers was found to be strongly dependent on the surrounding. It would be possible that the peptides change their conformation during the
binding process to the receptor and that already the ability to adopt a distinct secondary structure would favor one peptide with respect to other analogs.

Figure 3. Correlation of the amount of α-helicity with the affinity ($K_i$ values) at the $Y_1$- (A) and the $Y_4$- (B) receptor.

The 36 analogs were investigated by computer based analysis of the binding data. The analysis correlated amino acid sequence and affinity at the $Y_1$- and $Y_4$- receptor. Cross-validation resulted in 73% of correct prediction at the $Y_4$- and 60% at the $Y_1$-receptor. The relatively low value of 60% at the $Y_1$-receptor was conditioned by the number of tested sequences and would be raised, if the calculation would be based on more analogs. Besides the prediction of new, promising sequences, the computer program was able to analyze the sensitivity of an amino acid position with regard to amino acid exchange. This score-value, which defines the positions 34 and 32 as most sensitive, gives only a general aspect and neglects the individual chemical properties of amino acids that might be newly introduced. Introduction of the acidic Asp$^{26}$ (3) for example resulted in complete loss of affinity at the $Y_1$-receptor, although the score-value defined position 28 as rather unsensitive with respect to amino acid exchange. However, the computer based analysis of the sequences and binding data was found to be a helpful tool for the interpretation of the binding results and for the prediction of new, promising analogs, which recommends its further application, especially if big sets of data have to be analyzed.
3.5 Conclusion

36 monomeric and dimeric C-terminal analogs of NPY, based on the sequence \( I^{28}N^{29}P^{30}I^{31}Y^{32}R^{33}L^{34}R^{35}Y^{36}\text{-NH}_2 \) \(^{44} \), were tested on \( Y_1\)- and \( Y_4\)-receptors, in order to increase binding and receptor selectivity. An aromatic group in the side chain of position 32 was found to be crucial for high affinity to the \( Y_1\)- and the \( Y_4\)-receptor. The introduction of acidic amino acids (Asp, Glu) throughout the peptide resulted in complete loss of binding at the \( Y_1\)-receptor, while the \( Y_4\)-receptor was less affected, especially at position 34. \([P^{30},Y^{32},D^{34}]\text{NPY28-36} \) \(^{29} \) revealed most pronounced (> 2500-fold) \( Y_4\)-receptor preference. \( Y_1\)-receptor selectivity could not be correlated with sequential or structural motives, besides the aromatic group in position 32 and the absence of an acidic residue. This suggests a team-play of suitable direct ligand-receptor interaction and conformational predisposition for high \( Y_1\)-receptor selectivity. A series of analogs \( (7, 9, 17, 20 \text{ and } 25) \) revealed 11-fold to 37-fold preference to the \( Y_1\)-receptor, but highest (50-fold) preference to the \( Y_1\)-receptor interestingly was found for the cysteine containing compound \([P^{30},C^{31},F^{32},L^{34}]\text{NPY28-36} \) \(^{30} \). Dimerization of the monomers by disulfide coupling resulted in a significant (5- to 10-fold) raise of affinity at both, the \( Y_1\)- and the \( Y_4\)-subtype.

Analysis of the secondary structure of the peptides by circular dichroism spectrometry revealed a strong dependency on the surrounding and a general relationship between conformational properties and affinity at the \( Y_1\)- and \( Y_4\)-receptor could not be found.

Computer based analysis of the sequences and corresponding binding data revealed the sensitivity of positions 32 and 34 with respect to amino acid exchange. They might therefore play a more crucial role in ligand-receptor interaction than positions 28, 29 and 31. The computer based prediction of new analogs based on the synthesized compounds turned out to be a useful tool in the analysis of a large set of analogs and binding data and reoffers its use for further application.
3.6 Experimental

3.6.1 Materials

The N-terminal-Fmoc-protected amino acids were purchased from Alexis (Läufelfingen, Switzerland), Novabiochem (Läufelfingen, Switzerland) and Bachem (Bubendorf, Switzerland). The side-chain protecting groups were: tert-butyl for Asp, Glu and Tyr, trityl for Asn and Cys, Boc for Trp and d-Trp, 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg. The 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink Amide) resin was obtained from Novabiochem. Acetic acid (p.a.), N-hydroxybenzotriazole (HOBt), trifluor acetic acid (TFA), thioanisole, p-thiocresol, 1,2-ethanediol, piperidine, 1-methyl-2-pyrrolidinone, tert-butanol, DMF (puriss.), 2,2,2-trifluoroethanol (TFE), sodium hydrogenphosphate and potassium dihydrogenphosphate were obtained from Fluka (Buchs, Switzerland). N,N-diisopropylcarbodiimide (DIC) was purchased from Aldrich (Buchs, Switzerland). DMF (pure), chloroform, dichloromethane, methanol and diethyl ether were purchased from Scharlau (La Jota, Barcelona, Spain). Acetonitrile was obtained from Romil (Cambridge, England). Dulbecco’s modified eagle medium (DMEM) was obtained from BioWhittaker (Boehringer Ingelheim Bioproducts Partnership, Verviers, Belgium); fetal calf serum from BioWhittaker; Hepes from Fluka (Buchs, Switzerland). IPTG was purchased from Sigma (Deisenhofen, Germany), phosphate buffered saline (PBS) from Life Technologies (Basel, Switzerland), 75 cm² flasks from Techno Plastic-Products AG (Trasadingen, Switzerland).

3.6.2 Peptide Synthesis

The peptides were synthesized by automated multiple solid phase peptide synthesis on a peptide synthesizer (Syro, MultiSynTech, Bochum, Germany) using the Fmoc-strategy on Rink Amide resin (30 mg, resin loading 0.6 mmol/g) 48. The analogs based on the sequence INPlYRLRY-NH₂ (1) and are listed in Table 1. Special amino acids were Bpa = benzoylphenylalanine, Nal = naphthylalanine, hF
= homo-Phe, Aib = \(\alpha\)-amino-isobutyric acid, Cha = cyclohexylalanine. D-amino acids are indicated with small letters. The peptides were cleaved from the resin using a mixture of TFA/thioanisole/p-thiocresol (90:5:5 v/v), precipitated from ice-cold diethyl ether and lyophilised from water/tert.-butanol (3:1 w/w). For the cleavage of Cys-containing peptides, a cleavage mixture of TFA/thioanisole/1,2-ethanedithiol (90:7.5:2.5, v/v) was used. Dimerization of the cysteine containing peptides was achieved by air oxidation. 14 mg (6 \(\mu\)mol) of the crude peptide was dissolved in 10 ml of a 0.1 M ammonium hydrogen carbonate buffer and shaken for 40 h at room temperature. Subsequently the solvent was removed by lyophilization. The peptide was purified by preparative HPLC on a C-18 column (Waters, 5 \(\mu\)m, 25 x 300 mm) with a linear gradient of 10 to 30% A in B over 30 min (A = 0.08% TFA in acetonitrile, B = 0.1% TFA in water) at a flow rate of 15 ml/min. Characterisation of the peptides was achieved by electrospray ionization mass spectrometry (SSQ 710, Finnigan MAT, Bremen, Germany) and by analytical reversed-phase HPLC on a LiChrospher RP-18-column (5 \(\mu\)m, 3x 125 mm, Merck, Darmstadt, Germany) with 10 to 60% A in 30 min, flow rate: 0.6 ml/min. Analytical data were received as expected (data not shown).

### 3.6.3 Cell Culture

BHK cells (baby hamster kidney cells, transfected with \(hY_1\), \(hY_2\), \(hY_4\) or \(rY_4\)) were cultivated in DMEM supplemented with 10% (v/v) fetal calf serum, 1% (v/v) antibiotic-fungicide solution (penicillin 10000 U/ml, streptomycin 10 mg/ml and fungizone 25 \(\mu\)g/ml) and 1 mg/ml geneticin. Additionally, 1 mg/ml hygromycin and 24 hours before harvest 1 mM IPTG were added to the \(rY_4\) expressing BHK cells. Cells were maintained at 37\(^\circ\)C in a humidified atmosphere containing 5% \(CO_2\) and 95% air in 75 cm\(^2\) flasks (Techno Plastic-Products AG, Trasadingen, Switzerland). They were harvested at 100% confluency in phosphate buffered saline containing 0.02% EDTA.
3.6.4 Binding Assay

Membrane suspensions obtained from BHK cells (100 µl), transfected with hY₁-, hY₂-, hY₄- or rY₄-receptors, were prepared as previously described, and incubated with 25 pM ¹²⁵I-(Tyr¹)-NPY (hY₁, hY₂), 25 pM ¹²⁵I-hPP (hY₄) or 30 pM ¹²⁵I-hPP (rY₄) and different concentrations of the peptide in a total volume of 250 µl for 2 hours at room temperature, as recently described. The protein-bound radioactivity was determined in a γ-counter. The nonspecific binding is defined as the radioactivity bound in the presence of 1 µM NPY during the incubation period. Half-maximal inhibition of the specific binding of the radioligand of two to three separate experiments, each performed in triplicate, resulted in IC₅₀ values. Kᵣ-values were calculated according to the Cheng Prusoff equation (Table 1).

3.6.5 Circular Dichroism

The CD spectra were recorded on a JASCO J720 spectropolarimeter at 20 °C over a range of 190 to 250 nm as described. The peptides were dissolved in a) methanol, b) phosphate buffer (20 mM, pH 7.0) and c) phosphate buffer (20 mM, pH 7.0) : trifluorethanol (TFE) = 7 : 3 in a concentration of 150 µM. All CD spectra were corrected by subtraction of the CD spectrum of the solvent to eliminate the interference from cell, solvent and optical equipment. A low-path Fourier-transform filter was used to reduce high-frequency noise. The ellipticity was expressed as mean-residue molar ellipticity [Θ]ᵣ in deg cm² dmol⁻¹.
### 3.6.6 Supplementary Material

**Table.** Sequences and analytical data of the synthesized peptides. Special amino acids: Bpa = benzoylphenylalanine, Nal = naphthylalanine, hF = homo-Phe, Alb = α-amino-isobutyric acid, Cha = cyclohexylalanine. D-amino acids are indicated with small letters.

