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Characterisation of Neuropeptide Y Receptors by Antibodies

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SUMMARY

Neuropeptide Y (NPY) is one of the most abundant neurohormones in the mammalian peripheral and central nervous system. It consists of 36 amino acids and is C-terminally amidated. A variety of physiological effects have been attributed to NPY. Peripherally NPY mediates vasoconstriction through direct effects or through potentiation of other vasoconstrictors. One of the most striking central actions of NPY is the induction of food intake. Further central effects of NPY are memory retention processes and sedation. The broad physiological relevance of NPY gives reason for an increasing interest in NPY as a new target in drug discovery.

NPY exerts its effects via several receptor subtypes. Five distinct NPY receptors subtypes have been cloned and pharmacologically characterised. They have been named Y_1-, Y_2-, Y_4/PP_1-, Y_5- and y_6-receptor subtype. All subtypes belong to the large superfamily of G-protein-coupled, heptahelical receptors. The different receptor subtypes are localised in various tissues. Tissues with high density of NPY receptors are blood vessels, kidney, adrenal glands, nerve endings and brain in human, however distribution of the receptors is species specific. The typical signaling response of NPY receptors found in almost all tissues and cell types is the inhibition of adenylyl cyclase.

It has yet been impossible to structurally characterise G-protein coupled receptor proteins by cristallography or magnetic resonance. This limits our knowledge of the NPY receptor binding site, as well as on the molecular mechanism of action. Accordingly, alternative methods are required to characterise the receptors. The major aspect of this work was the characterisation of NPY receptors on a molecular level by antibodies.

Insights in the structural requirements of ligand-receptor interaction provide the knowledge base which facilitates drug design and a better comprehension of the complex physiological mechanisms that are associated with NPY. In this work, polyclonal antibodies raised against defined segments of the NPY receptor subtypes have been used for localisation, for identification and for characterisation of the structure affinity relationships of NPY and its receptors.

Synthetic fragments of the second and third extracellular loop of the Y_1-, Y_2-, Y_4- and Y_5-receptor subtype were used to generate selective anti-receptor antibodies. Sera were tested on intact receptors in ELISA assays and on solubilised receptors in Western blot experiments. Molecular mass was determined for each receptor protein. Because of the different glycosilation and fragmentation, several bands were stained for every receptor. For the Y_1-receptor, particularly bands at 73 kDa and 51 kDa were detected. The Y_2-receptor was stained at 58 kDa, 50 kDa and 35 kDa. Bands of 51 kDa and 35 kDa were found for the Y_4- and the Y_5-receptor. Selectivity was achieved for the solubilised Y_2-receptor with the antibody directed against the second extracellular loop. Serum Y_5 E2/2 recognised the intact Y_1- and Y_5-receptor and serum Y_5 E3 recognised the intact Y_1- and Y_4-receptor. In combination these to sera can differentiate between the four receptor subtypes (Chapter 2).

Some of the generated antibodies were also tested on intact cells expressing the Y_1-, Y_2-, and Y_5-receptor. Additionally, and in order to have another tool for receptor characterisation, Ala-substituted and centrally truncated NPY analogues were synthesised and binding was tested on the intact receptors. Sera directed against the second extracellular loop were selective for the Y_1-receptor (Y_1 E2/2) as well as for the Y_2-receptor (Y_2 E2/1). Two sera (Y_5 E2/2 and Y_5 E_3) recognised the Y_2-, and the Y_5-receptor. Accordingly, in combination these sera can differentiate between the intact Y_1-, Y_2-, and Y_5-receptor subtype on living cells. Furthermore, subtype selectivity was achieved for the Ala-substituted NPY analogues [A^{13}]-pNPY and [A^{27}]-pNPY at the Y_2-receptor (Chapter 3).

Synthetic fragments of the N-terminus, extracellular loops and C-terminus of the Y_1-receptor were used to generate anti-receptor antibodies. Solubilised membranes, containing the Y_1-receptor were separeted by SDS-PAGE and detected with the antibodies in subsequent Western blotting experiments. Two proteins with molecular masses of 73 kDa and 51 kDa were stained for the rat and the human Y_1-receptor. Competition with NPY showed that the binding of seven antibodies is strongly inhibited. Photoactivatable NPY-analogues were used to bind the hormone covalentely to its receptor. Competition efficiency strongly depended on the position of the crosslinker within the ligand. Based on this studies, a model for the ligand-receptor interaction was suggested. The N-

terminus of NPY seems to be very flexible as binding of several antibodies, raised against different parts of the receptor, is inhibited after crosslinking via position 1. Crosslinking on position 21 and 22 leads particularly to a loss of affinity of sera E2/4 and E3/2. Position 27 of NPY could possibly be close to E2/2 because crosslinking on this position most efficiently blocks sera E2/2 and E2/3 (Chapter 4).

The Y_2-receptor subtype was in addition to the studies with antibodies also characterised by photoactivatable biotinylated analogues of NPY, which were labeled with ^{3}H-propionate. Photoaffinity labeling of the receptor was followed by SDS-PAGE and detection of the bound radioactivity. Additionally, the molecular mass of the receptor was verified in a Western blot experiment with the anti Y_2 antibody (Y_2 E2/1). Two proteins with molecular masses of 58 +/- 4 kDa and 50 +/- 4 kDa correspondingly were detected in human neuroblastoma cells (SMS-KAN), which is endogenously expressing the Y_2-receptor subtype, and in CHO-Y_2 cells, which have been transfected with Y_2-receptor cDNA. Both proteins represent the Y_2-receptor subtype with different amounts of glycosylation, which was proved in digest experiments with endoglycosidase (Chapter 5).

This work proved that anti-receptor antibodies represent valuable tools in differentiation and localisation but also in characterisation of the binding site of NPY receptor subtypes. Knowledge of receptor subtype distribution in different tissues is important in order to understand the biological role of the receptors. The results of this work could allow selective detection on a protein level of NPY receptors in tissue. Combined with a better understanding of hormone-receptor interactions these results could be helpful in designing new drugs.

ZUSAMMENFASSUNG

Neuropeptid Y ist eines der am häufigsten vorkommenden Neurohormone des peripheren und zentralen Nervensystems der Säugetiere. Es besteht aus 36 Aminosäuren und ist C-terminal amidiert. NPY ist für verschiedenste physiologische Effekte verantwortlich. Peripher wirkt NPY direkt, sowie durch die Potenzierung der Aktivität weiterer Neurotransmitter, gefäßverengend. Eine der markantesten zentralen Aktivitäten von NPY ist die Regulation der Nahrungsaufnahme. Desweiteren führt NPY zentral zur Steigerung der Gedächtnisleistungen und zur Sedation. Aufgrund seiner vielfältigen Eigenschaften als Neurotransmitter zeigt die Arzneimittelfoschung ein grosses Interesse am NPY System.

NPY vermittelt seine zahlreichen Effekte über mehrere Rezeptor-Subtypen. Fünf davon wurden bereits kloniert und pharmakologisch charakterisiert. Man nennt sie Y_1-, Y_2-,.Y_4/PP_1- Y_5- und y_6-Rezeptor-Subtypen. Alle NPY Rezeptor-Subtypen gehören zur Familie der G-Protein-gekoppelten Rezeptoren mit sieben transmembranen Domänen. Die Rezeptoren sind in den verschiedensten Geweben lokalisiert. Hohe Dichten von NPY Rezeptoren weisen zum Beispiel die Blutgefässe, die Nieren, Nervenendigungen und das Gehirn auf, wobei die Verteilung Arten spezifisch ist. In fast allen Zellen und Geweben wurde die Inhibition der Adenylatcyclase als typische Signalantwort von NPY-Rezeptoren gefunden.

Die strukturelle Charakterisierung G-Protein-gekoppelter Rezeptor Proteine mittels Kristallographie oder Magnetresonanz ist bis heute nicht gelungen. Dies limitiert unser Wissen über die NPY-Rezeptor Bindungsstelle und die molekularen Mechanismen der Signalübetragen, was alternative Untersuchungsmethoden notwendig macht. Das Hauptziel dieser Arbeit war die Charakterisierung von Neuropeptid Y Rezeptor-Subtypen auf molekularer Ebene mit Hilfe von anti-Rezeptor Antikörpern.

Einblicke in die strukturellen Verhältnisse von Ligand und Rezeptor sowie die Identifizierung der einzelnen Rezeptor-Subtypen in Zellen und Geweben ermöglichen ein besseres Verständnis der komplexen physiologischen Mechanismen und damit in einem nächsten Schritt rationales Design von Arznei-

mitteln. In dieser Arbeit wurden polyklonale Antikörperseren gegen definierte Segmente der NPY Rezeptorsubtypen zur Lokalisation, Identifizierung und für die Untersuchung der Struktur-Affinitäts-Beziehungen von NPY und seinen Rezeptoren eingesetzt.

Um eine Unterscheidung zwischen Y_1-, Y_2-,.Y_4- und Y_5-Rezeptor-Subtyp zu ermöglichen, wurden synthetische Fragmente des zweiten und dritten extrazellulären Loops der Rezeptoren zur Herstellung von Antikörper verwendet. Die Seren wurden in ELISA-Tests an intakten Rezeptoren und in Western Blot Experimenten an solubilisierten Rezeptoren getestet. Die molekularen Massen aller Rezeptor Proteine konnten bestimmt werden. Für jeden Rezeptor wurden Banden bei mehreren Massen detektiert, was mit unterschiedlicher Glykosilierung und Fragmentierung der Rezeptor Proteine erklärt werden kann. Für den Y_1-Rezeptor wurde vor allem Banden bei 73 kDa und 51 kDa detektiert. Der Y_2-Rezeptor wurde bei 58 kDa, 50 kDa und 35 kDa angefärbt. Banden von 51 kDa und 35 kDa wurden für den Y_4-Rezeptor und Y_5-Rezeptor gefunden. Selektivität wurde am solubilisierten Y_2-Rezeptoren mit dem Serum gegen den zweiten extrazellulären Loop erreicht. Das Serum Y_5 E2/2 erkannte den intakten Y_1-und Y_5-Rezeptor und das Serum Y_5 E3 erkannte den intakten Y_1- und Y_4-Rezeptor. Durch Kombination dieser zwei Seren ist es möglich, die vier Rezeptorsubtypen zu unterscheiden (Chapter 2).

Einige der Antikörper wurden auch an intakten Zellen, die den Y_1-, Y_2-,. und Y_5-Rezeptor exprimieren, getestet. Um eine weitere Unterscheidungsmöglichkeit zu haben, wurden zusätzlich eine Serie von Ala-substituierten und eine Serie von im Mittelteil verkürzten NPY Analoga synthetisiert und an den intakten Rezeptoren auf Selektivität getestet. Für den Y_1-Rezeptor war das Serum gegen den zweiten extrazellulären Loop (Y_1 E2/2) selektiv. Für den Y_2-Rezeptor erwies sich ebenfalls das Serum gegen den zweiten extrazellulären Loop (Y_2 E2/1) als selektiv. Zwei Seren (Y_5 E2/2 und Y_5 E3) erkannten den Y_2- und den Y_5-Rezeptor. Damit können die Seren in Kombination den Y_1-, Y_2- und Y_5-Rezeptor unterscheiden. Bei den Ala-substituierten NPY Analoga wurde für [A^{13}]-pNPY und [A^{27}]-pNPY Subtypenselektivität für den Y_2-Rezeptor erreicht (Chapter 3).

Synthetische Fragmente aus dem N- und C-Terminus und aus den extrazellulären Loops der Y_1-Rezeptorsequenz wurden verwendet, um anti-

Rezeptor Antikörper herzustellen. Die Proteine von Membranpräparationen, die den Y_1-Rezeptor enthalten wurden mittels SDS-PAGE aufgetrennt und in Western Blot Experimenten mit den Antikörpern detektiert. Es konnten zwei Proteine mit den Massen von 73 kDa und 51 kDa für den Ratten und den humanen Rezeptor nachgewiesen werden. Kompetition der Seren mit NPY zeigte, dass sieben Antikörper stark inhibiert wurden. Es wurden photoaktivierbare NPY-Analoga verwendet, um das Hormon kovalent an den Y_1-Rezeptor zu binden. Die Kompetition der Seren hing dabei stark von der Position des Crosslinkers im Liganden ab. Aus den Daten dieser Verdrängungsversuche konnte ein Modell für die Ligand-Rezeptor Wechselwirkungen entwickelt werden. Der N-Terminus von NPY scheint sehr flexibel zu sein, da Crosslinking an Position 1 verschiedene Seren gegen verschiedene Stellen im Rezeptor blockierte. Crosslinking an Position 21 und 22 führt vor allem zu einem Affinitätsverlust der Seren gegen E2/4 und E3/2. Position 27 von NPY dürfte nahe von E2/2 liegen, weil Crosslinking an dieser Position zu einer starken Blockierung der Seren E2/2 und E2/3 führt (Chapter 4).

Die Charakterisierung des Y_2-Rezeptor-Subtypes wurde neben den Antikörpern auch mit Hilfe photoaktivierbarer, biotinylierter Analoga von NPY, die mit ^{3}H-Propionsäure modifiziert wurden, erreicht. Hierzu wurden entsprechende Membran-Präparationen durch Crosslinking kovalent mit dem Ligand verbunden, die Proteine mittels SDS-PAGE getrennt und die gebundene Radioaktivität gemessen. Parallel dazu wurden das Molekulargewicht des Rezeptors im Western Blot mit einem anti Y_2 Antikörper verifiziert. Es wurden jeweils zwei Proteine mit molekularen Massen von 58 +/- 4 kDa und 50 +/- 4 kDa identifiziert, und zwar sowohl in humanen Neroblastoma-Zellen (SMS-KAN), die den Y_2-Rezeptor endogen exprimieren, als auch in Eierstock-Zellen des Chinesischen Hamsters, die mit humaner cDNA transfiziert wurden. Diese beiden Proteine entsprechen zwei unterschiedlich stark glykosilierten Formen des Y_2-Rezeptor Subtyps, wie durch Verdau-Experimente nachgewiesen wurde (Chapter 5).