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<td>1218.9</td>
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<td>S-S([P30, C31, w32, L34] NPY28-36)₂</td>
<td>INPCwRLFY-NH₂</td>
<td>2436</td>
<td>2436.1</td>
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3.7 References


CHAPTER 3


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CHAPTER 4

Studies of the Human, Rat, and Guinea Pig Y4 Receptors Using Neuropeptide Y Analogues and Two Distinct Radioligands

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Peptides (2000), in press
Abbreviations

PP, pancreatic polypeptide
NPY, neuropeptide Y
PYY, peptide YY
gp, guinea pig
r, rat
h, human
p, porcine
b, bovine
CHO, Chinese hamster ovary
Fmoc, fluorenylmethoxycarbonyl
4.1 Abstract

The neuropeptide Y-family receptor Y4 differs extensively between human and rat in sequence, receptor binding, and anatomical distribution. We have investigated the differences in binding profile between the cloned human, rat, and guinea pig Y4 receptors using NPY analogues with single amino acid replacements or deletion of the central portion. The most striking result was the increase in affinity for the rat receptor, but not for human or guinea pig, when amino acid 34 was replaced with proline; [Ahx^{8-20},Pro^{34}]NPY bound to the rat Y4 receptor with 20-fold higher affinity than [Ahx^{8-20}]NPY. Also, the rat Y4 tolerates alanine in position 34 since p[Ala^{34}]NPY bound with similar affinity as pNPY while the affinity for hY4 and gpY4 decreased about 50-fold. Alanine substitutions in position 33, 35, and 36 as well as the large loop-deletion, [Ahx^{5-24}]NPY, reduced the binding to all three receptors more than 100-fold. NPY and PYY competed with $^{125}$I-hPP at Y4 receptors expressed in CHO cells according to a two-site model. This was investigated for gpY4 by saturation with either radiolabeled hPP or pPYY. The number of high-affinity binding-sites for $^{125}$I-pPYY was about 60% of the receptors recognized by $^{125}$I-hPP. Porcine [Ala^{34}]NPY and [Ahx^{8-20}]NPY bound to rY4 (but not to hY4 or gpY4) according to a two-site model. These results suggest that different full agonists can distinguish between different active conformations of the gpY4 receptor and that Y4 may display functional differences in vivo between human, guinea pig, and rat.

Keywords: pancreatic polypeptide (PP), neuropeptide Y (NPY), peptide YY (PYY), structure-activity relations, G-protein coupled receptor, Y4, alanine scan, two-site model, binding
4.2 Introduction

The neuropeptide Y (NPY) family of neuroendocrine peptides consists in mammals of NPY, pancreatic polypeptide (PP), and peptide YY (PYY). All three peptides share a common three-dimensional hairpin-like structure although the primary sequence is only about 50% identical between PP and NPY over the 36 amino acids. The peptides differ dramatically in evolutionary rate; whereas rat (r) and human (h) NPY are identical, PP differs at eight positions between these species. Guinea pig (gp) PP differs in four and ten positions compared to hPP and rPP, respectively, although the three species are approximately equally distant from each other in evolution.

There are presently five NPY-family receptors cloned in mammals (Y1, Y2, Y4, Y5, and y6). NPY and PYY bind with high affinity to the Y1, Y2, and Y5 receptors while PP binds with much lower affinity. In contrast, PP binds with higher affinity than NPY and PYY to the Y4 receptor, but the binding of NPY and PYY may still be in the physiological range, at least for the human and guinea pig Y4 receptors. The Y1 and Y2 receptors can be distinguished by amino-terminally truncated peptide analogues like NPY3-36 and NPY13-36 as these ligands bind to Y2 with high affinity while the affinity for the Y1 receptor decreases gradually with amino-terminal truncation. Pro34-substituted peptides like [Leu31,Pro34]NPY and [Leu31,Pro34]PYY are full agonists with high affinity for Y1, Y4, and Y5 receptors but display very low affinity for Y2.

When the PP-prefering receptor, Y4 (previously called PP1), was cloned its amino acid identity between human and rat was found to be as low as 75%. Our group has cloned the gpY4 receptor which displays higher identity to hY4 (83%) than to rY4 (74%). Despite the species differences in both receptor and ligand sequences, rPP, hPP and gpPP bind with similar affinity to Y4 from the three species. Furthermore, the binding properties reported for NPY and PYY to Y4 differ extensively between laboratories (see refs. 5. 9 for an extensive discussion about differences in pharmacology). Previous studies have identified the carboxy-terminus of the PP molecule as the major interaction point with the Y4 receptor. Interestingly, when position 34 of NPY was changed from glutamine to
proline, which is the corresponding amino acid in PP, the affinity for the rat Y4 receptor increased 200-fold \(^{17}\). Binding to the human and guinea pig receptors, however, were unaffected by this modification.

To further explore these species differences as well as the importance of the carboxy-terminus of the NPY-molecule, we have investigated the human, rat, and guinea pig Y4 receptors stably expressed in CHO cells using porcine NPY with alanine substitutions in positions 33-36. Influence of the central loop in binding to Y4 receptors was also investigated by three loop-deleted peptides: \([\text{Ahx}^{8-20}]\text{NPY}\) which binds with high affinity to both Y1 and Y2 receptors \(^{19}\), \([\text{Ahx}^{8-20},\text{Pro}^{34}]\text{NPY}\) which does not bind to Y2 receptors and \([\text{Ahx}^{5-24}]\text{NPY}\) which is Y2-selective \(^{1,3}\). In these peptides amino acids 8-20 or 5-24 of the NPY molecule have been replaced with 6-aminohexanoic acid (Ahx). We report here results that identify additional species differences in the binding-properties of the Y4 receptor as well as intriguing species differences in high-affinity binding sites for some peptide analogues.

4.3 Methods

4.3.1 Cloning

The rat receptor was cloned by PCR with degenerate primers. Subsequently, the hY4 and gpY4 receptors were cloned by screening of genomic DNA libraries, using rat Y4 as probe. Cloning and generation of stable cell lines for the Y4 receptors have been reported \(^{9,16,17}\).

4.3.2 Peptides

The peptides were synthesized by solid-phase peptide synthesis using Fmoc-strategy and tertiary butyl/trityl side-chain protection. Rink-amide resin was used for solid phase. Cleavage from the resin and side-chain deprotection was performed in one step using trifluoracetic acid. Peptides were precipitated from diethyl ether, containing 0.05% HCl in order to receive peptide hydrochlorides, and
lyophilized from water. Purification was performed by preparative HPLC and peptides were characterized by HPLC (purity) and mass spectrometry (identity) as described previously 1,2,19. Four alanine-substituted pNPY analogues were made: p[Ala33]NPY, p[Ala34]NPY, p[Ala35]NPY, and p[Ala36]NPY where each of the last four amino acids in the NPY sequence (Arg33, Gln34, Arg35 and Tyr36) has been changed, one in each analogue 4. Three loop-deleted peptides where parts of the loop were substituted with a 6-aminohexanoic acid bridge were also used: [Ahx8-20]NPY 19 where amino acids 8-20 were substituted. [Ahx8-20, Pro34]NPY is the same molecule as [Ahx8-20]NPY but with Gln34 changed to a proline. This peptide does not bind to Y2 receptors while [Ahx5-24]NPY where amino acids 5-24 are substituted is Y2-specific 1,19.

4.3.3 Binding

Competition assays were performed on cell homogenates as described in ref. 9 using 125I-hPP (2000 Ci/mmol, Eurodiagnostica, Malmö, Sweden) as radioligand. 100 nM bPP was used to define non-specific binding for all three receptors. Saturation assays were performed to the gpY4 receptor using both 125I-hPP and 125I-pPYY (4000 Ci/mmol, Amersham) in parallel. The reactions were incubated for eight hours at RT and terminated by rapid filtration through a GF/C filter using a Tomtec Mach III harvester. Radioactivity remaining on the filter was determined using a Wallac 1450 Microbeta counter. All results were analyzed using the Prism 2.0 software package (GraphPad, San Diego, CA). A two-site model for the competition-curves was used when this significantly improved the curve-fit (p<0.05; F-test). Protein concentrations were measured using Bio-Rad Protein Assay (Bio-Rad) with BSA as a standard.
Table 1. Potencies of NPY and NPY-related ligands to inhibit $^{125}$I-hPP binding to membranes prepared from CHO cells stably expressing the human, rat, and guinea pig Y4 receptors.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Receptor</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>human Y4</td>
<td>rat Y4</td>
<td>guinea pig Y4</td>
</tr>
<tr>
<td>pNPY*</td>
<td>0.20 ± 0.04$^{(\text{ref. 6})}$</td>
<td>0.18 ± 0.03$^{(\text{ref. 6})}$</td>
<td>0.7 ± 0.3$^{(\text{ref. 6})}$</td>
</tr>
<tr>
<td>pNPY</td>
<td>6 ± 3$^{(\text{ref. 6})}$</td>
<td>18 ± 4$^{(\text{ref. 6})}$</td>
<td>26 ± 11$^{(\text{ref. 6})}$</td>
</tr>
<tr>
<td>p[Ala$^{33}$]NPY</td>
<td>3000 ± 1000</td>
<td>400 ± 100</td>
<td>1900 ± 400</td>
</tr>
<tr>
<td>p[Ala$^{34}$]NPY*</td>
<td>-</td>
<td>0.12 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>p[Ala$^{34}$]NPY</td>
<td>9 ± 1</td>
<td>15 ± 8</td>
<td>30 ± 9</td>
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<tr>
<td>p[Ala$^{35}$]NPY</td>
<td>37 ± 7</td>
<td>170 ± 30</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>p[Ala$^{36}$]NPY</td>
<td>70 ± 20</td>
<td>70 ± 20</td>
<td>90 ± 40</td>
</tr>
<tr>
<td>[Ahx$^{9-20}$]NPY*</td>
<td>-</td>
<td>0.5 ± 0.2</td>
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<tr>
<td>[Ahx$^{9-20}$]NPY</td>
<td>58 ± 9</td>
<td>20 ± 10</td>
<td>40 ± 20</td>
</tr>
<tr>
<td>[Ahx$^{9-20}$.Pro$^{34}$]NPY</td>
<td>7 ± 1</td>
<td>0.027 ± 0.009</td>
<td>12 ± 6</td>
</tr>
<tr>
<td>[Ahx$^{5-24}$]NPY</td>
<td>250 ± 40</td>
<td>20 ± 7</td>
<td>400 ± 100</td>
</tr>
<tr>
<td>hPP</td>
<td>0.039 ± 0.003$^{(\text{ref. 6})}$</td>
<td>0.003 ± 0.002$^{(\text{ref. 6})}$</td>
<td>0.031 ± 0.004$^{(\text{ref. 6})}$</td>
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<tr>
<td>rPP</td>
<td>0.071 ± 0.005$^{(\text{ref. 6})}$</td>
<td>0.0027 ± 0.0009$^{(\text{ref. 6})}$</td>
<td>0.044 ± 0.005$^{(\text{ref. 6})}$</td>
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<tr>
<td>gpPP</td>
<td>0.06 ± 0.02$^{(\text{ref. 6})}$</td>
<td>0.007 ± 0.002$^{(\text{ref. 6})}$</td>
<td>0.046 ± 0.009$^{(\text{ref. 6})}$</td>
</tr>
</tbody>
</table>

Data represent inhibition constants ($K_i$ ± SEM, nM) of three to six experiments performed in duplicate. Non-specific binding was defined in the presence of 100 nM bPP. Non-linear regression analyses were carried out using the GraphPad Prism 2.0 Software. A star (*) indicates $K_i$-value for the high-affinity binding site.
Figure 1. Competition of $^{125}$I-hPP binding by various bPP, rPP, pNPY as well as three loop-deleted ligands to membranes prepared from CHO cells stably expressing with the human (a), guinea pig (b), and rat (c) Y4 receptors. Results shown are from one typical experiment performed in duplicate. Non-specific binding was defined in the presence of 100 nM bPP. Porcine NPY was found to compete according to a two-site model at all three receptors.
4.4 Results

$^{125}$I-hPP has been reported to identify a single high-affinity binding site with dissociation constants ($K_d$) of 31 ± 2 and 6.3 ± 0.4 pM at the human and rat Y4 receptors, respectively. At the guinea pig Y4 receptor the $K_d$ for $^{125}$I-hPP was 29 ± 3 pM (n=6). In the present study $^{125}$I-pPYY was also used as a radioligand in binding to the gpY4 receptor and gave a $K_d$ value of 500 ± 60 pM (n=3). However, the B-max for $^{125}$I-hPYY was only about 60% of that for $^{125}$I-hPP (400 ± 30 fmole/mg prot. compared to 670 ± 70 fmole/mg prot.). Each of the radioligands detected a single high-affinity binding site on these cells. $^{125}$I-pPYY (1.5 nM) showed no specific binding to the rY4 receptor and membranes from untransfected cells showed no specific binding of $^{125}$I-pPYY or $^{125}$I-hPP (data not shown).

The binding characteristics of NPY and NPY-related ligands to human, rat, and guinea pig Y4 receptors are shown in Table 1. All peptides tested, except where indicated, competed with $^{125}$I-hPP in a manner best fit by a one-site model. The Ala$^{33}$ substitution had the largest impact on binding to all three receptors. Porcine [Ala$^{33}$]NPY bound with affinities that were 2000- to 15000-fold lower than NPY while p[Ala$^{35}$]NPY and p[Ala$^{36}$]NPY lost affinity by 100- to 600-fold (Fig. 2). In contrast, the p[Ala$^{34}$]NPY analogue bound to the rat Y4 receptor with the same affinity as native pNPY and also displayed similar two-site binding while the high-affinity binding to the hY4 and gpY4 receptors was completely lost only leaving the low-affinity component.

The loop-deleted peptide [Ahx$^{8-20}$]NPY bound with lower affinity than the parent molecule. The affinities for hY4 and gpY4 were 58 and 40 nM, respectively. To the rat receptor, [Ahx$^{8-20}$]NPY bound according to a two-site model. The Pro$^{34}$-substituted [Ahx$^{8-20}$,Pro$^{34}$]NPY bound with higher affinity than [Ahx$^{8-20}$]NPY to all three receptors. However, the increase in affinity was most dramatic at the rat receptor where the affinity increased 20-fold compared to the high-affinity component for [Ahx$^{8-20}$]NPY. The Y2 selective [Ahx$^{5-24}$]NPY bound to hY4, rY4, and gpY4 receptors with a $K_i$-value of 250, 20, and 400 nM, respectively (i.e. 1200-, 100-, and 600-fold lower affinity than pNPY).
4.5 Discussion

As the Y4 receptor is one of the most rapidly evolving G-protein coupled receptors known, we undertook this study of the pharmacological properties in vitro for the human, rat, and guinea pig receptors with the primary aim to explore species differences in ligand binding. With such dramatic sequence differences, one would expect PP from various species to bind differently to Y4 receptors. To our surprise, previous studies showed that PP from cow, human, rat and guinea pig bound to all three receptors equally well. The only difference was that bovine (b)
PP bound with two- to three-fold higher affinity to the gpY4 receptor than human, rat, and guinea pig PP did. It has been shown that amino acids 2 and 3 at the amino-terminus of PP are involved in binding to the human Y4 receptor. Yet bPP13-36 displays only 13-fold lower affinity to hY4 than the intact peptide which identifies the carboxy-terminus as the major interaction point.

There are ten variable positions among these four PP sequences, eight of which are in the loop between positions 11 and 24. The affinity of NPY for the human, rat, and guinea pig Y4 receptors decreased dramatically when amino acids 5-24 are deleted, 1200-, 100, and 700-fold, respectively. Deletion of amino acids 8-20 affected the binding to the human and guinea pig receptors 300- and 60-fold while binding to the rat receptor was unaffected. Thus, the loop region does contribute to binding in some species.

The most striking result was the increase in affinity for the rat receptor when amino acid 34 of the NPY molecule (glutamine) was replaced with proline. Out of the four last amino acids 33-36, position 34 is the only one that differs between mammalian NPY and PP sequences (glutamine in NPY and proline in PP). [Ahx8-20,Pro34]NPY binds to the rY4 receptor with seven-fold higher affinity than pNPY and 20-fold higher than [Ahx8-20]NPY when only comparing the high-affinity binding (see below). The hY4 and gpY4 receptors bound the Pro34-substituted peptide [Ahx8-20,Pro34]NPY with eight and three times higher affinity than [Ahx8-20]NPY, respectively. Furthermore, alanine is accepted in position 34 by the rat receptor as the affinity of p[Ala34]NPY for the rY4 receptor was similar to that for NPY, while the affinity for hY4 and gpY4 receptors decreased about 40-fold.

As for the Y1 and Y2 receptors, the affinity for all three Y4 receptors decreased when either of the positions 33, 35, and 36 was changed to an alanine. When Arg33 was substituted the affinities dropped more than 2000-fold. When Arg35 and Tyr36 were substituted the effect on the guinea pig receptor was slightly less dramatic than on the human and rat receptors.

Interestingly, several ligands seem to bind to human, rat, and guinea pig Y4 receptors in a manner best fit by a two-site binding model. This was most clear when NPY and PYY were tested in competition with 125I-hPP. We report here that also p[Ala34]NPY and [Ahx8-20]NPY bound to the rat Y4 receptor according to a two-site model but not to the human and guinea pig Y4 receptors (Table 1, Fig. 2).
Thus the Ala^{34}-substitution and the deletion of 13 amino acids (8-20) of the NPY-loop completely abolished high-affinity binding to these receptors. To further explore the apparent two-site binding, we performed a saturation to the gpY4 receptor using either iodinated hPP or pPYY as radioligand. Indeed, each of the radioligands, ^{125}I-hPP and ^{125}I-pPYY, identified a single high-affinity binding site. The affinities were similar to those obtained in the competition studies for hPP and for pPYY if only the high-affinity component was taken into consideration. However, ^{125}I-hPP recognized a higher B-max than ^{125}I-pPYY. The saturation with ^{125}I-pPYY was performed up to a concentration of 2.5 nM which is insufficient to detect the low-affinity site previously shown to have an affinity (K) for pPYY of only 16 nM. Thus, the low-affinity site for PYY would not be detected by this experiment. Similar results have been published for the rat Y4 receptor where the B-max for PP was 20 times higher than for PYY. In that study the receptors were expressed transiently in COS-7 cells which yielded an extremely high B-max for PP of more than 5400 fmol/mg protein. The B-max for PYY, however, was only 280 fmol/mg protein which is similar to the levels in our study. It can therefore be speculated that a cell can only host a limited number of Y4 receptors in a high-affinity state, possibly due to G-protein depletion or loss of interaction with other cytoplasmic or membran-bound proteins. A very efficient expression system like that in ref. could thereby mask the high-affinity binding since up to 95% of the receptors bind the ligand with 100-fold lower affinity than the high-affinity receptors.

These data might explain some enigmatic results in the literature where different pharmacological profiles have been published for the same cloned receptor. For example, when ^{125}I-rPP was used as radioligand at the mouse Y4 receptor, NPY and PYY bound with IC_{50} values of 500 and 800 nM, respectively, whereas with ^{125}I-pPYY as radioligand the IC_{50} values were 3 nM for both peptides. The IC_{50} values for unlabeled human and rat PP were the same regardless of which radioligand was used. It seems as if ^{125}I-pPYY only labels a fraction of the total receptor pool that binds NPY and PYY with high affinity while ^{125}I-hPP labels all Y4 receptors regardless of what state they are in. Also the cloned mouse y6 receptor has been published as being both a PP-selective receptor as well as a receptor not binding PP at all. In a study of the impact of
different radioligands at chimeric opioid receptors, it was found that \(K_i\) values differed extensively when comparing data obtained using radiolabeled agonist or antagonist. Similarly to the present study the B-max values also differed between radioligands at several of the chimeric constructs.

PP and PYY are full agonists at the gpY4 receptor and would thus be expected to bind with high affinity only to the active state (that can couple to G-proteins) of the receptor as compared to an antagonist that recognizes both the active and inactive state with equally high affinity according to the traditional ternary complex model. Agonists that discriminate between receptor states have also been reported for the NK1 receptor stably expressed in CHO cells and for the VIP/PACAP receptors transiently expressed in COS-7 cells. Thus, some agonists can also be selective between different receptor conformations/states implying a multi-state model of agonist action. A theoretical three-state model with two active conformations was recently presented by Leff et al. The current lack of Y4-selective antagonists prevents us from investigating whether there still is a pool of gpY4 receptors that are not labeled by either radioligand.

Overall, the human and guinea pig Y4 receptors have more similar pharmacological properties while the rapidly evolving rat Y4 receptor is less sensitive to alanine replacements in the carboxy-terminus and deletions of the central loop. Our data show that although all three receptors bind PP from all three species with similar affinity, the rat Y4 receptor displays the most divergent binding properties also for the peptides tested here. These results corroborate our previous conclusion that the gpY4 receptor is a more appropriate animal model than the rat Y4 receptor.