Durch diese Arbeit konnte gezeigt werden, dass anti Rezeptor Antikörper wertvolle Hilfsmittel zur Unterscheidung und Lokalisierung aber auch zur Charakterisierung der Bindungsstellen von NPY Rezeptor Subtypen darstellen. Die Kenntnis der Verteilung verschiedener Rezeptor Subtypen in verschiedenen

Geweben ist wichtig, um die biologische Rolle der Rezeptoren zu verstehen. Die Resultate dieser Arbeit könnte die selektive Detektion von NPY-Rezeptoren auf Proteinebene in Gewebe ermöglichen. Zusammen mit dem besseren Verständnis der Hormon-Rezeptor Wechselwirkungen können die erzielten Resultate für die Entwicklung von Arzneistoffen hilfreich sein.

CHAPTER 1

Characterisation of G-Protein-coupled Receptors by Antibodies

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Abbreviations:

BHK, baby hamster kidney

BSA, bovine serum albumin

CHAPS, (3-[(cholamidopropyl)dimethyl-ammonio]-1-propane-sulfonate

CNS, central nervous system

ELISA, enzyme-linked immunosorbent assay

FITC, fluoresceinisothiocyanate

^{3}H-Tmd27, [N_{α}-biotinyl-Ahx_2, ^{3}H-propionyl-Lys^4, Ahx^{5-24}, (Tmd)Phe^{27}] NPY

^{3}H-Tmd36, [N_{α}-biotinyl-Ahx_2, ^{3}H-propionyl-Lys^4, (Tmd)Phe^{36}] NPY

Ig, immunoglobulin

KLH, keyhole limpet hemocyanin

LiDS, lithium dodecyl sulfate

Mabs, Monoclonal antibodies

NPY, neuropeptide Y

PAGE, polyacrylamide gel electrophoresis

PP, pancratic polypeptide

SDS, sodium dodecyl sulfate

TM, transmembrane segments

Tris, tris(hydroxymethyl)aminomethane

Triton X-100, polyoxyethylene [9-10] p-t-octyl phenol

Triton X-114, polyoxyethylene [7-8] p-t-octyl phenol

Tween 20, polyoxyethylene-sorbitan monolaurate

1.1 Abstract

G-protein-coupled receptors constitute a superfamily of integral membrane proteins encompassing hundreds of receptors for all types of chemical messengers, as well as, for example, the key molecules of our light and smell sensory systems, bioactive amines, peptide hormones, neurotransmitters and even proteins. Because of their complicated organisation with the characteristic seven transmembrane segments (7 TM) it has yet been impossible to structurally characterise any G-protein coupled receptor by crystallography or magnetic resonance. However, a number of indirect methods to study the structure and ligand binding of these proteins have been developed. Various studies have shown that antibodies produced against G-protein-coupled receptors are valuable tools. In this review we focus on the use of anti-receptor antibodies for the characterisation of membranes, cells and tissue, for mapping of the binding site, for purification by immunoaffinity chromatography and for biochemical studies of G-protein-coupled receptors. As an example we describe the characterisation of the G-protein-coupled neuropeptide Y receptor subtypes.

1.2 Introduction

The specificity and antigen-binding properties of antibodies have been investigated since the beginning of this century but within the last 20 years an enormous growth has been seen in their application. Antibodies are essential tools to identify, quantify and probe the structure and biological properties of antigenic molecules. The steady growth of experience in making specific antisera saw major new applications which evolved in the study of cell membrane components and this has again been greatly enhanced recently by the use of monoclonal antibodies [1]. Antibodies, generated by phage-display technology is rapidly maturing into a very effective tool for monoclonal antibody generation [2]. Assays for components of the cell surface are now available which utilize antibodies for binding to, and separating, membrane molecules, and for revealing their nature through affinity labeling of discrete entities of membrane eluates separated on polacrylamide gels [1].

Anti-receptor sera can be raised by immunisation with purified or enriched receptors or by immunisation with receptor fragments. They are used for molecular mass determinations by SDS/PAGE and subsequent Western blotting, receptor purification by affinity chromatography on antibody columns; investigations of the receptor localisation, regulation and desensitisation are only a few applications of anti-receptor sera. Moreover, if the binding site of a monoclonal antibody is known, or if the antibodies are directed against receptor fragments, then localisation of the functional groups will be possible by competition with ligands [3].

1.3 Generation of Antibodies

The generation of antibodies in animals is a very complex biological process. There is no prevalent protocol for antibody generation and a lot of experience (and sometimes also a little bit of luck) is necessary to obtain antisera with high affinity and specificity. However, polyclonal antibodies produced against G-protein-coupled receptors are valuable tools, especially in Western blot experiments and

ELISA (enzyme-linked immunosorbent assay). A polyclonal antibody serum contains a lot of different antibodies with different affinities against different epitopes of the antigen. This makes recognition of the antigen in Western blot experiments and ELISA assays highly probable. The amount of obtained serum is usually sufficient for the subsequent experiments. Polyclonal antisera are mostly produced in rabbits or chickens. Especially chicken are interesting because the antibodies can be isolated without bleeding from the yolk.

Several methods have been used to obtain anti-receptor antibodies. These range from simple immunisation with cells bearing receptors at their surface, injection of affinity-purified receptor to immunisation with synthetic peptides derived from the nucleotide sequence of receptor genes. Anti-receptor peptide antibodies may display properties similar to those of antibodies raised against the whole receptor protein. Fig. (**1**) shows a possible immunisation protocol for the generation of antibodies in rabbits.

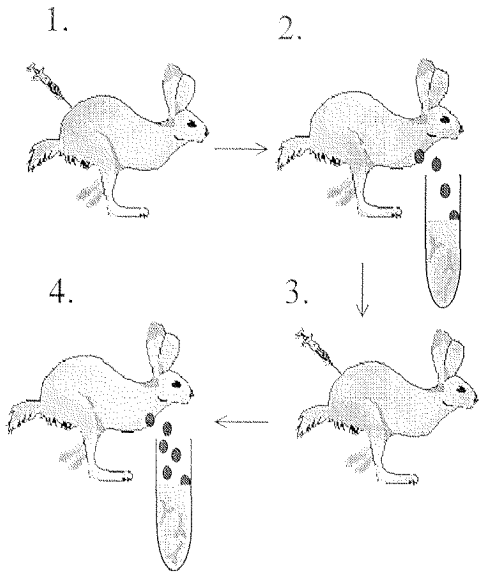

Figure 1. *Immunisation of rabbits. 1. A preliminary bleed should always be taken from each animal prior to immunisation. The animal is immunised with the antigen. After 14 days there should be a first booster-immunisation, after 28 days a second booster-immunisation. 2. First bleed after 35 days. 3. Third booster-immunisation after 56 days. 4. Final bleeding after 63 days.*

When the initial immunogen is extremely heterogeneous, as is the case with whole cells or membranes, most of the effort will be directed towards developing very specific methods for demonstrating the appearance of anti-receptor-antibodies. For many application immunisation with cells or membranes is not useful.

When the purified receptor is injected, every part of the immunogen may induce a response and antibodies may be directed to epitopes located in the extracellular, transmembrane, or intracellular regions. Specific procedures may be developed to obtain purified receptor, by classical protein chemistry or specific affinity chromatography. The quantities of protein obtained after several successive steps of purification are often limited, and usually difficult to manipulate without considerable loss of material. One way to circumvent this complication is to transfer the protein from polyacrylamid gel to a nitrocellulose membrane which is then introduced under the skin of the animal. More drastic is the direct injection of a portion of polyacrylamide gel containing the receptors. But these two methods are very stressing for the animals.

Immunisation with synthetic receptor peptides is a very useful method for generating anti-receptor antibodies. Antisera targeted against defined regions of the receptor can be used to detect functional groups that are involved in ligand-receptor interactions. The antibodies may also act as specific ligands by interacting with the binding site for agonists or antagonists. Such antibodies may actually compete with hormone and neurotransmitter, and this property provides a method for evaluating their affinity and specificity. Peptide sequences may be derived from the sequence of gene coding for the receptor. Those segments selected for immunisation should preferably be chosen from parts of the receptor protein likely to be exposed at the surface of the molecule, as may be deduced from hydropathicity plots. Antibodies against the peptide segments of intracellular regions are unsuitable for hormone receptor interaction studies but useful to study signal transduction processes and interaction with G-proteins. Before immunisation the peptides require coupling to larger protein carriers, because small molecules have only low intrinsic immunogenicity. The antibody response will be directed towards the carrier determinants as well, so a carrier that is irrelevant to future assays and/or that can be prepared easily as an adsorbent has

to be chosen. Convenient carriers are serum albumin of another species than the immunised animal (bovine serum albumin, BSA) or keyhole limpet hemocyanin (KLH).

Some immunological procedures can be carried out using the impure antibody solution, isolated from serum. But antibody enrichment is frequently required for satisfactory results, particularly if the antibodies are to be used for affinity chromatography. Purified antibodies can also be used for immunoassays, immunoblotting, enzyme conjugation or cell staining. Although conventional protein purification methods (precipitation or chromatography techniques) can be applied to antibody purification, specialised procedures employing the bacterial cell wall protein A and protein G offer a simple and highly specific separation [4]. Protein A and protein G specifically bind the Fc portion of immunoglobulins. Fig. (**2**) shows a model of an immunoglobulin molecule. Immunoaffinity purification of the antisera is not absolutely necessary and can be difficult due to different affinities of the antibodies to the antigen, which makes elution a problem.

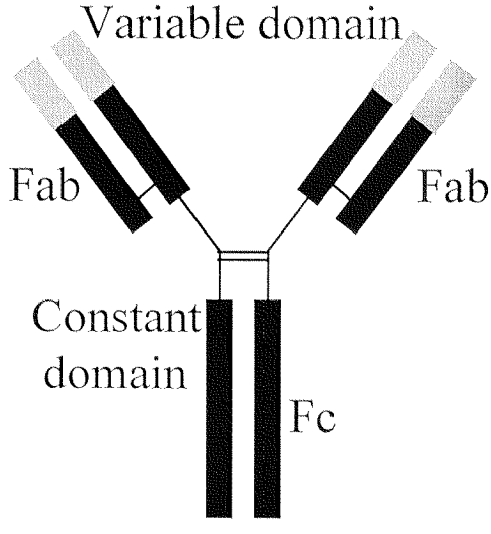

Figure 2: *Model of an immunoglobulin molecule. The molecule is build of a Fc portion with two disulfide bridges and two Fab portions with one disulfide bridge. The variable domain is represented in grey.*

Monoclonal antibodies (Mabs) are, in contrast to polyclonal antibodies, directed against a single epitope of an antigen, which makes them extremely selective. In general, the use of Mabs in experimental and clinical studies eliminates the problem of non-specific reactions of conventional antisera due to irrelevant antibodies or binding of non-immunoglobulin components. Production of Mabs is expendable but theoretically inexhaustible, while the immunoglobulin composition of a polyclonal serum is not reproducible.

The immunisation of an animal with antigen leads to clonal expansion of those B-lymphocytes which recognise antigen and their differentiation to antibody-secreting cells. The antibody-secreting lymphocytes are isolated from lymphoid tissue of the animal and fused with cells from a plasmacytoma cell line which represents a similar differentiation stage. The resultant hybrid cells retain the ability of one parent to secrete a particular antibody molecule and the continuous growth characteristics of the other parent (the plasmacytoma). The hybrid cells are cultured and isolated and again cultured. Culture supernatants are then screened for activity against the antigen. Cells with positive supernatants are cultured and again differentiated until a cell population consisting of a single clone is received. This cell clone is used for antibody production. The technique has been highly successful with mouse and rat cells; there has been less success with human hybridomas and little work has been done on other species [1,5,6].

Phage display technology to obtain antibodies is a new and very effective tool for monoclonal antibody generation. Antibody fragments of predetermined binding specificity have recently been constructed from repertoires of antibody V genes, bypassing hybridoma technology and even immunisation [7]. The V gene repertoires are harvested from populations of lymphocytes, or assembled in vitro, and cloned for display of associated heavy and light chain variable domains on the surface of filamentous bacteriophage. Rare phages are selected from the repertoire by binding to antigen; soluble antibody fragments are expressed from infected bacteria Fig. (**3**). The affinity of binding of selected antibodies can be improved by mutation. The process mimics immune selection, and antibodies with many different binding specificities have been isolated from the same phage repertoire. Thus, human antibody fragments have been isolated with specificities against both foreign and self antigens, including haptens, carbohydrates, secreted

and cell surface proteins, viral coat proteins and intracellular antigens from the lumen of the endoplasmic reticulum and the nucleus. Such antibodies have potential as reagents for research and therapy.

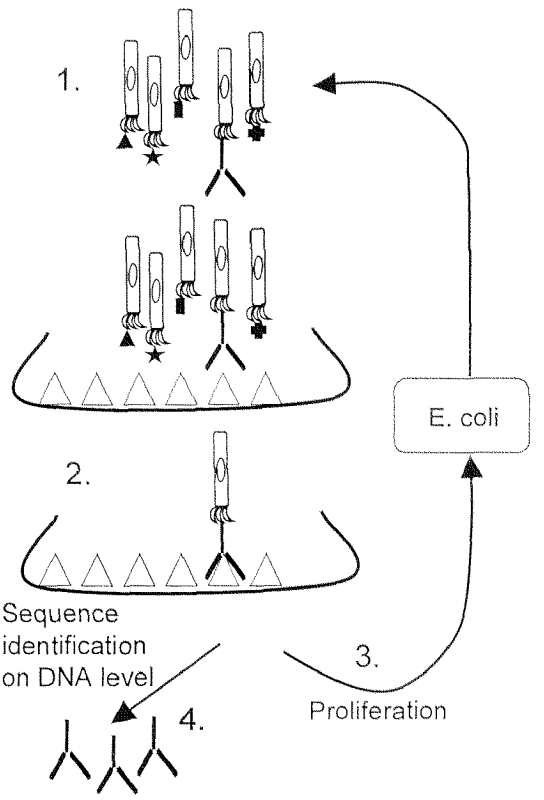

Figure 3. *Generation of monoclonal antibodies by phage display. 1. Phage surface display of antibody (fragments). 2. Antigen-driven selection. 3. Proliferation of phage in E.coli. 4. Sequence identification of the antibodies.*

1.4 Characterisation of Membranes

Antibodies produced against G-protein-coupled receptor can be tested on membranes containing the receptor. The membranes can be characterised either by an enzyme-linked immunosorbant assay (ELISA) or by immunoblotting (Western blot). For the ELISA assay the antibodies are added to plastic plates, that have been coated with the membranes to be tested before. Binding of the antibodies is detected by adding a second enzyme-linked antibody directed against the immunoglobulin of the first species followed by a colourimetric assay

for the enzyme [6]. For immunoblotting, the membranes are solubilised and separated by SDS/PAGE and subsequent Western blotting. Antibodies are added to the membrane. Binding is detected like in the ELISA assay by adding a second enzyme linked antibody Fig. (**4**).