ACKNOWLEDGEMENTS

We thank Dr. Nina Mohell (AstraZeneca Södertälje) for valuable comments of this paper. This work was supported by the Swedish Natural Science Research Council and the Swiss National Foundation (31-05081.97).
4.6 References


CHAPTER 5

On the Synthesis of Orexin A: A Novel One-Step Procedure to Obtain Peptides With Two Intramolecular Disulfide Bonds

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5.1 Abstract

Efficient strategy for the synthesis of orexin A, a recently discovered neuropeptide with two intramolecular disulfide bonds, was developed. Four different methods for the synthesis of peptides containing two disulfide bonds were compared and optimized with respect to reaction time, purity of the crude peptide and yield of the purified peptide. A new one-step cyclization method in solution is presented for fast, easy and high yield synthesis of orexin A, based on iodine oxidation in acetic acid/water and S-acetamidomethyl (S-Acm) and S-trityl (S-Trt) for side chain protection of cysteine. Disulfide formation without selective side chain protection leads to the formation of different mono- and bicyclic configurations of orexin A. These data stress the requirement of selective cysteine side chain protection in the synthesis of orexin A.

**Keywords:** orexin, hypocretin, disulfide, obesity, food intake, solid phase peptide synthesis, neuropeptides, cystine

**Short title:** Synthesis of Orexin A
5.2 Introduction

Two novel neuropeptides were discovered recently independently by two groups and identified as potent stimulators of food intake after intracerebroventricular administration \(^1,\ 2\). Orexin A (also called hypocretin 1, because of its homology to secretin \(^3\)) is a 33 amino acid residue peptide with two intramolecular disulfide bonds, connecting cysteine residues from positions 6 to 12 and 7 to 14, respectively. Orexin B consists of 28 residues and shares 46% identity with orexin A, which is mainly comprised at the C-terminus. The three-dimensional solution structure of orexin B, determined by 2D-NMR most recently, shows two α-helices, connected by a short linker sequence at position 20-23 \(^4\). The structure of orexin A is conserved among human, rat, mouse and cow, whereas rodent orexin B contains two amino acid substitutions compared to the human sequence: Pro instead Ser in position two and Asn instead of Ser in position 18. The orexins of Xenopus laevis show several amino acid exchanges, but the C-terminal decapeptide of orexin A and B and the positions next to the disulfide bonds in orexin A remain conserved (Fig. 1), which suggests some importance in biological activity \(^5\).

\[
\text{A} \\
\text{h/r/m-orexin} \quad \text{xenopus orexin A} \\
\text{UPLDCCRQKTCSRLYELLHGAGNHAAGILTL-NH}_2 \\
\text{APDCCRQKTCSRIYDILRGTNHAAGILTL-NH}_2 \\
\]

\[
\text{B} \\
\text{h-orexin B} \quad \text{r/m-orexin B} \quad \text{xenopus orexin B} \\
\text{RSGPPGLQGLRQLQQASGNHAAGILTM-NH}_2 \\
\text{RGPPGLQGLRQLQQANGNHAAGILTM-NH}_2 \\
\text{RSDFQTMQRLQGLQSGSNHAAGILTM-NH}_2 \\
\]

**Figure 1.** (A) Sequence of mature orexin A peptides. (B) Sequence of mature orexin-B peptides. Deviations from the human sequences are highlighted in grey. U = pyroglutamic acid.

The two peptide amides derive from the same 130 amino acid precursor, prepro-orexin, whose mRNA is produced in defined regions of the lateral and perifomical hypothalamus (LH and PFH) and upregulated upon fasting. Orexin
immunoreactive neurons are widely distributed in the brain, including the regions of the cerebral cortex, the medial groups of the thalamus, the circumventricular organs, the limbic system and the brain stem. Further investigations suggest the involvement of these peptides into many physiological and behavioural activities that are involved in or associate with feeding behaviour, and other functions like modulation of neuroendocrine function or sleep-wake cycle.

Orexin A and B are endogenous ligands of two closely related heptahelical G-protein-coupled receptors termed OX₁ and OX₂. Orexin A shows higher affinity to OX₁, whereas the affinity of the two peptides to OX₂ is in the same range.

Up to now, no structure-activity relationships of any of the two orexin peptides have been reported, nor any potent subtype selective antagonist or agonist is known to characterise the physiological and pharmacological role of the different receptors.

Solid phase peptide synthesis is a fast and convenient strategy to frequently obtain peptides of more than 40 amino acids. However, it is still a challenge to synthesise long peptides with intramolecular cycles, one or multiple disulfide bonds or sterically hindered sequences. The aim of this work was to establish and compare synthesis methods for fast and easy synthesis of orexin A by solid phase peptide synthesis based on the Fmoc-strategy. The obtained peptides were examined by HPLC and electrospray ionisation mass spectrometry (ESI-MS).

5.3 Experimental Procedures

5.3.1 Materials

The Nα-Fmoc-protected amino acids were purchased from Alexix (Läufelfingen, Switzerland). The side-chain protecting groups were: tert-butyl for Asp, Glu, Ser, Thr and Tyr, Boc for Lys, trityl for Asn, Gln and His, 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg. Cys was protected by Trt or Acm, according to the synthesis strategy. The 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink Amide) resin was obtained from Novabiochem (Läufelfingen, Switzerland). acetic acid (p.a.), N-hydroxybenzotriazole (HOBT),
TFA, thioanisole, p-thiocresol, trimethylbromsilane, 1,2-ethanedithiol, piperidine, 1-methyl-2-pyrrolidinone, tert-butanol, DMF (puriss.), sodium hydrogenphosphate, potassium dihydrogenphosphate and iodine were obtained from Fluka (Buchs, Switzerland). N,N’-diisopropylcarbodiimide (DIC) was purchased from Aldrich (Buchs, Switzerland). DMF (pure), chloroform, dichloromethane, methanol and diethylether were purchased from Scharlau (La Jota, Barcelona, Spain). Acetonitrile was obtained from Romil (Cambridge, England) and tetrachloromethane (puriss) from Merck (Dietikon, Switzerland). N-(3-Maleinimidopropionyl)-biocytin (MPB) was synthesized according to 14.

5.3.2 Synthesis Methods

5.3.2.1 General Procedure

Orexin A was synthesized by four different methods of solid phase-technique in order to optimise reaction time, purity of the crude peptide and yield of the purified peptide (see Scheme 1). The peptides were synthesized by automated multiple solid phase peptide synthesis on a peptide synthesizer (Syro, MultiSynTech, Bochum, Germany) using Rink Amide resin (30 mg, resin loading 0.6 mmol/g). Amino acids were attached by Fmoc-strategy in a double coupling procedure with ten-fold excess of Fmoc-amino acid, HOBt, DIC in DMF (2 x 40 min). The Fmoc-deprotection step was accomplished with 40% piperidine in DMF for 3 min, 20% piperidine for 7 min and finally 40% piperidine for 5 min.
Scheme 1. Synthesis strategies of Orexin A: (1.) Two-step cyclization in solution; (1.a) Air oxidation in 0.1 M ammonium hydrogencarbonate; (1.b) Iodine oxidation in 50% acetic acid. (2.) Selective disulfide formation by one-step cyclization on the resin; (2.a) Cyclization by iodine treatement in methanol/chloroform (1:1); (2.b) Cleavage from the resin. (3.) Selective disulfide formation by one-step cyclization in solution by iodine oxidation in aqueous acetic acid. (4.) Unselective disulfide formation by iodine oxidation in 50% acetic acid might lead to different configurations of mono- or bicyclic structures. Four possible oxidation products are indicated.
5.3.2.2 Synthesis of Orexin A

5.3.2.2.1 Two-step cyclization of orexin A in solution
The linear resin-bound peptide was synthesized as described above with Cys(Acm) in positions 6 and 12 and Cys(Trt) in positions 7 and 14. The peptide was cleaved from the resin with a cleavage-cocktail of trifluoroacetic acid : thioanisole : ethandithiole (90:7:3), precipitated from ice-cold diethylether, collected by centrifugation, washed four times in diethylether and characterized by reversed phase HPLC and mass spectrometry. The peptide (32 mg/9 μmol) was dissolved in 0.1 M ammoniumhydrogencarbonat (50 ml) to give a 1.8x10^{-4} M solution and vigorously shaken at room temperature for 72 hours to obtain the first disulfide bond by air oxidation. The reaction was terminated after control by analytical RP-HPLC and mass spectrometry. After lyophilization, the peptide was dissolved in 50% acetic acid (10^{-3} M) and iodine (50 mM) was added. The solution was shaken for 60 minutes and the iodine was extracted with tetrachlormethane six times. The aqueous phase was three-fold diluted with water and lyophilized. Purification of the peptide was achieved by preparative HPLC on a C-18 column (Waters, 5 μm, 25 x 300 mm) with a linear gradient of 20 to 40% A in B over 50 min at a flow rate of 15 ml/min. the relevant fractions were collected. The crude (see Fig. 2) and the pure (data not shown) peptide were characterized by electrospray ionisation mass spectrometry (SSQ 710, Finnigan MAT, Bremen, Germany) and by analytical reversed-phase HPLC on a LiChrospher RP-18-coloumn (5 μm,3x 125 mm, Merck, Darmstadt, Germany) using 0,08% TFA in acetonitrile (A) and 0.1% TFA in water (B) as eluting system (20 to 40% A over 30 min at a flow rate of 0.6 ml/min).

5.3.2.2.2 One-step cyclization of orexin A on the resin
The linear resin bound peptide was synthesized as described above. Side chain protection groups were identical to method 1. 15 equivalents of iodine (0.135 mmol, 34.3 mg) were solved in 2 ml of methanol : chloroform (1:1). This solution was given to the pre-swollen resin (94 mg, resin loading 0,6 mmol/g) and shaken for 4 hours. The resin was washed with methanol, dichlormethane, DMF and
diethylether (4 x 1 ml each). The peptide was cleaved from the resin with a mixture of trifluoroacetic acid : thioanisole : water (90 : 5 : 5) for 3 hours. The fully deprotected peptide was precipitated from ice-cold diethylether, centrifuged and washed in diethylether four times. The crude peptide was dissolved in tert-butanol: water (1:3 v/v), lyophilized and characterized by mass spectrometry and by analytical reversed-phase HPLC. The mass spectrum of the crude product showed the expected masses of orexin A (expected: 3560 Da, found: 3560.4 ± 1.0) (Fig. 3). Purification of the peptide was performed by preparative HPLC and the pure peptide was characterized by HPLC and mass spectrometry. Analytical data were found as expected (M.W. expected: 3560 Da, M.W. found: 3560.5 ± 1.1 Da).