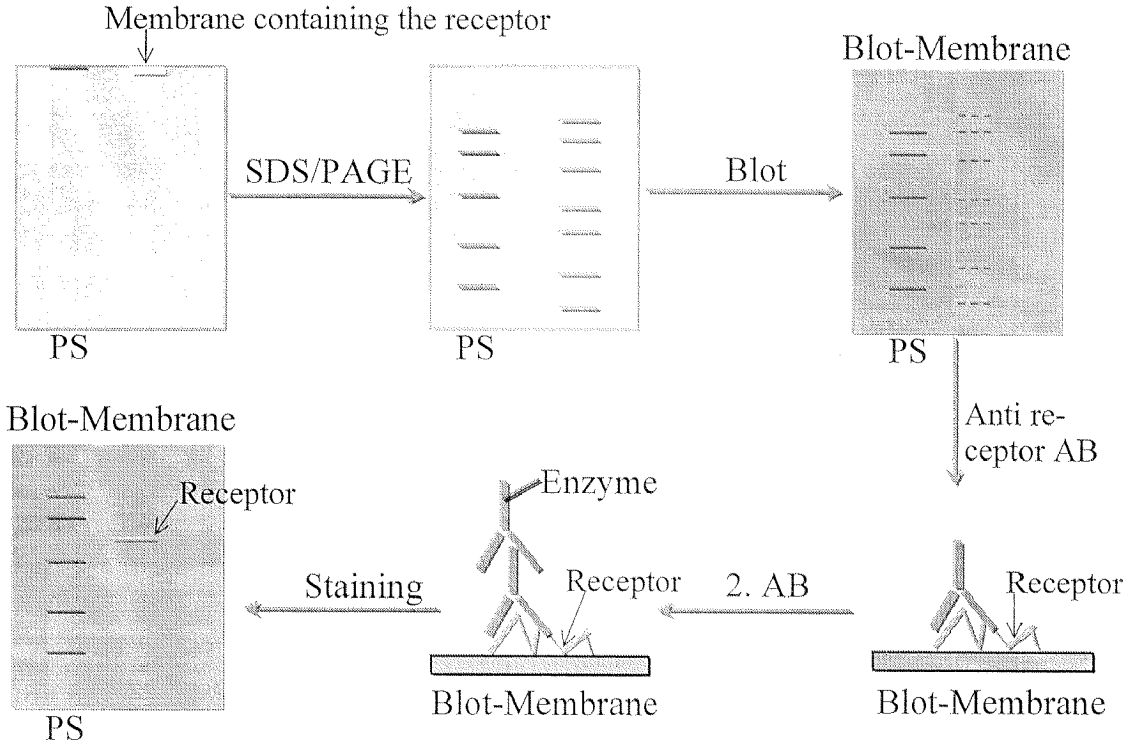

Figure 4. *Characterisation of G-protein-coupled receptor containing membranes by immunoblotting. The proteins are separated by SDS/PAGE and subsequently transferred to a membrane which makes the proteins accessible for antibodies. The membrane is incubated with the anti receptor antibody (1. AB), washed and incubated with a second, enzyme linked antibody(2. AB). The receptor is stained with an enzymatic dye reaction. A protein standard mixture (PS) allows to determine the molecular weight of the sample proteins.*

Both methods are suitable to detect a given G-protein-coupled receptor in membranes. In the ELISA the antibodies are tested against intact receptors, whereas in Western blots they are tested against solubilised receptor. There often is a big difference in recognition of the intact and the solubilised receptors. For

antibodies generated by immunisation with receptor fragments either not all synthetic receptor fragments develop the same secondary structure as in the intact receptor, or otherwise there is steric hindrance in the intact receptor which prevents antibodies from binding [8].

The ELISA is versatile and easy to perform. Plastic microtitration plates allow simple in situ optical density measurement with special ELISA-readers. Western blot studies are more time-consuming than ELISA assays. But Western blot gives the possibility of molecular mass determination of the receptor protein. Moreover, the receptor proteins can be destained and are accessable to proteolytic or chemical digest and subsequent elution from membrane. The receptor fragments can than be analysed by mass spectrometry.

1.5 Characterisation of Cells and Tissue (Immunofluorescence)

The possibility of covalently attaching fluorescence dyes to antibodies make them a good tool to investigate the regional distribution of G-protein-coupled receptors in cells and tissues. Binding of the antibodies is mostly detected by adding a second fluorescence dye-linked antibody directed against the immunoglobulin of the first species. Binding of the antibodies can than be detected with a fluorescence microscope with a suitable filter system. Fluorescence dyes can also be directly linked to the specific antibody but this immuno-fluorescent staining procedure is less sensitive than the indirect method. Fluorescein is still the most important fluorochrome. Fluorescein conjugates show an absorption maximum of 495 nm. Other widespread fluorochromes are rhodamine (absorption maximum of 555 nm) and Texas Red (absorption maximum of 596 nm). The use of to different fluorochromes allows the technique of double staining. This is a useful method for localizing specific staining in complex tissue or for identifying cell types in preparations of mixed cells. Antibody-mediated localization studies are often advantageous to mRNA localization, because the localization of the mature receptor protein must not necessarily fit with the localization of the mRNA [9].

Recently, a G-protein coupled, extracellular calcium/polyvalent cation–sensing receptor was identified in rat kidney [10]. The cellular and regional distribution of

the receptor protein was examined by immunofluorescence microscopy using a polyclonal antiserum raised against a 22 amino acid region of the NH_2-terminus of the receptor. Basolateral staining was, among others, detected in macula densa cells identified by co-staining with an antibody to brain nitric oxide synthase and in distal convoluted tubule cells distinguished by co-staining for the apical thiazide-sensitive Na^+-Cl^- cotransporter. For the double staining two secondary antibodies were used: The first fluorescence-labeled secondary antibody was rhodamine Red-X-conjucated Fab fragment anti-rabbit IgG, and the second secondary antibody was FITC-conjugated anti-rabbit IgG.

The development of laser-scanning confocal microscopes has given a considerable impact on studies of cellular structure and organisation. It enables structures to be sectioned optically and has the potential to reveal features that are completely obscured by conventional fluorescence microscopy.

1.6 Purification of G-Protein-coupled Receptors

Affinity chromatography is a wide spread method in protein purification. Antibodies raised against G-protein-coupled receptors allow affinity purification using antibody columns or immunoprecipitation, which both are extremely powerful methods of protein purification. Monoclonal antibodies are more useful than polyclonal antibodies for immunoaffinity protein purification. Monoclonal antibodies represent a homogenous antibody population with specificity for a single binding site on the protein of interest, thus allowing well-defined binding and more uniform protein elution. Nevertheless, antibodies from a polyclonal source can sometimes prove to be useful for affinity purification [4].

An immunoaffinity column requires that the antibody first is covalently attached to a matrix. Widely used is the immobilisation of the antibody with cyanogen bromide-activated sepharose that is commercially available. The receptor must be solubilised before it is applied to the Sepharose column.

Detergents are used most often for the extraction and purification of membrane proteins, which otherwise are usually insoluble in aqueous solution. An important property of detergents is the formation of micelles, which are clusters of detergent molecules. Solubilized membrane proteins form mixed micelles with detergent. A

number of classes of detergents may be used for the solubilisation and stabilisation of membrane proteins. Most important are the ionic detergents sodium or lithium dodecyl sulfate (SDS, LiDS), the nonionic detergents Triton X-100 (polyoxyethylene [9-10] p-t-octyl phenol), Triton X-114 (polyoxyethylene [7-8] p-t-octyl phenol), octyl glucoside (1-O-n-octyl-β-D-glucopyranoside) and Tween 20 (PEG [20] sorbitan monolaurate) and the zwitterionic detergent CHAPS (3-[(cholamidopropyl)dimethyl-ammonio]-1-propane-sulfonate). Detergents are summarised in Table 1. Ionic detergents have the dis-advantage of being highly denaturing. However, they permit the separation of proteins into their monomeric forms, facilitating molecular weight determination. Nonionic detergents have uncharged hydrophilic head groups. As a result, they are less likely to disrupt protein-protein interactions and are particularly useful for isolating functional protein complexes. Nonionic detergents are far less denaturing than ionic detergents; thus, protein aggregation may occur in the presence of these detergents [4].

***TABLE 1**: Characteristics of common detergents for solubilisation of membrane proteins. The exact concentration of the detergent for solubilisation has to be determined for every membrane protein.*

Detergent	Molecular Weight [Da]	Concentration for Solubilisation	Critical Micelle Concentration
Sodium Dodecyl Sulfate	289	variable	0.23 %
Lithium Dodecyl Sulfate	272	> 10 mg/mg protein	0.2 %
Triton X-100	about 628	0.2-0.6 mg/mg protein	0.02 %
Triton X-114	about 543	variable	0.02 %
Tween 20	1230	variable	0.006 %
Octyl Glucoside	292	20 - 45 mM	0.5 %
CHAPS	615	6 - 10 mM	0.5 %

The solubilised receptors bind selectively to the immobilised antibodies, the contaminants can be washed away. The receptors are then eluted by a decrease in pH, a change in ionic strength or with saturating concentrations of a competing substrate, which is the most specific method and allows well defined separation of the protein without otherwise perturb the column conditions. Elution from an immunoaffinity column can be difficult if the nature of the protein binding is not understood. If a specific ligand is not available which can disrupt the antibody-receptor interaction, nonspecific conditions for disrupting protein interactions are required. [4] Immunoaffinity purification of G-protein-coupled receptors is summarised in Fig. (**5**).

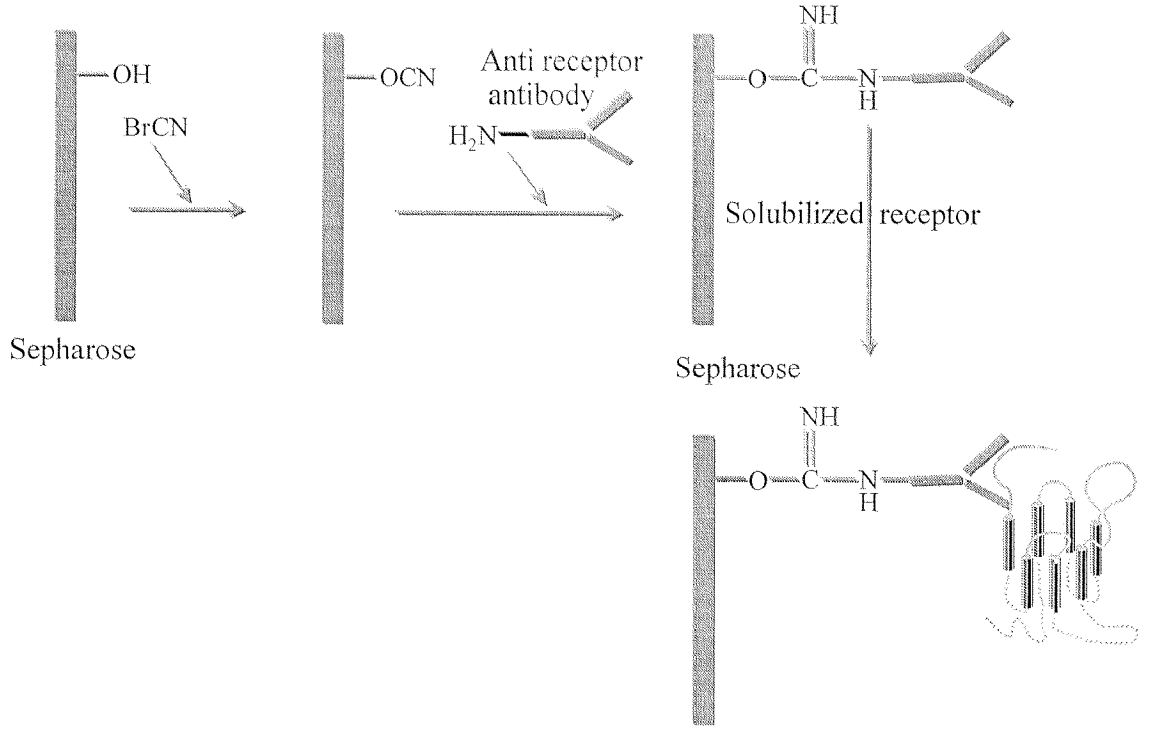

Figure 5. *Anti receptor antibodies are covalently bound by an amino group to cyanogen bromide-activated sepharose. The solubilised receptors bind to the immobilised antibodies, contaminants are washed away. The receptors can be eluted from the column by a decrease in pH, a change in ionic strength or with saturating concentrations of a competing substrate.*

A receptor-specific antibody may permit quantitative isolation of a receptor by immunoprecipitation. Protein A or protein G coupled to beads of Sepharose offers a solid matrix for removing the antibody-antigen complex from the protein extract. Alternatively, antibodies can be directly coupled to beads and remove the receptor from the extract [4-5].

1.7 Anti-Receptor Antibodies in Biochemical Studies

Binding of ligands to G-protein coupled receptors is investigated with several methods. Syntheses of analogues of a high-affinity ligand, site directed mutagenesis in transmembrane regions and extracelluar loops and covalent cross-linking of the ligand with the receptor are important techniques. Other valuable tools are anti-receptor antibodies. If the binding site of a monoclonal antibody is known, or if the antibodies are obtained against receptor fragments, then localisation of the functional groups will be possible by competition with ligands (for review see [11]). The use of anti-receptor antibodies and photoaffinity labelling to characterise the binding site can be combined [12]. Fig. (**6**) shows schematically the mapping of the binding site of a G-protein coupled receptor by using a combination of photoaffinity labelling and anti-receptor anti-bodies.

Antibodies raised against receptors may act as specific ligands by interacting with the binding site for agonists or antagonists. Such antibodies may actually compete with hormone and neurotransmitters, and this property provides a method for evaluating their affinity and specificity. However, it is often observed that the large immunoglobulin molecules, while binding specifically to the receptor, are unable to competitively inhibit the effect of the natural ligands. By binding to the receptor, antibodies may trigger mechanisms that normally are activated by hormones and neurotransmitters: receptor redistribution, stimulation or inhibition of adenylate cyclase, receptor internalisation, receptor desensitisation, inhibition of receptor-mediated cell-cell interactions, etc. Some of these phenomena may be due to the multivalent immunoglobulin molecules that cause aggregation of the receptor, rather than any direct effect on the ligand-binding site. Hence, formal proof for direct interaction at the ligand binding site requires the use of monovalent Fab antibody fragments [6].

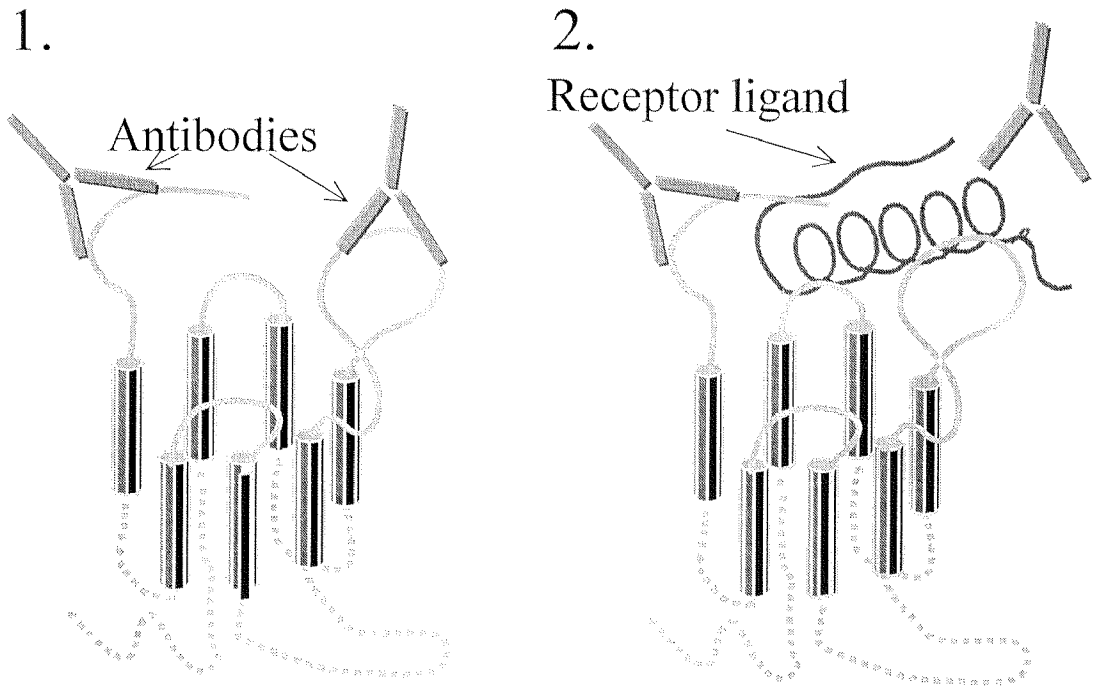

Figure 6. *1. Mapping of the binding site. Antibodies obtained against receptor fragments bind to their epitopes in the receptor. 2. Ligand binds to the receptor. Binding of antibodies against binding site epitopes is inhibited. A model of the ligand-receptor interaction can be derived from these data, combined with cross-linking data.*

Recently, antibodies directed against a peptide corresponding to the second loop of the human β_2-adrenergic receptor were generated in rabbits [13]. The resulting antibodies were affinity-purified and shown to be monospecific for the target receptor. They were able to stimulate the L-type Ca^{2+} channels in whole-cell patch-clamp experiments on isolated adult guinea-pig cardiomyocytes. This effect was similar to that obtained by the specific β_2-adrenergic agonist zinterol. The antibody effects could be blocked with a specific β_2-adrenergic inverse agonist but not with the neutral antagonist alprenolol. These results suggests that the antibodies recognise the active conformation of the β_2-adrenergic receptor.

Redistribution of receptors within the plasma membrane as well as between the plasma membrane and various cell compartments presents an important way of regulating the cellular responsivness to their cognate agonists. Recently, immunocytochemical methods have been applied to localise the bradykinin B_2

receptor and to examine its agonist induced redistribution in A431 cells [14]. The bradykinin B_2 receptor has been classified as a prototypical member of the superfamily of G-protein-coupled receptors. Interaction of the B_2 receptor with its major agonist, bradykinin, mediates physiological effects such as vasodilation, change in vascular permeability, contraction of smooth muscle cells, and pain sensations. There is little knowledge about specific cellular locations of the B_2 receptor in its active or inactive state and the cellular dynamics of the receptor molecule accompagning the signalling event. Ligand-induced activation of the B_2 receptor is followed by a rapid desensitisation which leaves cells and organs refractory to agonist challenge for a period of 10 to 20 minutes. The molecular events underlying the B_2 receptor desensitisation are starting to be understood: ligand-induced phosphorylation of the receptor is one key event that mediates B_2 receptor desensitisation and down-regulation. The physical redistribution of the receptor into cell compartments is likely to participate in ligand-induced desensitisation of the B_2 receptor. Recent biochemical studies showed an association of the stimulated receptor with caveolin-1, the marker of plasmalemmal caveolae. Due to the lack of specific antibodies the mechanisms regulating the B_2 receptor activity and availability have been studied exlusively at the pharmacological and biochemical level. The recent advent of anti-peptide antibodies directed to the various extra- and intracellular domains of the bradykinin B_2 receptor has now allowed the functional dissection and the immunohistochemical localisation of the receptor at the cellular level. In the described study [14], polyclonal antibodies to extracellular domain-2 of the B_2 receptor were used for immunocytochemical analyses of the subcellular distribution of the receptor in native cells, and for the identification of cellular compartments involved in receptor redistribution in agonist-stimulated cells. In situ labeling with antibodies to ectodomain-2 of the receptor, which do not interfere with bradykinin binding of the receptor, showed a random distribution of the B_2 receptor on the plasma membrane. Stimulation of cells with 20 nM bradykinin markedly reduced the accessibility of the antibody to its corresponding epitope in non-permeabilised cells. Immun-electron microscopy revealed the presence of receptors in membrane-near vesicles that are surrounded by an electron-transparent halo. Fluorescence microscopic double labeling co-localised the B_2

receptor protein with caveolin-1 by a convergent pattern of punctate staining. At the ultrastructural level the B_2 receptor protein was found in vesicles that bear the immunolable of caveolin-1 and display the morphological characteristics of caveolae. From that data it was concluded that stimulation of B_2 receptors result in their redistribution and sequestration in caveolae, an event that is likely to be implicated in receptor signalling and/or desensitisation. The localisation of B_2 receptors in endosome-like structures after prolonged exposure to bradykinin might indicate that the internalisation through caveolae may communicate with other endocytotic pathways of A431 cells [14].

Phosphorylation of G-protein coupled receptors is considered an important step during their desensitisation. Recently, pretreatment of SK-N-BE cells (a pertinent model for the studies of the human δ-opioid receptor) with the opiod agonist etorphine increased time dependently the rate of phosphorylation of a 51-kDa membrane protein [15]. Immunological characterization of this protein with an antibody, raised against the amino terminal region of the cloned human δ-opioid receptor, revealed that it corresponded to the δ-opioid receptor. During prolonged treatment with etorphine, phosphorylation increased as early as 15 min to reach a maximum within 1 h. Phosphorylation and desensitisation of adenyl cyclase inhibition paralleled closely and okadaic acid inhibited the resensitisation, which strongly suggests that phosphorylation of the δ-opioid receptor play a prominent role in its rapid desensitisation. The increase of phosphorylation of the δ-opioid receptor, as well as its desensitisation, was not affected by H7, an inhibitor of protein kinase A and protein kinase C, but was drastically reduced by heparin or Zn^{2+}, known to act as G-protein coupled receptor kinase inhibitors. These results on endogenously expressed human δ-opioid receptors show, that a close link exists between receptor phosphorylation and agonist-promoted desensitization and that desensitization involves G-protein coupled receptor kinase.

The effect on internalisation of an antibody against the epitope EYMPME, cloned to the amino terminus of the human muscarinic cholinergic receptor hm1, was recently studied [16]. The antibody directed against the tag induced internalisation of the hm1 receptor within minutes after exposure of human embryonic kidney 293 cells transfected with the tagged receptor. This antibody-induced internalisation was reversible following removal of the antibody. In

contrast to hm1 internalisation induced by the agonist carbachol, internalisation induced by antibody is not blocked by the muscarinic antagonist atropine. The mechanism of antibody-mediated internalisation did not appear to involve receptor dimerisation by the antibodies, as Fab fragments derived from the antibody also induced internalisation. The pathway of antibody–induced internalisation, similar to the agonist-induced process, was mediated by clathrin-coated vesicles. Furthermore, antibody treatment did not result in any second messenger production, as was measured by phosphoinositide accumulation. This data show that internalisation of a G-protein-coupled receptor can be triggered by interaction of the amino terminus of the receptor with an exogenous ligand and can occur independently of second messenger production. This suggest that the receptor can exist in multiple conformations, each mediating distinct downstream events.

Antibodies are also useful in characterising the assembly of G-proteins from their subunits and the role of different G-protein families in certain processes. Recently, heterotrimeric G proteins were purified from bovine brain by immunoaffinity chromatography on immobilised anti G-protein monoclonal antibody 3C2 [17]. Release of $\beta\gamma$ subunits was effectuated by exposure of immobilised trimeric G proteins to $MgAlF_4$. The resultant $\beta\gamma$ subunits were pure and biologically active. Following immunisation of mice with purified $\beta\gamma$ subunits, monoclonal antibodies, directed against the G_β-subunit, showed a broad species cross-reactivity. Characterisation of the epitope that was recognised by one such a monoclonal antibody, ARC9, indicated the involvement of the extreme COOH-terminus, as assessed by its reactivity on β subunits lacking the COOH-terminal 15 residues, obtained by in vitro translation. Although native $\beta\gamma$ subunits were used as immunogen, all obtained monoclonal antibodies failed to recognise the assembled $\beta\gamma$ subunits, and were specific for free β subunits. This property is useful in characterising the assembly of G proteins from their subunits in living cells.

1.8 Characterisation of Neuropeptide Y Receptors by Antibodies

1.8.1 Neuropeptide Y – The Ligand

Neuropeptide Y (NPY) is a 36 amino acid peptide amide and a member of the pancreatic polypeptide hormone family. NPY is predominantly located within neurons of the central and sympathetic nervous systems [18-19]. It is one of the most abundant neurohormones in brain and is localised in high concentrations within the hypothalamus [20]. A variety of biologic effects have been attributed to NPY due to its widespread distribution throughout the CNS. It induces a strong increase in food intake and has marked effects on peripheral metabolism, such as decrease of brown adipose tissue, thermogenisis, and increase of lipoprotein lipase activity that promotes white fat lipid storage. NPY has also profound effects on secretion of lutenising hormone as well as on growth hormone and insulin release. These observations suggest an important role of NPY in the pathophysiology of obesity and diabetes. It has also been reported that NPY facilitates learning and memory retention processes [21], modulates locomotor behaviors [22-23], produces hypothermia [24-25], inhibits sexual behavior [26], shifts the circadian rhythms [27-28], modulates cardiorespiratory parameters [29] generates anxiolytic effects [30], and inhibits alcohol consumption and resistance [31].

In sympathetic nerves NPY is co-localised with noradrenaline. Prejunctionally, NPY regulates its own release and inhibits the release of noradrenaline. Postsynaptically, NPY evokes an increase in blood pressure by direct vasoconstrictory effects or by potentiating those of other vasoconstrictors such as angiotensin II and noradrenaline (for reviews see, [32-36]).

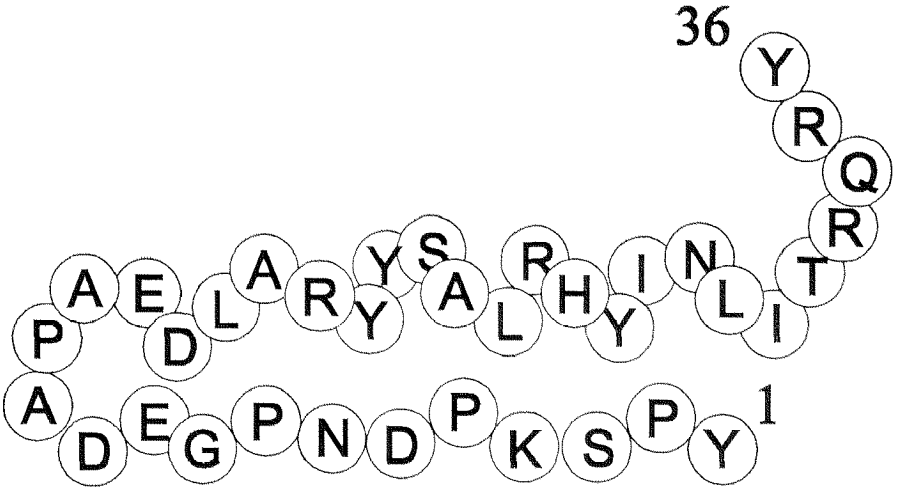

Figure 7: *Sequence and schematic structure of the hairpin folded pNPY*

The broad physiological relevance of NPY is reflected by the multiplicity of its receptors and gives reason for an increasing interest in NPY as a new target in drug discovery. Five distinct NPY receptors subtypes have been cloned and pharmacologically characterised. They have been named Y_1- [37-39], Y_2- [40-42], Y_4/PP_1- [43-44], Y_5- [45] and y_6- [46] receptor subtype. Sequence comparisons show that the receptors Y_1, Y_4, and y_6 are more closely related to each other than to the receptors Y_2 and Y_5.