5.3.2.2.3 One-step cyclization of orexin A in solution
The linear peptide was synthesized as described above, using Cys(Acm) in positions 6 and 12 and Cys(Trt) in positions 7 and 14. The peptide was cleaved from the resin with trifluoroacetic acid : thioanisole : 1,2-ethanedithiole (90:7:3), precipitated from ice-cold diethylether, washed four times in diethylether and characterized by electrospray ionisation mass spectrometry and by analytical reversed-phase HPLC. For cyclization 0.009 mmol (32 mg) of the peptide were dissolved in 9 ml acetic acid (10⁻³ M) and a solution of 10 equivalents iodine (90 μmol, 23 mg) in 150 μl methanol was added for fast building of the first disulfide bond. After 60 min of stirring, 2.25 ml water was added to accelerate the half-time of S-Acm cleavage. The solution was shaken for another 90 min. To follow the cleavage of the S-Acm group and the building of the second disulfide bond, the solution was monitored by mass spectrometry 0, 20, 40 and 90 minutes after the addition of water (see Fig. 4). After completeness of the reaction, 9 ml of water was added and the iodine was extracted with tetrachlormethane (6 x 20 ml). The aqueous phase was diluted three-fold with water and lyophilized. The crude peptide was characterized by analytical HPLC (Fig. 5 D). The peptide was purified by preparative HPLC and characterized by ESI-MS and reversed phase HPLC on a LiChrospher RP-18 column. Analytical data were found as expected (M.W. expected: 3560 Da, M.W. found: 3560.2 ± 0.6 Da).
5.3.2.2.4 Orexin A synthesis by unselective disulfide formation

Reduced orexin A was synthesized as described above, using the S-trityl protecting group for the four cysteines. The peptide was cleaved from the resin with trifluoroacetic acid: thioanisole:1,2-ethandithiol (90:7:3), precipitated from ice-cold diethylether, washed four times with diethylether, lyophilized and characterized with mass spectrometry and reversed phase HPLC. 32 mg of the peptide (0.009 mmol) were solved in 4.5 ml water. 4.5 ml acetic acid and 11.4 mg iodine (5 equivalents) in 200 μl methanol were added and the solution shaken for two hours. The solution was diluted to twice the volume with water and the iodine was extracted with tetrachloromethane (6 times, equal volume). The aqueous phase was lyophilized and the peptide characterized by mass spectrometry and reversed phase HPLC (LiChrospher RP-18-column, 5 μm, 3x 125 mm, Merck, Darmstadt, Germany) using 0.08% TFA in acetonitrile (A) and 0.1% TFA in water (B) as eluting system (20 to 40% A over 30 min at a flow rate of 0.6 ml/min). The mass spectra showed the correct masses (expected: 3560 Da; found: 3560.3 ± 0.8 Da), the HPLC chromatogram revealed a mixture of at least three major products, a double peak at RT = 21.28 min, 21.72 min and a third peak at 22.61 min (Fig. 5).

To control the shift in retention time of the oxidized orexin A, a co-injection with reduced orexin A was performed, using an HPLC system as follows: RP-18 column (LiChrospher 100, 5 μm, 250 x 4 mm, Merck, Darmstadt, Germany), linear gradient of 20 to 40% A in B over 40 minutes, flow rate of 0.6 ml/min (A: 0.08% TFA in acetonitrile; B: 0.1 % TFA in water).

5.3.2.2.5 Synthesis of Orexin A analogue with 6-14 and 7-12 disulfide bonds

The peptide was synthesized using the one-step cyclization method as described above. Cysteine protecting groups were chosen as follows: Cys(Acm) in positions 6 and 14 and Cys(Trt) in positions 7 and 12, respectively. The peptide was purified by preparative reversed phase HPLC and characterized by mass spectrometry and analytical HPLC. Analytical data were found as expected (M.W.-expected: 3560 Da, M.W.-found: 3559.8 ± 1.07 Da).
5.3.3 Control of complete oxidation and correct folding of orexin A

Completeness of oxidation was investigated by mass spectrometry. 5 μg of the purified orexin A (one-step cyclization in solution) were solved in 70 μl water : tert.-butanol (1:3) and subsequently 5 μg of N-(3-maleinimidopropionyl)-biocytin (MPB) was added. After 2 hours of shaking, the product was characterized by mass spectrometry. For control the same experiment was performed with reduced orexin A.

The mass spectra, that were measured after the reaction of MPB with orexin A, still showed the mass of oxidized orexin A (expected: 3560 Da, found: 3559.9 ± 0.7 Da), whereas for the reduced orexin A the addition of four MPB was found as expected (expected: 5658 Da, found: 5657.2 ± 0.3 Da).

Control of the correct bridging of orexin A was achieved by HPLC co-injection of orexin A (one-step cyclization in solution) with the wrongly bridged orexin A analogue and each of them with the unselectively oxidized orexin A (see Fig. 6). The following HPLC system was used; LiChrospher 100, 5 μm, 250 x 4 mm, Merck, Darmstadt, Germany and a linear gradient of 20 to 40% A in B within 40 minutes (A: 0.08% TFA in acetonitrile; B: 0.1 % TFA in water).

5.4 Results

5.4.1 Synthesis of orexin A

Orexin A is a 33 amino acid peptide with two intramolecular disulfide bonds, connecting the four cysteine residues from positions 6 to 12 and 7 to 14, respectively. The peptide was synthesized by four different methods of solid-phase technique to optimise reaction time, purity of the crude peptide and yield of the purified peptide (see Scheme 1). Selective and unselective formation of the two disulfide bonds was performed and compared (Table 1). Subsequently, the peptides were purified by preparative HPLC in each case in a single step. Electrospray ionisation mass spectrometry and analytical HPLC was used to confirm peptide purity and identity.
5.4.1.1 Two-step synthesis of orexin A in solution

During the cleavage of the peptide from the resin under reductive conditions (1,2-ethanediol), all side chains were removed, except of S-Acm in positions 6 and 12. The linear orexin A showed the expected mass of 3706 Da as demonstrated by mass spectrometry and a purity of about 85% (data not shown). Complete building of the first disulfide bridge after 72 hours of air oxidation in 0.1 M ammoniumhydrogencarbonat was verified by HPLC and mass spectrometry (data not shown). After lyophilization of the peptide, cleavage of the S-Acm group and connection of the second disulfide bond was performed by iodine oxidation in 50% acetic acid. Analytical HPLC and mass spectrum of the oxidized peptide are shown in Figure 2. The main peak of the HPLC chromatogram (retention time (RT) of 19.8 min) derived from orexin A and the smaller peak at RT = 20.8 min corresponded to orexin A with two S-Acm groups, as proven by mass spectrometry of the peak fractions. A big heap of polymers and side products led to the peaks of longer retention time (>23 min). The peptide was purified by preparative HPLC and the pure peptide was characterized by analytical HPLC and mass spectrometry (data not shown). The overall yield from peptide-resin was only about 10%, because of the too high amount of polymerisation products and incomplete cleavage of the Acm group.

Figure 2. HPLC and mass spectrum of crude orexin A after two-step cyclization in solution. Expected mass: 3560 Da, found main product: 3561.4 ± 0.6 Da.
5.4.1.2 One-step cyclization of orexin A on the resin

The selective formation of the two disulfide bonds on the resin in one step was achieved by the use of the iodine-labile cysteine protecting groups S-acetamidomethyl (Acm) and S-trityl (Trt) with Cys(Acm) in positions 6 and 12 and Cys(Trt) in positions 7 and 14. The advantage of these protecting groups in peptide synthesis is, that their halftime in the presence of iodine differs dramatically, depending on the solvent. This allows selective formation of two disulfide bonds by selection of suitable reaction conditions. Remarkable for the selective formation of the two disulfide bonds in solution is, that the S-Acm group is stable to trifluoroacetic acid cleavage, whereas S-Trt is cleaved by trifluoroacetic acid during cleavage of the peptide from the resin.

The HPLC of the crude peptide, that was cleaved from the resin after cyclization, showed a main product at a retention time (RT) of 20.6 min, corresponding to the desired orexin A (see Figure 3). A smaller peak at RT = 21.45 originated from the peptide, still contained the two S-Acm protecting groups, as verified by mass spectrometry (data not shown). The big peaks at higher RT derive from a large amount of side products and polymers. Purification of the crude peptide by preparative HPLC led to highly pure product. The overall yield of this synthesis method from peptide-resin was in the range of 10%.

Figure 3. HPLC and mass spectrum of the crude product of the one-step orexin A synthesis on the resin. Expected mass: 3560 Da, found: 3560.4 ± 1.0 Da.
5.4.1.3 One-step cyclization of orexin A in solution

In order to improve the yield of orexin A synthesis by avoiding polymer formation, selective disulfide formation was performed in highly diluted solution. The peptide was cleaved from the resin under reductive conditions (1,2-ethanediol). The S-Acm groups remained bound to the peptide whereas all other protecting groups were cleaved. The linear orexin A intermediate showed a purity of >95% (HPLC) and the expected mass of 3706 Da was verified by mass spectrometry. The deprotection of the S-Acm group by iodine was monitored by mass spectrometry 0, 60, 80, 100, and 150 minutes after iodine addition. One hour after iodine addition, 20 % of water was added to the solution in order to accelerate the speed of S-Acm deprotection dramatically. The mass spectra, that follow S-Acm deprotection and the HPLC of the crude orexin A after lyophilization demonstrate the completeness of the oxidation procedure (Figure 4). The lyophilized peptide was purified by preparative RP-HPLC and the relevant fractions investigated by analytical HPLC and mass spectrometry. The overall yield from peptide resin was 35%.
5.4 Results

Figure 4. Mass spectrum of orexin A, (A) 0, (B) 60, (C) 150 minutes after dissolving the peptide in acetic acid and adding 10 equivalents of iodine. After 60 minutes of reaction time ("time B"), 20% water was added to the solution to accelerate S-Acm deprotection. (A) linear orexin A with two S-Acm groups on Cys 6 and 12: correct mass 3706 Da, found: 3706.5 ± 0.3 Da. (B) Partially oxidized orexin A. (C) Fully oxidized orexin A: correct mass 3560 Da, found: 3560.5 ± 1.1. (D) HPLC of the crude orexin A.