The different receptor subtypes are localised in various tissues, in the central nervous system as well as in the periphery. Tissues with high density of NPY receptors are blood vessels, kidney, adrenal glands, colon, heart, pancreas, intestine, nerve endings and brain. While their distribution appears to be species specific, all subtypes belong to the large superfamily of G-protein-coupled, heptahelical receptors [11]. Their signal transduction pathways appear to be similar, and no clear and consistent alignment of a specific receptor subtype with a distinct transduction pathway has been identified. NPY receptors act via pertussis toxin sensitive G-proteins in almost every cell type studied, i.e. via members of the G_i and G_0 family. In a small number of cases pertussis toxin insensitive responses to NPY have been found, mostly involving presynaptically localized receptors [47-51], while it remains unclear whether this is more likely due to a distinct signaling mechanism or to failure of pertussis toxin to fully inactivate its substrates. The typical signaling response of NPY receptors found in almost all tissues and cell

types is the inhibition of adenylyl cyclase, whereas inhibition of Ca^{2+} channels, activation or inhibition of K^+ channels and mobilisation of Ca^{2+} from intracellular stores is only found in some [52]. Further responses appear to be induced in some cases, like activation of phospholipase A_2 and D and activation of a tyrosine kinase.

1.8.2 Why Antibodies for the Characterisation of Neuropeptide Y Receptors?

Neuropeptide Y receptor subtypes have been characterised so far by means of pharmacological experiments and investigations on the mRNA level. Several questions on the protein level like mass determination and localisation functionality are not yet solved. Furthermore, it has yet been impossible to structurally characterise G-protein coupled receptor proteins with the characteristic seven transmembrane segments by christallography or magnetic resonance which limits our knowledge of the NPY receptor binding site, as well as on the molecular mechanism of action. Accordingly, alternative methods are required, to characterise the receptors on the protein level and to learn more about the structure of the receptor protein and the binding of NPY to its receptor subtypes. Anti-receptor antibodies are a very powerful tool, that can provide answers to many of the above mentioned questions.

1.8.3 Mapping of the Binding Site of the Y_1-receptor

In order to map the binding sites of the Y_1-receptor, recently 18 peptides of the extracellular segment oft the receptor were selected to gain antibodies [12]. Ten of them recognised the receptor expressed on intact cells as well as on membranes that have been prepared. Interference of the antibody binding epitope with the NPY binding site was tested on Y_1-receptor expressing cell membranes. One serum directed against the N-terminus and one serum, that binds to the second extracellular loop were inhibited by NPY to a high extend. Photoaffinity analogs of

NPY have been used on stably rat Y_1-receptor-expressing cells to further characterise the binding epitopes of the antibodies in competition experiments. Fig. (**8**) shows the possible position of the photolabile amino acid in the NPY sequence. In order to irreversibly block the NPY binding site, the cells were incubated with the photolabile NPY analogues and irradiated to couple the NPY covalently to the receptor protein. Antisera were then tested on the crosslinked receptors. The position of the crosslinker had impact on the extend to which it blocked the access of the antibodies to the binding site. Fig. (**9**) shows as an example a crosslinking experiment with the photolabile amino acid in position 36 of the NPY. From the antibody interaction studies a schematic model of the NPY-Y_1-receptor was suggested.

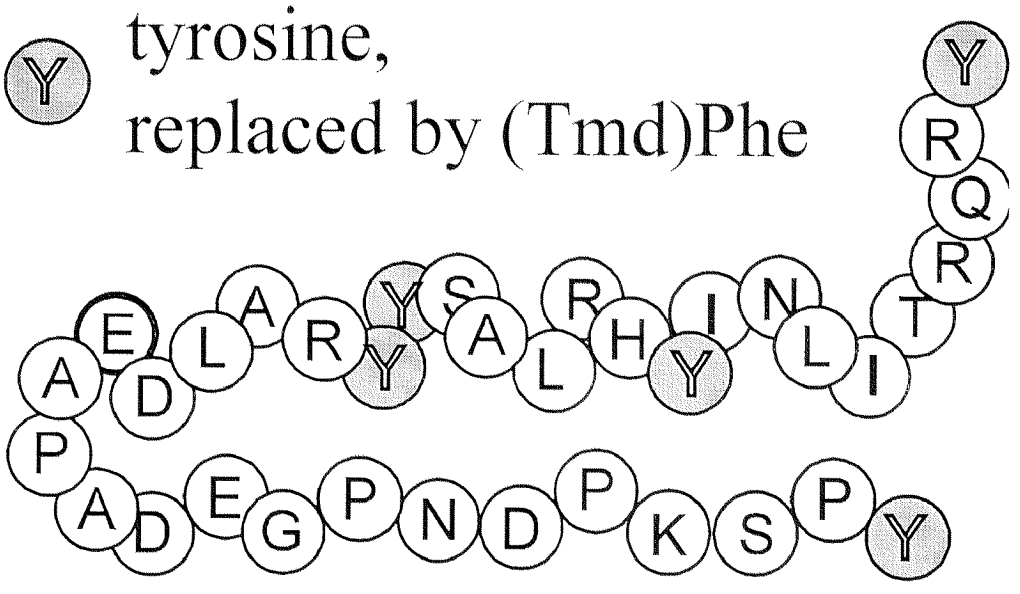

Figure 8: *Positions of the replacement of Tyr by 4'-(3-trifluoromethyl)-3-diazirine-3-yl-phenylalanine ((Tmd)Phe).*

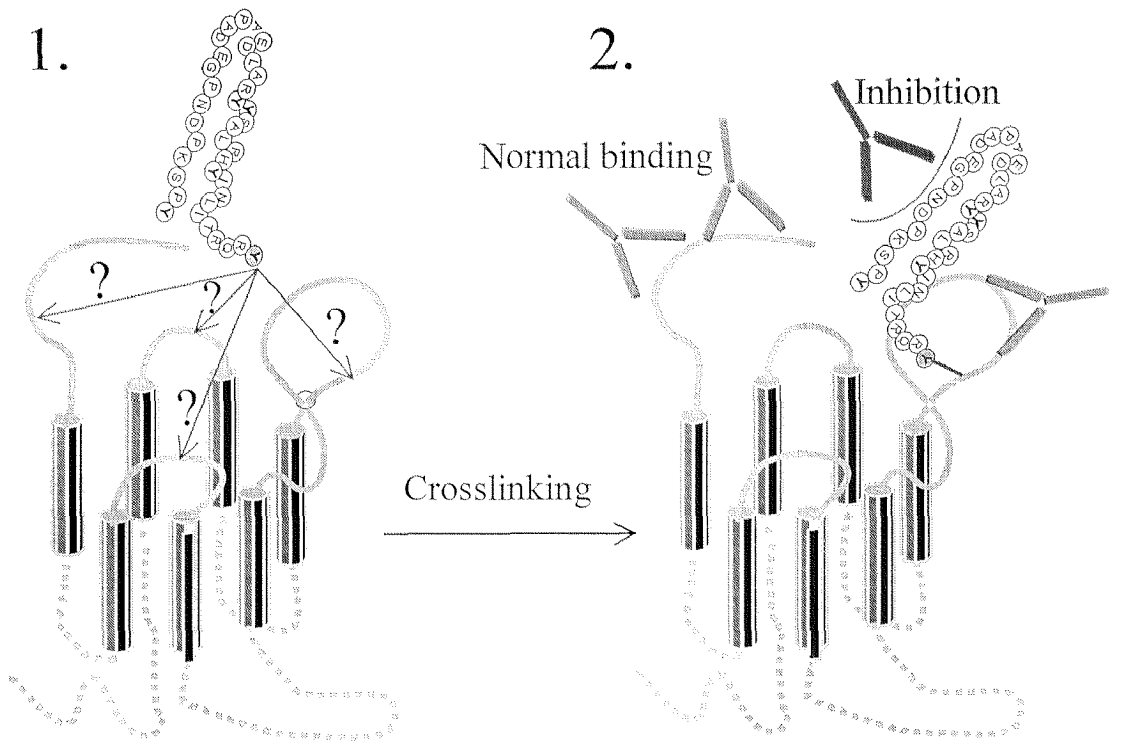

Figure 9. *Crosslinking experiment with the photolabile amino acid in position 36 of the NPY.* ***1.*** *NPY binds to the receptor. Irradiation causes covalent binding of Tmd(Phe)36 to the nearest amino acid of the receptor protein.* **2.** *Anti-sera against the extracellular segments of the receptor are tested. An antibody against the second extracellular loop is inhibited. From toset data and the data of the other crosslinking positions a schematic model of the ligand-receptor interactions can be derived.*

1.8.4 Characterisation of Neuropeptide Y Receptor Subtypes

In order to characterise several neuropeptide Y receptor subtypes, antibodies were recently raised against the second and the third extracellular loop of the receptors [8]. Antibodies against the extracellular loops of a receptor protein may recognise also intact cells or tissue. Because of possible post-translational modifications like glycosylation and palmitoylation, characterisation of the receptor on the protein level is important. A receptor subtype identified on the mRNA level may or may not be functionally expressed. Moreover, since rapid axonal transport has been suggested for the Y_1-receptor [53] mRNA localisation must not necessarily fit with the localisation of the mature protein. In order to determine the

molecular mass of the receptor proteins, Western blot experiments have been performed with Y_1-, Y_2-, Y_4 and Y_5-receptor expressing cell membranes Fig. (**10**). An antibody against the second extracellular loop of the Y_2-receptor identified selectively the Y_2-receptor with two bands of a molecular weight of 61 and 50 kDa Fig. (**11**). The two bands might be explained by receptor degradation, different glycosylation of receptors or different transcription products [12]. The molecular mass of the Y_2-receptor has additionally been determined by photoaffinity labelling [54]. SMS-KAN cell membranes were crosslinked using the ^3H labelled photoactivatable NPY analogues Tmd27 and Tmd36. SDS-PAGE was performed subsequently, followed by cutting the gels in slices and determination of the radioactivity of each gel slice. When compared to the mass determination via Western blotting, the masses identified by photoaffinity labelling with two different photoactivatable analogues were in good agreement.

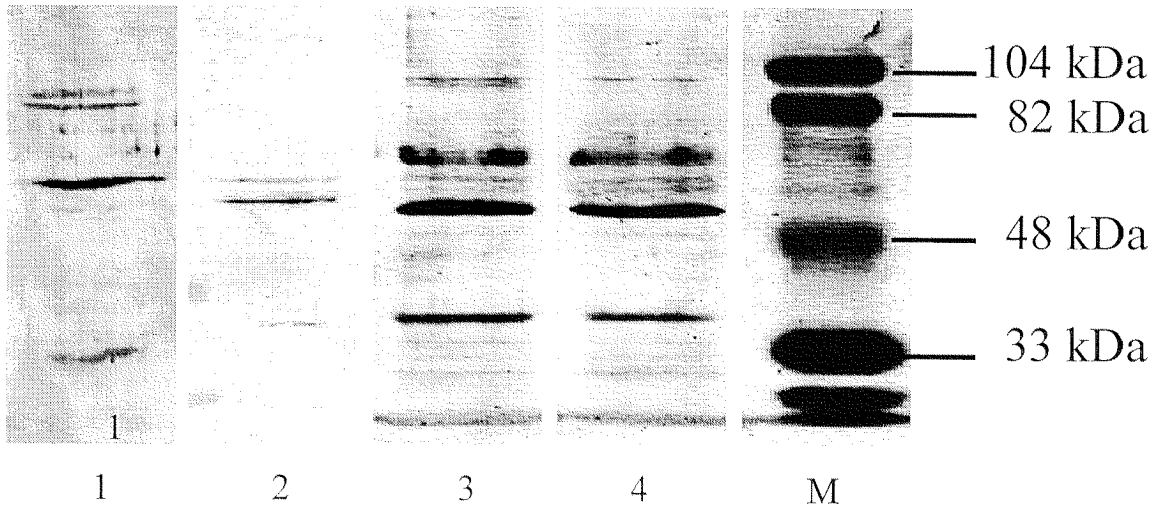

***Figure 10**: Western blot staining of Y-receptor subtypes by antibodies. 1: Y_1 expressing SK-N-MC cell membranes. 2: Y_2 expressing SMS-KAN cell membranes. 3: Y_4expressing BHK cells membranes. 4. Y_5expressing BHK cell membranes. M = SDS-PAGE Standard.*

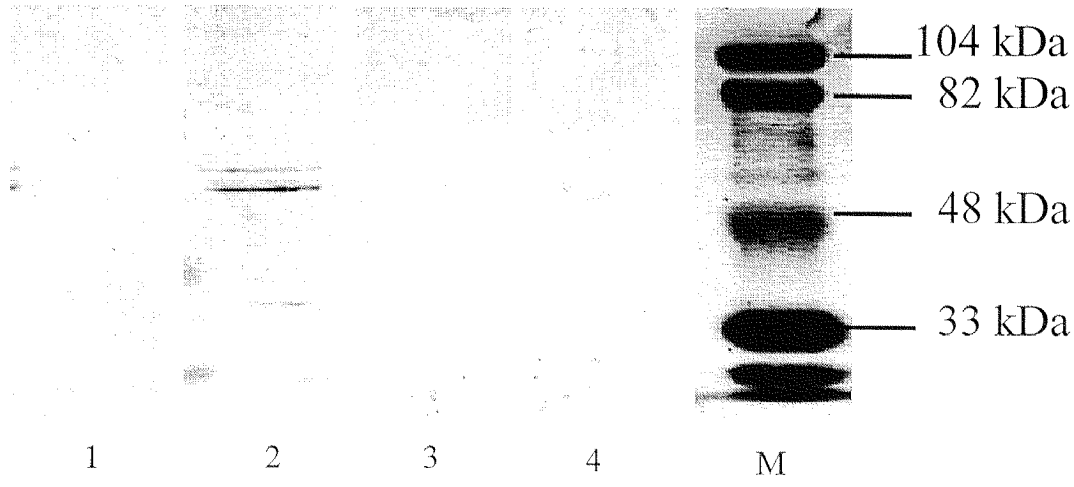

***Figure 11**: Western blot staining by a selective sera against the second extracellular loop of the Y_2-receptor. 1: Y_1 expressing SK-N-MC cell membranes. 2: Y_2 expressing SMS-KAN cell membranes. 3: Y_4 expressing BHK cells membranes. 4. Y_5 expressing BHK cell membranes. M = SDS-PAGE Standard*

Most sera stained several receptor subtypes in the Western blot experiments. The extracellular loops of the NPY receptors showed high homology, which makes it difficult to achieve subtype selectivity. Because of possible glycosylation [11] the N-terminal segment was not suitable for immunisation. The first extracellular loop showed high homology in many G-protein coupled receptors, accordingly no segments of this loop were chosen for immunisation. In order to use the antibodies also for studies with intact cells or tissue, no peptide of the transmembrane or of the intracellular part of the receptor was selected. Selection of the suitable receptor segments for immunisation is quite difficult. In order to obtain more selective antibodies, the use of smaller fragments of the extracellular loops or the N-terminus would be a possibility. But small peptides have often not the same secondary structure as the segments in the receptor and might be too small to trigger proper immune reaction.

1.8.5 Immunofluorescence Experiments

Immunofluorescence experiments were performed in order to determine the selectivity of the antibodies for the Y-receptors in receptor expressing intact cells. The cells were incubated with the antisera, washed and incubated with a fluorescein coupled anti rabbit secondary antibody. Binding of the antibodies was detected with a fluorescence microscope and measured with a fluorescence counter. The antibodies against the second extracellular loop of the Y_2-receptor selectively recognised the Y_2-receptor also in the intact cells. The same sera did not recognise the intact Y_2-receptor in ELISA with receptor expressing cell membrane. This suggests that the Y_2-receptor might not be stable during preparation for the ELISA essay. This hypothesis is further supported by the fact, that the Y_2-receptor is also recognised by the antibodies in Western blot experiments [8].

The anti neuropeptide Y receptors antisera represent valuable tools in several applications, for example in localisation studies, in combination with cross linking experiments and in affinity purification of the Y-receptors even though selectivity was not always achieved. The selectivity problem can be bypassed in using several antisera in combination.

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CHAPTER 2

Comparison of Antibodies directed against Receptor Segments of NPY-Receptors

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2.1 Abstract

The Y_1-, Y_2-, Y_4- and Y_5-receptor, which belong to the rhodopsin-like G-protein coupled, 7 transmembrane helix spanning receptors, bind the 36-mer neuromodulator NPY (neuropeptide Y) with nanomolar affinity. Synthetic fragments of the second (E2) and third (E3) extracellular loop were used to generate subtype selective anti-receptor antibodies against the Y-receptors. As investigated on intact receptors by ELISA and on solubilized receptors by SDS-PAGE and subsequent Western blotting, subtype selectivity was only partly achieved. Nevertheless, selectivity can be obtained by using several antisera in combination. These antibodies represent tools for molecular mass determination, receptor purification by affinity chromatography with antibody-columns and receptor localisation studies.

2.2 Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide that is widely distributed both, peripherally as well as centrally. Similar to many other neurotransmitters NPY elicits diverse physiological effects, e. g. induction of food intake and potent vasoconstriction (1-4). Accordingly, NPY is a neuronal and endocrine messenger that exerts its effects via several receptor subtypes of which the so called Y_1-, Y_2-, Y_4-, Y_5- and y_6-receptor have been cloned (5).

Various studies have shown that antibodies produced against hormone receptors are valuable tools (6-10). Anti-receptor antisera can be raised by immunisation with purified, enriched receptors (6) or by immunisation with receptor fragments (7-10). They are used for molecular mass determinations by SDS-PAGE and subsequent Western blotting. Receptor purification by affinity chromatography on antibody-columns (9) and investigations of the receptor localisation (11), regulation (12) and desensitisation (13) are only a few applications of anti-receptor-antibodies (14). Functional groups that are involved in ligand/receptor interaction can be detected by antisera targeted against defined regions of the receptor (10, 13, 15). Antibodies specifically recognising the NPY receptor subtypes could be used to determine the localisation pattern and the quantity of the receptor protein expression as well as to determine the topology and function of a receptor subtype.

In this study, we compare antibodies raised against the second and the third extracellular loop of several NPY receptor subtypes. We concentrated on the extracellular loops to obtain antibodies which also react with receptors in intact cells or tissue.

2.3 Materials and Methods

2.3.1 Synthesis and Characterisation of the Segments of Y-Receptors

The peptides for immunisation were synthesised by automated multiple solid phase peptide synthesis using a robot system (Syro, MultiSynTech, Bochum, Germany). In order to obtain peptide amides, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin was used for anchoring. The polymer matrix was polystyrene-1%-divinylbenzene (30 mg; 15 μmol). The side chain protection was chosen as follows: Trityl side chain protection for Asn, Gln and His, *tert*-butyl for Asp, Glu, Ser, Thr and Tyr, *tert*-butoxycarbonyl for Lys and Trp, and 2,2,5,7,8-pentamethyl-6-chromansulfonyl for Arg. Double coupling procedures were performed with diisopropylcarbodiimide/1-hydroxy-benzotriazole activation, 7-fold excess and a coupling time of 40 min.

The peptide amides were cleaved with trifluoroacetic acid/thioanisole/thio-cresol within 3 h and precipitated from cold diethyl ether. The products were collected by centrifugation and resuspended twice in diethyl ether. Finally they were lyophilized from water/tert. butyl alcohol (1:2).

Analysis of the peptides was performed on nucleolsil C-18 column, 5 μm, 3 x 125 mm (Merck-Hitachi) by a gradient of 15 - 60 % or 25 - 75 % acetonitrile over 30 min. Correct mass was identified by ion-spray mass spectrometry (SSQ710, Finnigan, San Jose, CA). The sequence and the chemical characterization of the peptides are summarized in Table 1.

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

Name	Sequenz	Mass calc. [Da]	Mass exp. [Da]
Y_1 E2/3	N V S L A A F K D K Y V C F D K F P S	2179.6	2178
Y_2 E2/1	I F R E Y S L I E I I P D F E I V A F	2313.7	2314
Y_4 E2/1	A N S I L E N V F H K N H S K A L E C	2152.5	2153
Y_5 E2/1	V F H S L V E L Q E T F G S A L L S S R	2219.6	2220

Y_5 E2/2	L L S S R Y L C V E S W P S D Y R I A F	2490.9	2491
Y_6 E2	L P T D I Y T H Q V A C V E I W P S K L N	2425.8	2426
Y_4 E3	L E D W H H E A I P I C H G N L I	1995.3	1996
Y_5 E3	H V V T D F N D N L I S N R H F K L V	2267.6	2268

2.3.2 Preparation of the Conjugates and Immunisation

For the immunisation the peptides were coupled to keyhole limpet hemocyanin (KLH) (Biotrend, Cologne, Germany). Polyclonal antibodies against the KLH-coupled receptor fragments were obtained by immunisation of chicken (Y_1 E2/3) and rabbits (Biotrend, Cologne, Germany).

2.3.3 Titer Determination of the Peptides

In order to determine the titer of antibodies raised against the peptide-KLH conjugates, the peptides were covalently coupled to ELISA plates (Nunc Immuno Modules, CovaLink NH) by incubation of the peptide (150 µl, 0.2 mg/ml in 60% DMSO) for two hours at room temperature after the addition of 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (50 µl/per well, 1.23 mg EDC/ml in water). After three washing steps with PBS-0.05% Tween 20 blocking was achieved with 1% BSA in PBS-0.05% Tween 20 at 4°C over night. The antibody solutions were diluted 1:10, 1:30, 1:100 and 1:300 with 0.2% BSA in PBS and incubated at 37°C for 90 min (100 µl/well). The second antibody was diluted 1:5000 (goat anti-rabbit POD) (SIGMA) with 0.2% BSA in PBS, incubated at 37°C for 1 h. The plates were washed 4 times with PBS-0.05% Tween 20. Staining was carried out with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Fluka) in citrate-phosphate buffer (0.1% ABTS, 0.003% H_2O_2, 100 mM citric acid, 100 mM K_2HPO_4, pH 4.1). The reagent (150 µl/well) was incubated for 30 min in the dark and the absorption at 405 nm subsequently measured in an ELISA-reader (Thermomax, Molecular Devices, Menlo Park, CA). Each value was determined at least 3 times and the average value ± S.E.M. was given. For the negative control, the preimmune serum was treated in parallel using the same dilutions. Furthermore in one series the plate was not coated with peptide but blocked with 1% BSA in

PBS-0.05% Tween 20 (=blank control). As positive control, one series was directly coated with serum. The specific absorption values were determined as follows: Average of the antibody values minus average values of preimmune sera. Since the blank values ranged below the values of preimmune sera, they were not considered for determination of the specific reaction. The determination of unspecific reaction resulted from the mean values of preimmune sera minus average of the blank values.

2.3.4 Investigation of Antibodies on Prepared Membrane

Affinity of the anti-receptor antibodies was investigated at Y_1-receptor expressing SK-N-MC cells (neuroblastoma, hY_1), SMS-KAN cells (neuro-blastoma, hY_2) and transfected BHK cells (baby hamster kidney cells, rY_4 and rY_5). Membrane solutions were diluted to 10 µg protein/ml in MEM medium (Gibco, Life Technologies, Basel, Switzerland), 25 mM Hepes (Sigma) and 3.75 mM $CaCl_2$. The ELISA plates (Nunc-Immuno Plate MaxiSorp™) were incubated with the membrane suspension (100 µl/well) overnight at 37°C then blocked with 2% BSA in PBS buffer at 37°C for 2h. Incubation of the antibody solutions as well as detection and performance of the control experiments was performed as described above. Different concentrations of the antibody solutions were assayed (1:30 and 1:100).

2.3.5 Immunoblot

Y_1-receptor expressing BHK cells (baby hamster kidney cells), SMS-KAN cells (neuroblastoma, hY_2) and BHK cells (baby hamster kidney cells, rY_4 and rY_5) were prepared by incubation for 7 min at 95°C in an urea sample buffer (8 M urea, 63 mM Tris/HCl pH 6.8, 2% SDS, 5% mercaptoethanol, 0.01% bromophenol blue) and were separated on a 12% polyacrylamide gel according to Lämmli (16). After blotting 60 min (350 mA/20 V) in a semi-dry electrophoretic transfer cell (Biorad) onto nitrocellulose membrane in a transfer buffer containing 25 mM Tris/HCl pH 8.3, 150 mM glycine, 20% methanol, the blots were blocked over night with TBS/Tween (0.5% Tween 20 in TBS/HCl pH 7.4) containing 1% BSA. The blots

were incubated with antibodies for 90 min with gentle shaking, washed three times with TBS/Tween, incubated for 60 min with alkaline phosphatase-bound second antibodies, and washed three times again. Immunoreactivity was detected with 5-bromo-4-chloro-indolyl-phosphate (Sigma, Buchs, Switzerland).

2.4 Results

2.4.1 Synthesis of the Receptor Segments and Characterisation of the Sera

The size of the synthetic peptides ranged between 17 and 21 amino acids in order to increase the probability that the peptides fold into a secondary structure. The receptor segments and the corresponding sequence of the peptides is summarised in Table 1.

The total absorption (405 nm) of the antibodies tested against the peptides ranged from 0.15 to 0.45. The values of preimmune sera ranged from 0.10 to 0.36. They were subtracted from the total absorption to obtain specific binding. The blank values were under 0.05. All antisera showed specific binding, the most specific binding was found for the antisera Y_1 E2/3, Y_5 E2/2 and Y_6 E2.

2.4.2 ELISA Test on Membranes Containing Y-Receptors

In order to determine those antipeptide-antibodies that recognize not only the peptide derived from the receptor, but the receptor itself as well, ELISA plates were coated with membranes. The selectivity of the antibodies for the Y-receptors was determined using membranes prepared from Y_1-receptor expressing SK-N-MC cells (neuroblastoma, hY_1), SMS-KAN cells (neuroblastoma, hY_2) and BHK cells (baby hamster kidney cells, rY_4 and rY_5). The specific interaction was determined in dilutions of 1:10, 1:30, 1:100 and 1:300. The best ratio between total binding versus preimmune serum binding was found for the antibody dilution of 1:30. Whereas Y_2 E2/1 and Y_4 E2/1 did not identify any Y-receptor subtype, Y_5 E2/1 bound the Y_5- but also the Y_1- and Y_4-receptor. Y_5 E2/2 stained the Y_1- and Y_5-receptor. Y_6 E2 recognized the Y_4- and to some extend also the Y_5-receptor. Y_4 E3 stained the Y_1- and Y_4-receptor and quite weak also the Y_5-receptor. Y_5 E3

bound to the Y_1- and Y_4-receptor. No antibody serum recognized the Y_2-receptor very well (Fig.1).