Figure 5. Mass spectrum and HPLC of unselectively oxidized orexin A. The HPLC shows multiple peaks, indicating different configurations. The mass spectrum gives the expected masses (expected: 3560 Da, found: 3560.3 ± 0.8 Da).
5.4.1.4 Orexin A synthesis by unselective disulfide formation

Many peptides that are found in nature and show multiple disulfide bonds, have a preferred conformational structure that might lead to a selective disulfide bond formation even under unselective oxidation conditions. The peptide was synthesized by iodine oxidation of linear orexin A in 50% acetic acid using a concentration of $10^{-3}$ M. After 2 hours of reaction time, the iodine was extracted and the peptide was lyophilized. HPLC interestingly showed three major peaks: a double peak at RT = 21.28 min, 21.72 min and a third peak at 22.61 min, whereas the mass spectrum showed the expected masses (Fig. 5). In order to identify the peaks, co-injection of the obtained mixture was performed with reduced orexin A, first. All three major peaks revealed a shorter retention time (32.1 min, 32.7 min, 33.9 min) than reduced orexin A (36.2 min), suggesting that a partial oxidation had taken place. The formation of two different configurations was proven by co-injection of the mixture with orexin A (disulfide bonds Cys6-12/7-14) and an analogue with opposite disulfide bonds (Cys6-14/7-12). Both peaks of the double peak of unselectively oxidized orexin A could be raised exclusively, by co-injection with orexin A and its analogue with reverse disulfide bonds, respectively (Fig. 6).
Table 1. Comparison of the cyclization strategies for two intramolecular disulfide bonds in orexin A synthesis.

<table>
<thead>
<tr>
<th>method</th>
<th>yield of purified orexin A</th>
<th>time exposure</th>
<th>instrumental and material effort</th>
</tr>
</thead>
<tbody>
<tr>
<td>two-step cyclization in solution</td>
<td>~10% many side products, polymerisation</td>
<td>several days</td>
<td>high, peptide has to be lyophilized twice, toxicity of CCl₄, used for I₂ extraction</td>
</tr>
<tr>
<td>one-step cyclization on the resin</td>
<td>~10% high polymerisation</td>
<td>1 day</td>
<td>very low, easily to handle, easy removal of chemicals</td>
</tr>
<tr>
<td>one-step cyclization in solution</td>
<td>~30-35%</td>
<td>1 day</td>
<td>low, toxicity of CCl₄, used for I₂ extraction</td>
</tr>
<tr>
<td>unselective oxidation in solution</td>
<td>n. d. building of different configurations</td>
<td>1 day</td>
<td>low, toxicity of CCl₄, used for I₂ extraction</td>
</tr>
</tbody>
</table>

Figure 6. (A) Co-injection of orexin A synthesized by one-step cyclization in solution and unselectively oxidized orexin A leads to discrete enlargement of the peak at RT = 32.1. (B) Co-injection of the wrongly connected orexin A analogue (Cys6-14/7-12) and unselectively oxidized orexin A leads to discrete enlargement of the peak at RT = 31.5. This result indicates the building of different orexin A conformations during unselective orexin A oxidation.
5.4.2 Control of complete oxidation and correct folding of orexin A

Control of the complete oxidation was performed by reaction of oxidized (one-step cyclization in solution) and reduced orexin A with MPB. MPB reacts selectively with free thiol groups and therefore can be used as specific biotinylating reagent for detection of SH groups in protein- and peptide-chemistry. As expected, the mass spectra, that were measured after the reaction of MPB with the peptides, showed no addition of MPB to the oxidized orexin A, and an addition of four MPB to the reduced orexin A, according to the number of free thiol groups of the peptides. This confirms the complete oxidation of orexin A synthesized by one-step cyclization in solution and the applicability of MPB to detect free thiol groups simply by solving it in an aqueous solution together with the peptide and subsequent analysis by mass spectrometry or dot blot.

For verification of the correct building of the disulfide bonds during orexin A synthesis, an orexin A analogue with wrongly connected cysteines (Cys6-14/7-12) was synthesized. This peptide was co-injected with previously synthesized orexin A (one-step cyclization in solution). The retention time differed significantly, and co-injection led to a double-peak in HPLC similar to the double-peak of the unselectively oxidized orexin A (Fig. 5). Interestingly, the wrongly connected orexin A analogue displayed the shorter retention time.

This result, together with the proof of complete oxidation, suggests on the one hand the correct folding of orexin A during one-step cyclization in solution was obtained. On the other hand, it shows, that unselective oxidation of orexin A leads to a mixture of different configurations, which indicates the necessity of regioselective cysteine pairing strategies in the synthesis of correctly folded orexin A.

5.5 Discussion

The disulfide bond is one of the weakest types of covalent bonding in peptides and proteins. Intra-chain disulfide bonds serve to confer a discrete three
dimensional structure and conformational stability. Disulfide bond formation reaction is a key step in the synthesis of cystine-containing peptides. Air oxidation and iodine oxidation are amongst the general methods that are employed, as demonstrated in recent reviews 16, 19. Air oxidation is one of the mildest methods to build a disulfide bond, but usually requires a very long time up to several days 20. Hydrophobic or basic peptides tend to aggregate and precipitate, since the reaction has to be carried out in aqueous solution of slightly basic pH and high dilution to prevent polymerisation 21, 22. In contrast, disulfide bonds can be formed within a short time (minutes to hours), using iodine oxidation. Iodine oxidation needs particularly controlled conditions, since several nucleophilic amino acids, e.g. Met, Tyr, His and Trp, are susceptible to iodine and frequently overoxidized. Application of the S-Acm and S-Trt protecting groups allow the regioselective synthesis of two intramolecular disulfide bonds because of their significant differences in half-time depending on the solvent (see 15). This fact was used to optimize the synthesis strategy of peptides with two intra-chain disulfide bonds on the example of the recently discovered neuropeptide orexin A. The common way to synthesize two intramolecular disulfide bonds is to liberate two cysteine side chains, followed by mild oxidation to the disulfide. Formation of the second cystine is most frequently done by iodine oxidation of an S-Acm protected pair of cysteines, because S-Acm is acid stable, hydrophilic and can be converted directly to cystine without interfering with existing disulfide bonds 23. The combination of S-Trt and S-Acm as Cys-protecting groups, as performed in the presented work, has the advantage, that S-Trt can be cleaved by trifluoroacetic acid and iodine, whereas S-Acm is stable to trifluoroacetic acid cleavage. Because S-Trt can be selectively removed during cleavage of the peptide from the resin, whereas S-Acm remained bound, the different half-times of the cleavage reaction under iodine oxidation allowed selective disulfide formation of the peptide still bound on the resin.

The commonly used two-step cyclization, with initial air-oxidation of the S-Trt deprotected peptide followed by iodine oxidation of the Cys(Acm), interestingly resulted in a quite low yield of about 10% because of polymerisation and incomplete Acm deprotection. Details in the experimental procedure seemed to play a very important role for the correct building of the two disulfide bonds in high
quantities. The choice of other protecting groups, like the combination of S-tert.-butylsulfonyl groups (StBu) with S-Acm or S-2,4,6-trimethoxybenzyl (Tmob) with S-Acm, as it was performed in the synthesis of conotoxin 24, 25 might open other possibilities in two-step cyclization of two intramolecular disulfide bonds. The TFA stable StBu protecting group gives on the one hand the possibility to obtain a fully Cys side-chain protected peptide in solution, on the other hand an additional cleavage step (thiols 26 or trialkylphosphines 27, 28) is necessary to remove the StBu groups from the peptide. Accordingly, the use of S-Trt or Tmob in combination with S-Acm is favoured. Nevertheless, StBu is a well known Cys protecting group and successfully used in synthesis of single or multipe disulfide bonds 29.

Because air oxidation requires a long reaction time of up to several days, our optimisation steps led to the combination of Cys(Acm) and Cys(Trt), by applying their different behaviour under iodine oxidation.

The most elegant method, with respect to the handling, is cyclization of the peptide on the resin, because chemicals and reagents can be removed easily. Iodine-cleavage of S-Trt from the peptide is about 500 fold faster than S-Acm cleavage, using a solvent mixture of methanol : chloroform (1:1) 15. This allows selective formation of two disulfide bonds on the resin in a so called one-step cyclization. After only 4 hours of iodine oxidation, the disulfide formation was complete. Unfortunately, the yield of this synthesis method was only in the range of 10%. The use of other peptide resins, e.g. NovaSyn TG Sieber Resin with low resin loading, could not improve the result, neither the exchange of S-Acm and S-Trt in order to close the disulfide bridges in the other succession. Despite high polymerisation rate and formation of side products, the low yield might also be caused by the solvent, since chloroform is known to suppress disulfide formation 30.

To keep the short reaction time and simultaneously raise the yield by avoiding polymerisation, the one-step cyclization was transformed from resin into solution with surprisingly good results. The TFA labile S-Trt group was removed during cleavage of the peptide from the resin. Selective building of the first disulfide bridge of orexin A was achieved by performing the iodine oxidation reaction in acetic acid, where the half time of S-Acm is about 45 min 15. Addition of 20%
water after one hour raised the S-Acm half time up to 50-60 seconds what shortened the reaction time dramatically for the building of the second disulfide bond. The progress of the S-Acm cleavage and the oxidation reaction could be easily followed by mass spectrometry. This synthesis method provided high yield (~35%) of the correctly folded orexin A as proven by the synthesis of the wrongly connected orexin A analogue, associated with short reaction time, easy handling and the possibility of on-line control of the reaction.

It is important to note, that best synthesis results for peptides containing multiple disulfide bonds might be obtained by random oxidation, because the desired biologically active isomer is generally the most thermodynamically stable. However, unselective iodine oxidation of orexin A in 50% acetic acid led to the building of at least three different products (see Fig. 5). Two of them corresponded to orexin A and its wrongly connected analogue. The third one with a HPLC retention time between the reduced open chained orexin A and the two double bridged analogues, might derive from partial oxidation (only one disulfide bridge closed) or from a bicyclic analogue, connecting Cys6-7/12-14. Studies on air oxidation of bis-cysteinyl-peptides H-Cys-(Gly)$_n$-Cys-OH with $n = 0$-$15$ have shown that under conditions of high dilution ($10^{-3}$ – $10^{-4}$M) and $n \geq 4$, oxidation products are largely dictated by the probability of collision of the thiol groups and intrachain-bridged monomers were predominant, whereas for $n < 4$ formation of dimers and oligomers besides intrachain monomers was observed due to insufficient flexibility of the peptide chain $^{19}$. Nevertheless, it is possible to form even small disulfide loops, whereby the equilibrium constant $K_c$ for loop closing increases in the rank order $n = 1, 3, 0, 5, 4$ and $2^{31}$.