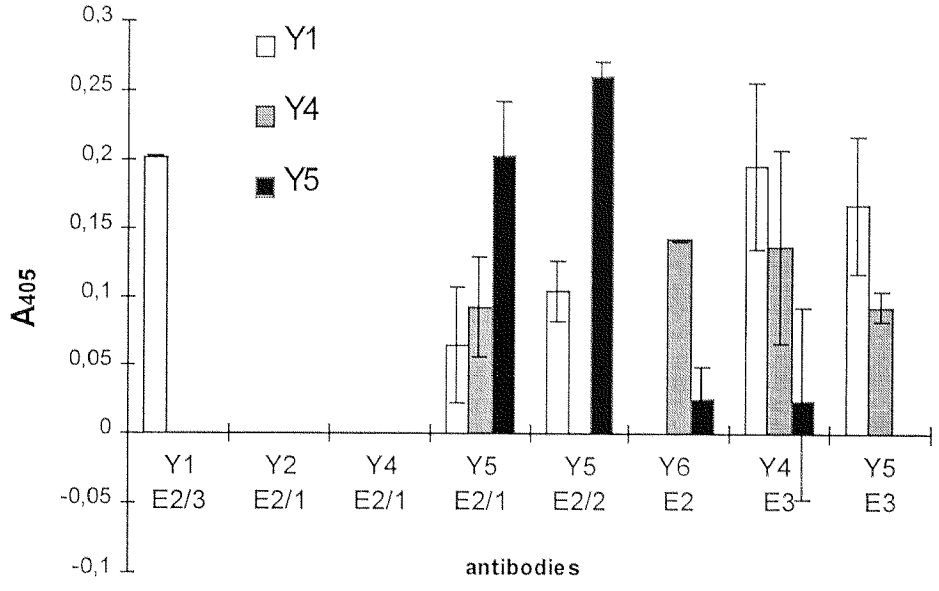


Figure 1: *Binding of the antibodies to the Y-receptors. Membranes were prepared from Y_1-receptor expressing SK-N-MC cells (neuroblastoma, hY_1), and BHK cells (baby hamster kidney cells, rY_4 and rY_5). Each bar represents the specific activity (total binding - binding of the preimmune serum) tested in the anti-membrane ELISA (dilution of the antisera 1:30). For E 2/3 the binding to Y_4 and Y_5 was not tested.*

2.4.3 Immunoblot

Western blots were performed in order to determine the receptor proteins that are recognised in a solubilized state. Membranes from SMS-KAN cells (neuroblastoma, hY_2) and transfected BHK cells (baby hamster kidney cells, hY_1, rY_4 and rY_5) were solubilized and proteins separated on a gel following subsequent Western blotting. Untransfected CHO (Chinese hamster ovarian) cells were used as negative controls.

Antibody Y_1 E2/3 stained the Y_1-receptor with a band of an apparent molecular weight of 73 kDa, the Y_2-receptor with major bands at 50 and 35 kDa, the Y_4-

receptor at 51 kDa and the Y_5-receptor at 51, 35 and 30 kDa (Fig. 2). Antibody Y_2 E2/1 identified only the Y_2-receptor with two bands of a molecular weight of 61 and 50 kDa (Fig. 2). Antibody Y_4 E2/1 marked bands at 73, 51, 40 and 35 kDa for the Y_1-receptor and weaker bands at 51 and 35 kDa for the Y_4- and Y_5-receptor. Two bands at 60 and 30 kDa were stained in the CHO membranes (Fig. 2). Antibody Y_5 E2/1 bound to the Y_2-receptor as a major band at 50 kDa and as a very weak band to the Y_4- and Y_5-receptor at 48 and 51 kDa. A band at 35 kDa was stained in the CHO membranes. Antibody Y_5 E2/2 stained the Y_1-receptor at 35 kDa the Y_2-receptor at 61 and 50 kDa and the Y_5-receptor with several weak bands. Antibody Y_6 E2 recognised theY_4- and Y_5-receptor at an apparent molecular weight of 35 kDa. Two bands at 45 and 40 kDa were stained in the CHO membranes. Antibody Y_4 E3 bound to the Y_2-receptor with two bands at 50 and 61 kDa and to the Y_4- and Y_5-receptor with major bands at 51 and 35 kDa. Bands at 80, 66, 55 and 30 kDa were stained in the CHO membranes (Fig. 2). Antibody Y_5 E3 identified the Y_1-receptor at 73 and 51 kDa, the Y_2-, Y_4- and Y_5-receptor at 35 kDa. No bands were stained in CHO membranes, as for Y_2 E2/1 and Y_5 E2/2 (Table 2).

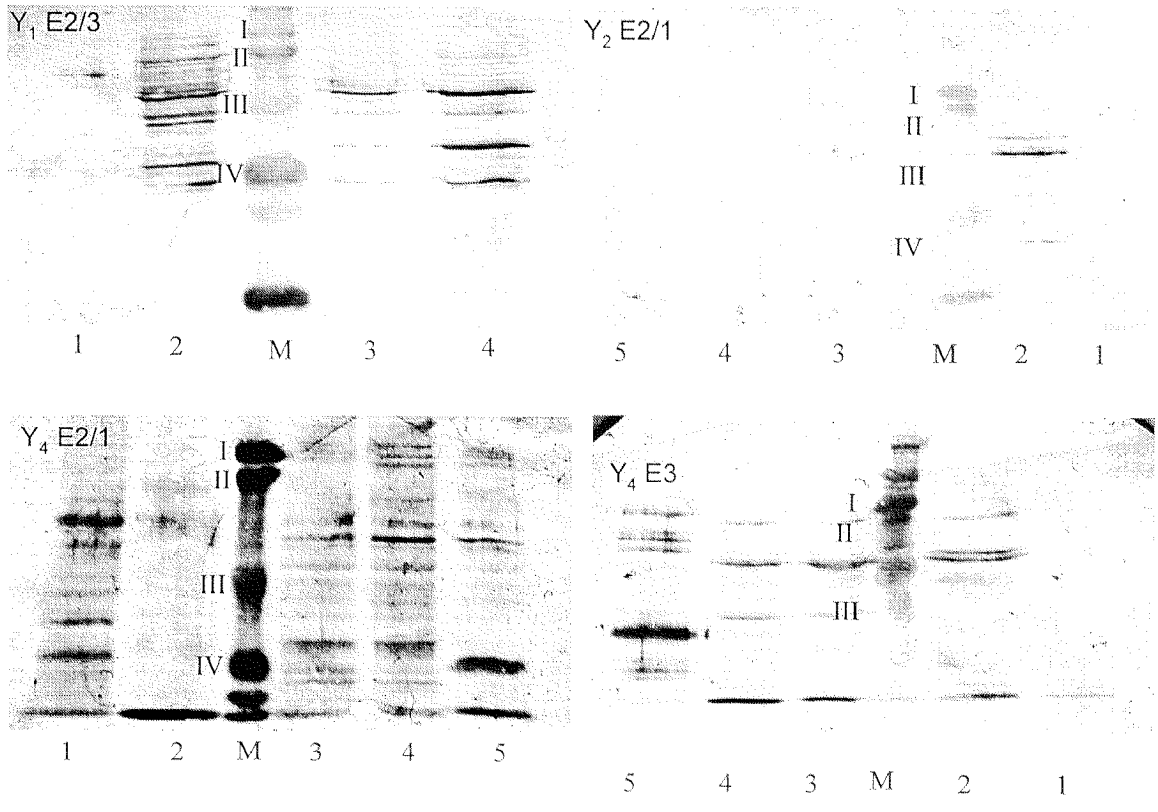


***Figure. 2**_ Western blot experiments. Lanes: 1 = BHK hY_1; 2 = SMS-KAN hY_2; 3 = BHK rY_4; 4 = BHK rY_5; 5 = CHO; M = SDS-PAGE Standard (Biorad), I = 104 kDa, II = 82 kDa, III = 48 kDa, IV = 33 kDa*

***Table 2**: Staining and detected masses of the Western blot experiments. - no staining; + weak staining; ++ moderate staining; +++ strong staining*

antibody	Y_1-receptor SK-N-MC	Y_2-receptor SMS-KAN	Y_4-receptor BHK	Y_5-receptor BHK
Y_1 E2/3	++ (73 kDa)	unspecific	+ (51 kDa)	+++ (51,35,30 kDa)
Y_2 E2/1	-	++ (61, 50 kDa)	-	-
Y_4 E2/1	+++ (73, 51, 40, 35 kDa)	-	++ (51, 35 kDa)	++ (51, 35 kDa)
Y_5 E2/1	-	++ (50 kDa)	+ (51 kDa)	+ (51 kDa)
Y_5 E2/2	+ (35 kDa)	+ (61, 50 kDa)	-	+ unspecific
Y_6 E2	-	-	++ (35 kDa)	++ (35 kDa)
Y_4 E3	-	+++ (50, 35 kDa)	+++ (51, 35 kDa)	+++ (51, 35 kDa)
Y_5 E3	++ (73, 51 kDa)	+ (35 kDa)	+ (35 kDa)	+ (35 kDa)

2.5 Discussion

The major aim of this work was to generate subtype-selective, polyclonal antibodies, which have been raised against synthetic receptor fragments of the neuropeptide Y receptor subtypes. We concentrated on the extracellular part of the receptors subtypes, the second (E2) and third (E3) extracellular loop, to obtain

antibodies which can be used with intact cells as well. Peptides of this size can already exhibit secondary structures (17, 18), so that antibodies can be expected to recognise the secondary structures and therefore the intact receptors, too.
The extracellular loops of the NPY receptors show high homology, which makes it difficult to achieve subtype selectivity. Because of possible glycosylation (19) the N-terminal segment has not been included. The first extracellular loop shows high homology in many G-protein coupled receptors, accordingly we did not choose any segments for immunisation of this loop. We intended to use the antibodies also with intact cells or tissue, therefore we did not select any peptide of the transmembrane and intracellular part of the receptor.

All antisera showed specific binding in the ELISA against the synthetic receptor fragments, however, partly with low titers. This probably is rather due to a low or wrong covalent binding of the peptides on the preactivated microtiter plates than to low affinity, because most of the antibodies recognise the receptor much better (Fig. 2).

Antibody Y_1 E2/3 reacts selectively with the Y_1-receptor in the ELISA performed with membranes and shows no crossreactivity to the Y_2-receptor. This can be expected, as the E2-loops of the Y_1- and Y_2-receptor do not share much similarity (Table 3). The antibodies Y_5 E2/1 and Y_5 E2/2 show only low selectivity. This result can be explained by the high similarity of the E2-loop of the Y_5-receptor with the sequences of the other receptor subtypes (Table 3). Obviously, this homology is high enough to produce crossreactivity between the antibodies and the receptor subtypes. In order to obtain more selective antibodies, we recommend now to use smaller fragments of the E2-loop of the Y_5-receptor, for example: LQETFGSALLS. But small peptides have often not the same secondary structure as the segments in the receptor and might be too small to trigger proper immune reaction. Furthermore, this segment is adjacent to Cys, that forms an internal disulfide bridge to the E1-loop and might not be accessible to antibodies for sterical reasons. In spite of the high homology of the E2-loop to the Y_4- and Y_5-receptor the intact Y_2-receptor was not recognised by any antibody, which suggests that the Y_2-receptor might not be stable during the preparation. This hypothesis is further supported by the fact, that the Y_2 E2/1, which is the only fully selective

antibody in the Western blot experiments, does not react with the intact Y_2-receptor as well.

The two antibodies raised against the third extracellular loops show very high crossreactivity. The sequence similarity is very high as well and obviously makes it impossible to obtain specific antibodies (Table 4). Thus, the E3-loop seems to be unsuitable for raising subtype selective antibodies against Y-receptors.

Table 3: *E2-Loop segments of NPY-receptor subtypes. Identical positions are marked in bold, receptor segments used for immunisation are underlined.*

receptor	E2 Loop
hY_1	IY---QVMTDEPFQNVTLD-AYK-DKYVCFDQFPSD--S-HRLSY
	Y_1 E 2/3
hY_2	IFREYSLIEIIPDFEIVA----------CTEKWPGEEKSIYGTVY
	Y_2 E 2/1
hY_4	A---NSILENVFHKNHSKALEFLADKVVCTESWP---LAHHRTIY
	Y_4 E 2/1
hY_5	VF--HSLVELQETFGSALLS----SRYLCVESWPSD--S-YRIAF
	Y_5 E 2/1 Y_5 E 2/2
my_6	LFLSYHLTNEPFHNLSLPTDIYTHQVACVEIWPSKLNKLNQLLF
	Y_6 E2

Except for antibody Y_2 E2/1, all sera stained several bands of the solubilized membranes. This might be explained by receptor degradation, different glycosylation of receptors or different transcription products (20). According to our studies, all receptor subtypes have an apparent molecular weight of about 35 kDa, which might be due to the non-glycosylated protein. Higher masses of 50-51 kDa were identified for all subtypes as well, and a 73 kDa protein was further stained with anti-Y_1 receptor antibodies.

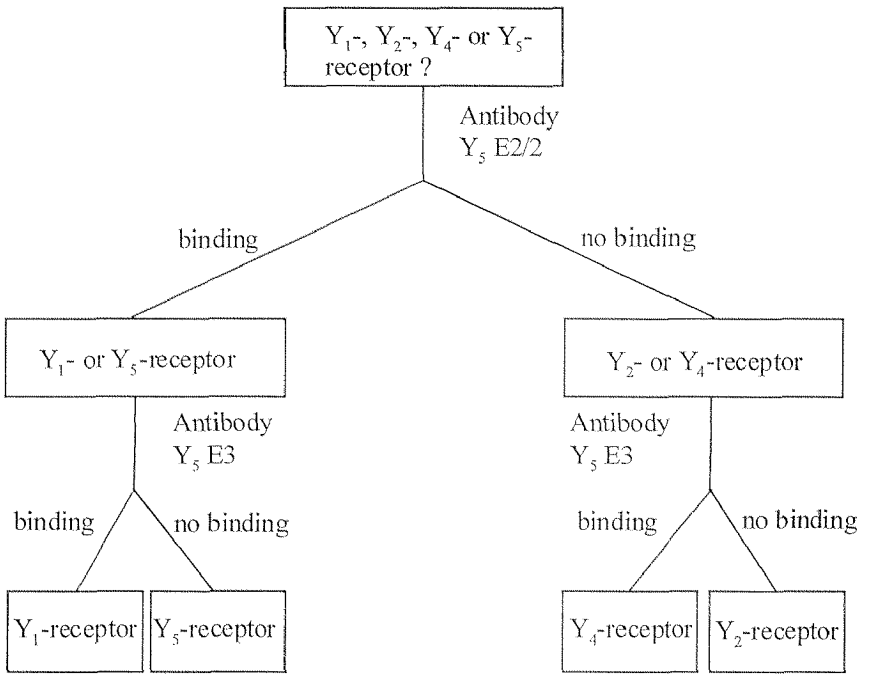
Obviously there often is a big difference in recognition of the intact and the solubilized receptors. As in Western blots, the proteins are denaturated, the

unspecificity might be expected, because of the similarity of the segments. However, in intact receptors, higher specificity has been found. Either not all synthetic receptor fragments develop the same secondary structure as in the intact receptor, or otherwise there is steric hindrance in the intact receptor which prevents antibodies from binding.