This work compares four different synthesis methods for the synthesis of peptides with multiple disulfide bonds and presents a one-step cyclization method for fast and easy synthesis of orexin A with high yield. Furthermore, this work indicates the building of different mono- or bicyclic configurations of orexin A after unselective disulfide formation, indicating the necessity of selective formation of the two disulfide bonds. The exact structure of the different products that are build after unselective oxidation besides orexin A and its wrongly connected analogue and whether milder oxidation conditions might lead to a higher yield of the correctly folded orexin A, remain topic of further investigation.
5.6 References


CHAPTER 6

Structure-Affinity studies of truncated Orexin A and B Analogs at OX_1 and OX_2 receptors
6.1 Abstract

Orexin A and B (also known as hypocretin 1 and 2), two recently discovered neuropeptides, play an important role in food intake, sleep-wake cycle and further neuroendocrine functions. Orexins are endogenous ligands of two G-protein-coupled receptors, termed OX₁ and OX₂. This work presents structure-affinity relationships of orexin fragments at the OX₁- and OX₂-receptors, suggesting the importance of the C-terminal part of the orexin peptides for ligand-receptor interaction. Orexin B(18-28), the C-terminal undecapeptide of orexin B, is presented as the first high affinity peptide antagonist at the OX₁-receptor. Binding experiments on SK-N-MC cells, a human neuroblastoma cell line, revealed the existence of an orexin-receptor. Differences in the binding profile of orexin A and B fragments at the OX₁-/OX₂-receptors and the receptor on SK-N-MC cells give raise to the suggestion of a third orexin receptor subtype. The presented results provide important information for the production of biomimetica and receptor selective agonists or antagonists, essential tools for pharmacological and biochemical investigaitons.

Key words: Orexin; Hypocretin; Obesity; Neuropeptides; Food intake; Ligand-receptor interaction
6.2 Introduction

Orexin A and B, two novel neuropeptides that are also known as hypocretin 1 and 2, were recently discovered independently by two groups and identified as potent stimulators of food intake after intracerebroventricular administration 1-3. Further investigations revealed a broad involvement of these peptides into the regulation of many physiological and behavioural activities that are associated with feeding behaviour 4-7, into the modulation of neuroendocrine function 8-9 and the sleep-wake cycle 10. Both peptide amides derive from prepro-orexin, a precursor protein produced in defined regions of the lateral and perifornical hypothalamus (LH and PFH), whose mRNA is upregulated upon fasting. Orexin immunoreactive neurons are, however, widely distributed in the brain, including regions of the cerebral cortex, the medial groups of the thalamus, the circumventricular organs, the limbic system and the brain stam 11,12.

Orexin A is also called hypocretin 1 because of its homology to secretin. It consists of 33 amino acids, is C-terminally amidated and contains two intramolecular disulfide bonds, that connect cysteine residues from positions 6 to 12 and 7 to 14, respectively. Orexin B consists of 28 residues and shares 46% identity with orexin A, which is mainly comprised at the C-terminus. The three-dimensional solution structure of orexin B was determined by 2D-NMR recently and shows two α-helices, connected by a short linker sequence at position 20-23 13. The structure of orexin A is conserved among human, rat, mouse and cow, whereas rodent orexin B contains two amino acid substitutions compared to the human sequence: Pro instead of Ser in position two and Asn instead of Ser in position 18. Xenopus laevis has orexins that differ slightly from the human sequence, but the C-terminal decapeptide of orexin A and B and the positions next to the disulfide bonds in orexin A remain conserved (Figure 1), which suggests some importance in biological activity of these peptide regions 14.
Orexin A and B are endogenous ligands of two closely related (64% amino acid identity) heptahelical G-protein-coupled receptors, termed OX1 and OX2. They probably couple to Gq, because intracellular Ca\(^{2+}\) concentrations are increased after activation of the receptors. Orexin A shows higher affinity to OX1, whereas the affinity of the two peptides to OX2 is in the same range.

Up to now, no structure-activity relationships of any of the two orexin peptides have been reported, nor any potent subtype selective antagonist or agonist is known to characterise the physiological and pharmacological role of the different receptors. We report here the synthesis and biological evaluation of C- and N-terminal analogs of orexin A and B in order to identify the minimal sequence, which is necessary for receptor binding. A selection of the peptides was examined for functional properties, i.e. receptor-mediated changes in intracellular Ca\(^{2+}\), using fluorometric imaging technique. An alanin-scan of the C-terminal undecapeptide of orexin A was performed to identify amino acid side-chains that are essential for binding.

6.3 Experimental Procedures

6.3.1 Materials

N\(^{6}\)-Fmoc-protected amino acids were purchased from Alexix (Läufelfingen, Switzerland). The side-chain protecting groups were: tert-butyl for Asp, Glu, Ser,
Thr and Tyr, Boc for Lys, trityl for Cys, Asn, Gln and His, 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg. The 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink Amide) resin and the Wang resin were obtained from Novabiochem (Läufelfingen, Switzerland). N-hydroxybenzotriazole (HOBt), TFA, thioanisole, p-thiocresol, trimethylsilylbromide, 1,2-ethanedithiol, piperidine, tert-butanol, 1,1,1-trifluoroethanol (TFE) and DMF (puriss.) were obtained from Fluka (Buchs, Switzerland). N,N'-diisopropylcarbodiimide (DIC) was purchased from Aldrich (Buchs, Switzerland). DMF (pure) and diethylether were purchased from Scharlau (La Jota, Barcelona, Spain). Acetonitrile was obtained from Romil (Cambridge, England). Orexin A was purchased from Boehringer Ingelheim (Biberach, Germany).

### 6.3.2 Peptide Synthesis

A set of orexin A and B analogs (see Table 1) was synthesized by automated multiple solid-phase peptide synthesis on a peptide synthesizer (Syro, MultiSynTech, Bochum, Germany) using Rink Amide or Wang resin (30 mg, resin loading 0.6 mmol/g). Amino acids were attached by Fmoc-strategy in a double coupling procedure, using a ten-fold excess of Fmoc-amino acid, HOBt and DIC in DMF and a reaction time of 40 minutes per coupling. Fmoc-deprotection was accomplished with 40% piperidine in DMF for 3 min, 20% piperidine for 7 min and finally 40% piperidine another 5 min. The orexin A analogs and non-methionine orexin B analogs were cleaved from the resin with a mixture of TFA/thioanisole/p-thiocresol (90:5:5, v/v), precipitated from ice-cold diethylether, collected by centrifugation and washed four times in diethylether. The methionine containing orexin B analogs were cleaved from the resin using a mixture of TFA/thioanisol/ethanedithiol (90:7:3, v/v), precipitated and washed as described. Partial oxidation of the methionine residue was reduced by dissolving the peptide (15 mg, 0.014 mmol) in 1 ml TFA, followed by addition of ethanedithiol (15.7 μl, 0.2 mol/l) and trimethylsilylbromide (13 μl, 0.1 mol/l) \(^{15}\). The solution was shaken 40 minutes at room temperature and the peptide was precipitated and washed as described. Purification of the peptide was achieved by preparative HPLC on a C18-column (Waters, 5μm, 25 x 300 mm) with a linear gradient of 10 to 30% (A) in
(B) (A: 0.08% TFA in acetonitrile, B: 0.1% TFA in water) and a flow rate of 15 ml/min. The peptides were dissolved in tert. butanol/water (1:3) and lyophilized. Dimerization of orexin A(13-33) and simultaneous cleavage of potential TFA-esters was achieved by dissolving 8 mg (3.6 µmol) of the peptide in 1 ml methanol/piperidine (9:1). After one hour of shaking at room temperature, the peptide was precipitated in ice-cold diethyl ether, resolved in 50 µl TFA, re-precipitated in diethyl ether, dissolved in water and lyophilized.

Analytical characterisation of the peptides was achieved by electrospray ionisation mass spectrometry (SSQ 710, Finnigan MAT, Bremen, Germany) and by analytical reversed-phase HPLC on a LiChrospher RP18-column (5µm, 3 x 125 mm, Merck, Darmstadt, Germany) using a linear gradient of 10 to 40% (A) or 5 to 50% (A) over 30 min ((A) = 0.08% TFA in acetonitrile; (B) 0.1% TFA in water). Analytical data were found as expected (data not shown).

6.3.3 Cell Culture, Binding Assays and Functional Assay

Binding experiments of all analogs were performed in co-operation with Boehringer Ingelheim at Biberach (Germany). The OX₁- and OX₂-receptors were cloned and expressed in CHO cells. Additional binding experiments were done on SK-N-MC cells, a human neuroblastoma cell line, which was found to express orexin binding receptors. Competition assays were performed, using ¹²⁵I-orexin B as radioligand and step wise dilutions of the peptide analogs. IC₅₀ values were determined as half-maximal inhibition of the specific binding of the radioligand. Functional properties of five selected peptides, including orexin A, orexin B (28), [L28]-orexin B (30), orexin B(1-27) (37) and orexin B(18-28) (41), were investigated at the OX₁-receptor by measurement of the intracellular Ca²⁺ concentration, using fluorometric imaging technique.

6.3.4 Circular Dichroism

Conformational properties of orexin A and B and the two short analogs orexin A(23-33) and orexin B(18-28) were investigated by circular dichroism spectroscopy using a JASCO model J720 spectropolarimeter over a range of 190-
250 or 280 nm at 20°C in a N₂ atmosphere. The peptides were dissolved in phosphate buffered salines (20 mM) at neutral pH, that contained 0%, 30%, 50% or 70 % TFE and in pure TFE, in a concentration range of 200-300 μM for the shortened analogs and 30 μM for the mature peptides. Each measurement was repeated tree times using a thermostatable sample cell with a path of 0.02 cm and the following parameters: response time of 2 s, scan speed of 20 nm/min, sensitivity of 10 mdeg, step resolution of 0.2 nm and band width of 2 nm. The CD spectrum of the solvent was subtracted from the CD spectra of the peptide solutions to eliminate the interference from cell, solvent and optical equipment. High frequency noise was reduced by means of a low-path Fourier-transform filter. The ellipticity was expressed as the mean-residue molar ellipticity [θ]₀ in deg cm² dmol⁻¹.
6.4 Results and Discussion

6.4.1 Synthesis of the Peptides

The peptides were synthesized by automated multiple peptide synthesis on a Rink Amide resin to directly obtain the peptide amides after cleavage of the peptides from the resins. The only peptide acid, orexin B(1-28)-OH, was synthesized on Wang resin. Most peptides were obtained in sufficient purity. The C-terminal orexin B fragments contained methionine, a residue that is highly sensitive to oxidation during synthesis\(^\text{16}\) and has the tendency to add back intramolecularly to resin-bound carbocations through its thioether moiety\(^\text{17}\). The purity of 60% of the crude peptide after cleavage from the resin could not be raised satisfactory by subsequent Met(0)-reduction with trimethylsilylbromide and 1,2-ethanedithiol\(^\text{15}\). Purification of the peptides by preparative HPLC was inevitable and resulted in products of >95% purity.

6.4.2 Biological and Structural Characterization of the Peptides

The affinity of the peptides was tested on CHO cell-lines transfected with the OX\(_1\) and OX\(_2\) receptors and on SK-N-MC cells, a human neuroblastoma cell-line. IC\(_{50}\) values are listed in Table 1. h-Orexin B (28) bound with IC\(_{50}\) values of 107 nM, 98 nM and 76 nM at SK-N-MC cells, OX\(_1\)- and OX\(_2\)-receptors, respectively. The secondary structures of orexin A and B were investigated by circular dichroism spectroscopy (CD) in aqueous solution on neutral pH and in 30% TFE (Figure 2 (A)). The content of \(\alpha\)-helical structure was very low in aqueous solution for both, orexin A and B, as followed by analysis of the spectra by a secondary structure estimation program (Jasco, J-700 for Windows) (see Table 2). Addition of 30% TFE markedly raised \(\alpha\)-helicity to 20-30%, which was expected because TFE is known to favor \(\alpha\)-helical structure. The solution structure of orexin B was recently solved by 2D-NMR to consist of two \(\alpha\)-helices connected with a short linker in both H\(_2\)O and 30% trifluorethanol solutions\(^\text{13}\). This high helical content of orexin B
could not be confirmed in the present work, at least in aqueous solution. However, the agonistic effect of orexin A and B at OX₁-receptors was shown by a functional assay (Figure 3).

6.4.3 Full length analogs

A first set of analogs and fragments was based on orexin A (1-27), a second set on orexin B (29-42). The affinity of full length analogs was dependent on the kind of variation. Reduced orexin A (1), which means that the four cysteine residues are not connected by disulfide bridges, interestingly revealed very low binding at all of the three tested systems, displaying IC₅₀ values >1000 nM. The exchange of Cys⁶,¹² (2) or Cys⁷,¹⁴ (3), respectively, by α-amino isobutyric acid (α-Abu) even resulted in complete loss of affinity. This suggests the crucial role of the correct disulfide bridging and folding of orexin A for high affinity receptor interaction. C-terminal modification of the orexin B peptide amide to the peptide acid (29) resulted in a 6-fold reduction of binding at SK-N-MC cells, 3-fold reduction at the OX₁-receptor and, surprisingly, almost unchanged affinity at the OX₂-receptor. Exchange of the C-terminal methionine residue by leucine (corresponding residue of orexin A) did not alter binding nor functional activity, suggesting that the N-terminal methionine is sufficiently replaced by leucine.

6.4.4 N-terminal Fragments

N-terminal fragments of orexin A and B (4, 5, 31-34) and the centrally truncated [1-5-Ahx-19-33]-orexin A (6) completely or almost completely lost binding. Only very long N-terminal fragments of orexin B (35-37) revealed weak binding. Interestingly, orexin B(1-27) even bound to OX₁-receptors with an IC₅₀ value of 65 nM, which is about 30 nM higher than orexin B itself. However, its functional properties were lost, suggesting the crucial role of an intact C-terminus for receptor activation.
Table 1. Affinity of h-orexin B and of orexin A and B fragments and analogs at the OX₁- and OX₂- receptors and at SK-N-MC-cells. Ahx = 6-amino hexanoic acid, α-Abu = α-amino isobutyric acid

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<th>OX₂ receptor IC₅₀ [nM]</th>
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<td>&gt;10'000</td>
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<td>h-Orexin B(18-28)</td>
<td>40</td>
<td>44 ± 9</td>
<td>39 ± 19</td>
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6.4.5 C-terminal Fragments

C-terminal fragments of orexin A and B (7-16, 38-41) bound according the rule: the shorter the peptide, the better the binding. Optimal binding was found for the C-terminal deca- and undecapeptides (13, 14, 41) with $IC_{50}$ values <45 nM at all receptors. Further truncation again raised binding to 69 nM for orexin A(25-33) (15) and 1000 nM for orexin A(26-33) (16). The increased affinity of the C-terminal undecapeptide of orexin B (41) came along with a loss of function and a loss of $\alpha$-helical structure, which could only be raised to 11% in pure TFE (Figure 2B). Therefore, $\alpha$-helicity is suggested to be involved in receptor activation and we present orexin B(18-28) as first high affinity peptide antagonist at the OX$_1$-receptor.

Dimerization of C-terminal fragments by disulfide coupling did not result in higher binding, as shown for S-S[orexin A(13-33)]$_2$ (10). An alanin-scan of orexin A(23-33) (17-27) revealed remained affinity for all analogs, with $IC_{50}$ values between 13 nM and 36 nM. This suggests, that single amino acid exchanges within the C-terminal undecapeptide of orexin A do not severely affect binding. However, positions 24, 30 and 32 of orexin A(23-33) (18, 24, 26) were found to be slightly more sensitive with regard to amino acid exchange, displaying $IC_{50}$-values >30 nM.

Orexin A fragments generally revealed higher binding at the OX$_1$-receptor, than at OX$_2$. This was most significant for compounds 12 and 13 (orexin A(19-33) and orexin A(23-33)), which showed about 4-fold higher binding at the OX$_1$- than at the OX$_2$-receptor. Contrarily, orexin B fragments generally displayed higher binding at the OX$_2$-receptor. The only exception was orexin B(1-27) (37), which bound to OX$_1$- and OX$_2$-receptors with IC50-values of 65 nM and 170 nM, respectively.
Table 2. Calculated secondary structure components [%] for orexin A and B in water and 30% TFE and for orexin A(23-33) dissolved in (a) H₂O, pH 7.0, (b) 50% TFE, (c) 70% TFE, (d) pure TFE

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<tr>
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<th>α-helix</th>
<th>β-sheet</th>
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<td>29</td>
<td>49</td>
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<td>0</td>
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Figure 2. (A) CD spectra of orexin A and B in aqueous solution (pH = 7.0) containing no or 30% TFE. (B) CD spectra of orexin A(23-33) dissolved in (a) H₂O, pH 7.0, (b) 50% TFE, (c) 70% TFE, (d) pure TFA

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<td>n.d.</td>
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<tr>
<td>Orexin B(18-28)</td>
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Figure 3. Functional assay of five selected compounds on OX₁-receptors by fluorometric imaging technique.
6.5 Conclusion

N- and C-terminal fragments and analogs of orexin A and B have been tested for binding at OX₁-, OX₂- receptors and on SK-N-MC-cells and, partly, for functional property on OX₁-receptors. The results indicate the importance of the C-terminal part of the orexins for ligand-receptor interaction, because N-terminal fragments almost completely lost binding. Highest receptor affinity was found for the C-terminal deca- and undecapeptides, which are almost conserved among all orexins. Receptor selectivity is suggested to be driven by the amino acid sequence, that follows N-terminally to the C-terminal decapeptide, because C-terminal orexin A fragments were found to generally display higher affinity at the OX₁- than at the OX₂-receptor, and vice versa for C-terminal orexin B fragments. This is in coincidence with the results of Sakurai et al., who found a preference of orexin A to the OX₁-receptor. Despite high affinity, functional investigation revealed no effect on the intracellular Ca²⁺-concentration of the C-terminal undecapeptide orexin B(18-28) at OX₁-receptors, which therefore has to be suggested as first small high affinity peptide antagonist at the OX₁-receptor. Likewise antagonistic effect at the OX₁-receptor was found for orexin B(1-27), suggesting an important role of the C-terminal residue for receptor activation. Interestingly, the C-terminal methionine of orexin B could be replaced by leucine, the C-terminal amino acid of orexin A, without loss of its agonistic property. An alanin-scan of the C-terminal undecapeptide of orexin A showed no irreplaceability for any residue.

Examination of the conformational properties of orexin A, orexin B and their C-terminal undecapeptides revealed a partial formation of α-helical structure in TFE-containing solutions in case of the mature peptides. This α-helical conformation might be involved in activation of the receptor, because C-terminal fragments didn’t adopt helical structure nor agonistic property, despite very high binding. In addition, it is shown here for the first time, that SK-N-MC cells, a human neuroblastoma cell line that is established for example for examination of NPY Y1 receptors, express receptors of the orexin family. It was not possible to clearly assign the OX₁- or OX₂-receptor subtype to SK-N-MC-cells, regarding the binding
of the examined orexin fragments and analogs. Mainly orexin B fragments bound significantly (2- to 18-fold) weaker at SK-N-MC-cells than at the OX$_1$- and OX$_2$- receptor, which gives raise to the suggestion of a third orexin receptor subtype.
6.6 References


PUBLICATIONS AND ORAL PRESENTATIONS

PUBLICATIONS


POSTERS


Pohl R., Machova Z., Söll R., Brakch N., Grouzmann E. and Annette G. Beck-Sickinger (9/2000) Pro-NPY and truncated analogues are substrates for prohormone convertase PC1/3. 26th European Peptide Symposium, Montpellier, France
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Orexin A and B: Synthesis and Role in the Regulation of Food Intake (10/99) Presentation of Ph. D. works, Department of Applied Biosciences, ETH Zurich, Switzerland.
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Ein herzliches Danke ...


Den Old-Docs Dr. Peter Weber, Dr. Nik Ingenhoven und Dr. Christophe Eckard danke ich für ihre Vorreiter-Rollen im Labor und vor allem auch für all den Spass in der Freizeit bei Wanderungen, Badminton- und Squash-Maches.