Table 4: *E3-Loop segments of NPY-receptor subtypes. Identical positions are marked in bold, receptor segments used for immunisation are underlined.*

receptor	E3 Loop
hY_1	N T V F D W N H Q I I A T C N H N L L
hY_2	Q L A V D I D S E V L D L K E Y K L I
hY_4	N S L E D W H H E A I P I C H G N L I
	Y_4 E3
hY_5	H V V T D F N D N L I S N R H F K L V
	Y_5 E3

The goal of this work was the production of subtype selective antibodies. This was only partly achieved with the described methods. Nevertheless, the obtained antisera represent valuable tools in several applications, for example in localisation studies and affinity purification of the Y-receptors. Selectivity can be achieved in using several antisera in combination. For example with the antisera Y_5 E2/2 and Y_5 E3 the intact Y_1-, Y_2-, Y_4-, and Y_5-receptors can be distinguished as outlined in Figure 3.



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3 Chapter

CHAPTER 3

Characterisation of Neuropeptide Y Receptor Subtypes by synthetic NPY Analogues and by anti-Receptor Antibodies

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Abbreviations:

BHK, baby hamster kidney
Boc, *tert*-butyloxycarbonyl
BSA, bovine serum albumin
CT, C-terminal segment
DMEM, Dulbecco's modified Eagle's medium
DMF, dimethyl-formamide
E1-3, extracellular loops 1-3
EDTA, ethylenediamin-etetraacetic acid
ELISA, enzyme linked immunosorbent assay
FCA, Freund's complete adjuvant
FCS, fetal calf serum
Fmoc, fluorenyl-9-methoxycarbonyl
FITC, 4(5)-fluoresceinisothiocyanate
HPLC, high performance liquid chromatography
hPP, pancreatic polypeptide human
Ig, immune globulin
KLH, keyhole limpet hemocyanin
MEM, minimum essential medium
NPY, neuropeptide Y
NT, N-terminal segment
PBS, phosphate buffered saline
pNPY, neuropeptide Y porcine
Pmc, pentamethylchromanesufonyl
TBS, Tris buffered saline; tBu, *tert*-butyl
(Tmd)Phe, 4'-(3-trifluoromethyl)-3-diazirine-3-yl-phenylalanine
Trt, trityl; TFA, trifluoroacetic acid

3.1 Abstract

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

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

3.2 Introduction

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

It is not yet fully understood which NPY receptor subtype is related to a certain physiological effect. Available evidence suggests for example that the Y1- or Y5-receptor or even both receptors together regulate the food intake behaviour [2]. Furthermore, the receptor subtypes are distributed heterogeneously in various tissues. Therefore, receptor subtype selective tools are very important for

understanding the biological role and distribution of the receptors and can provide important knowledge for drug design.

Each receptor subtype recognises specific parts of the hormone folded in an unique conformation. Thus, different receptor subtypes can be characterised by selectively increasing the affinity/activity of peptide analogues in cells or tissue [28]. Generally, full length analogues are obtained by D-amino acid- or L-alanine-scan or by further amino acid replacements [29]. Frequently, the native ligands are shortened by deletion of the C- or N-terminal parts, or by central truncation [30]. Moreover, analogues with agonistic or antagonistic activity can be a powerful tool to investigate the biological function of a receptor subtype.

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

In this study we describe synthetic NPY analogues tested in binding assays against the Y-receptors and we compare the binding of antibodies raised against the second and the third extracellular loop of NPY Y1-, Y2- and Y5-receptor subtypes to intact cells in immuno-fluorescence assays. Both methods are applied in order to get subtype selectivity and to obtain tools to localise the different receptor subtypes.

3.3 Materials and Methods

3.3.1 Synthesis and Characterisation of the NPY Analogues and Segments of Y-Receptors

The NPY analogues and the peptides for immunisation were synthesised by automated multiple solid phase peptide synthesis using a robot system (Syro, MultiSynTech, Bochum, Germany). In order to obtain the C-terminally amidated

peptides, the chain assembly was performed on a 0.015 mmol scale using 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Novabiochem, Läufelfingen, Switzerland) and Fmoc/*tert*-butyl chemistry. The side chain protection of the Fmoc-amino acids (Alexis, Läufelfingen, Switzerland) was chosen as follows: Trt for Asn, Gln and His, *tert*-butyl for Asp, Glu, Ser, Thr and Tyr, Boc for Lys and Trp, and 2,2,5,7,8-pentamethylchroman-6-sulfonyl for Arg. A double coupling procedure was performed with diisopropylcarbodiimide/1-hydroxy-benzotriazole (Fluka, Buchs, Switzerland) activation, 7-fold excess and a coupling time of 40 min. Fmoc removal was accomplished by 30% piperidine (Fluka) in DMF for 15 min. The peptide amides were simultaneously cleaved from the resin and deprotected with TFA/thioanisole/thiocresol (Fluka) for 2.5 hours, and collected by precipitation from diethyl ether.

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

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

Name	Sequence	$Mass_{calc}$ [Da]	$Mass_{exp}$ [Da]	Position in the receptor
Y_1 E2/2	Q I L T D E P F Q N V S L A A F K D K	2163.5	2165	76-94 (rat)
Y_2 E2/1	I F R E Y S L I E I I P D F E I V A F	2313.7	2314	75-93[a] (human)
Y_5 E2/2	L L S S R Y L C V E S W P S D S Y R I A F	2491.9	2491	186-206 (human)
Y_5 E3	H V V T D F N D N L I S N R H F K L V	2267.6	2268	278-296 (human)

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

TABLE 2: *Analytical data of amino acid mono-substituted and centrally truncated NPY analogues and of centrally truncated hPP analogues. In brackets the affinity of the peptide divided by the affinity of pNPY is given.*

Peptide	$Mass_{calc.}$ [Da]	$Mass_{exp}$ [Da]	IC_{50}							
			hY_1 [nM]	$\frac{IC_{50}(Pep)}{IC_{50}(NPY)}$	hY_2 [nM]	$\frac{IC_{50}(Pep)}{IC_{50}(NPY)}$	hY_4 [nM]	$\frac{IC_{50}(Pep)}{IC_{50}(NPY)}$	hY_5 [nM]	$\frac{IC_{50}(Pep)}{IC_{50}(NPY)}$
pNPY	4253.7	4253	0.23	(1)	0.04	(1)	5.5	(1)	0.8	(1)
[A^1]-pNPY	4161.7	4162	21	(91)	0.2	(5)	5.8	(1.1)	2.2	(2.8)
[A^2]-pNPY	4227.7	4224	114	(496)	0.3	(8)	7.8	(1.4)	5.5	(7)
[A^5]-pNPY	4227.7	4228	228	(991)	24	(600)	25	(4.5)	32	(40)
[A^8]-pNPY	4227.7	4225	32	(139)	0.7	(18)	60	(11)	55	(69)
[A^{11}]-pNPY	4209.7	4210	8.0	(35)	0.2	(5)	3.1	(0.6)	0.5	(0.6)
[A^{13}]-pNPY	4227.7	4226	7.5	(33)	0.1	(3)	37	(6.7)	17	(21)
[A^{19}]-pNPY	4168.6	4169	282	(1226)	1.6	(40)	4.1	(0.7)	1.4	(1.8)
[A^{20}]-pNPY	4161.7	4160	71	(309)	1.2	(30)	161	(29)	19	(24)
[A^{21}]-pNPY	4161.7	4160	5.5	(24)	0.2	(5)	66	(12)	32	(40)
[A^{25}]-pNPY	4168.7	4167	11	(48)	0.7	(18)	201	(37)	80	(100)
[A^{27}]-pNPY	4161.7	4159	250	(1087)	1.4	(35)	340	(62)	370	(463)
[A^{32}]-pNPY	4223.8	4221	723	(3143)	45	(1125)	380	(69)	7.7	(9.5)
[A^{33}]-pNPY	4168.7	4167	>1000	(>4348)	54	(1350)	>1000	(>182)	94	(118)

[A^{34}]-pNPY	4196.7	4195	94	(409)	6.0	(150)	7.4	(1.3)	1.3	(1.6)
[A^{35}]-pNPY	4168.7	4167	>1000	(>4348)	>1000	(>25000)	>1000	(>182)	>1000	(>1250)
[A^{36}]-pNPY	4161.7	4158	970	(4217)	48	(1200)	141	(26)	68	(85)
[L^{34}]-pNPY	4238.8	4238					0.3	(0.05)	1.8	(2.3)
[D-P^{34}]-pNPY	4222.8	4222	266	(1157)			271	(49)	156	(195)
[Ahx^{5-24}]-NPY	2220.6	2219	>1000	(>4348)	1.4	(35)	600	(109)	795	(994)
[$Ahx^{5-24,}$ P^{34}]-NPY	2189.6	2190	>1000	(>4348)			514	(93)	>10000	(>12500)
[Ahx^{8-20}]-NPY	2981.4	2980	28	(122)			67	(12)	31	(39)
[Ahx^{8-19}]-NPY	3144.6	3144	46	(200)			95	(17)	19	(24)
[Ahx^{9-20}]-NPY	3078.6	3078	74	(322)			108	(20)	29	(36)
[Ahx^{9-17}]-NPY	3469.0	3470	13	(57)			45	(8)	11	(14)
hPP	4181.8	4181	>1000	(>4348)	>1000	(>25000)	0.04	(0.007)	24	(30)
[Ahx^{5-24}]-hPP	2161.6	2162	>500	(>2174)	>1000	(>25000)	144	(26)	>1000	(>1250)
[Ahx^{5-20}]-hPP	2532.0	2531	>1500	(>6522)	>1000	(>25000)	216	(39)	>7000	(>8750)
[Y^{5-20}]-hPP	2582.0	2582	>500	(>2174)	>1000	(>25000)	27	(5)	>5000	(>6250)

3.3.2 Preparation of the Conjugates and Immunisation

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

3.3.3 Titer Determination of the Receptor Segment Peptides [38]

In order to determine the titer of the antibodies raised against the peptide-KLH conjugates, the peptides were covalently coupled to preactivated ELISA plates (Life Technologies, Basel, Switzerland). The antibody solutions were diluted and incubated at 37 °C for 90 min. The plates were washed 4 times and the secondary antibody was incubated for 1 hour. After washing the plates 4 times, staining was carried out with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and the absorption was subsequently measured at 405 nm in an ELISA-reader.

3.3.4 Cell Culture

SK-N-MC cells (neuroblastoma, hY_1) were cultivated in MEM earl's salt (Life Technologies, Basel, Switzerland) supplemented with 10% (v/v) fetal calf serum (AMIMED, Allschwil, Switzerland), 4 mM L-glutamine, 1% (v/v) non-essential amino acids (Life Technologies) and 1 mM sodium pyruvate (Life Technologies). SMS-KAN cells (neuroblastoma, hY_2) were cultivated in Nut-mix HAM's F-12/DMEM 1:1 (Life Technologies) supplemented with 15% fetal calf serum, 1% (v/v) L-glutamine and 1% (v/v) non-essential amino acids. BHK cells (baby hamster kidney cells, transfected with hY_4, rY_5 and hY_5) were cultivated in DMEM (Life Technologies) supplemented with 10% (v/v) fetal calf serum, 1% (v/v) antibiotic-fungicide solution (penicilin 10000 U/ml, streptomycin 10 mg/ml and fungizone 25 µg/ml; AMIMED) and 1 mg/ml geneticin (G418; SIGMA, Buchs, Switzerland). Additionally, 1 mg/ml Hygromicin and 24 hours before harvest 1 mM IPTG was added to the rY_4 expressing BHK cells. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air in 75 cm^2 flasks

(Techno Plastic-Products AG, Trasadingen, Switzerland). They were harvested at 100% confluency in phosphate buffered saline (PBS; Life Technologies) containing 0.02% EDTA.

3.3.5 Binding Potency of NPY Analogues

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

3.3.6 Immunofluorescence

Affinity of the anti-receptor antibodies was investigated at Y_1-receptor expressing SK-N-MC cells (neuroblastoma, hY_1), SMS-KAN cells (neuroblastoma, hY_2) and transfected BHK cells (baby hamster kidney cells, rY_5). About 500000 cells per well were added to 12 well tissue culture test plates (Techno Plastic-Products) and grown to confluency. Antisera were diluted 1:50 in the corresponding cell culture media, 1.5 ml antisera solution was added per well. Tissue culture test plates were incubated for 90 min at 37 °C and washed twice with media. Goat anti-rabbit 4(5)-fluoresceinisothiocyanate (FITC) conjucated IgG (SIGMA, Buchs, Switzerland) was diluted 1:80 in media, 1 ml antisera solution was added per well. Tissue culture test plates were incubated for 1 hour at 37 °C and washed twice with media. The cells were viewed in a fluorescence microscope (Zeiss, D-Oberkochen; Axiovert 35, Filters: excitation: BP 450–490 nm, beamsplitter FT 510, emission LP 520). As positive control, one lane of each plate was directly incubated with goat anti-rabbit FITC coupled IgG. For the negative control, anti-

receptor antisera were preincubated for 1 hour with the corresponding antigen peptide in concentrations of 10^{-5} M or 10^{-4} M. Incubation of the antibody solutions as well as detection was performed as described above.

3.4 Results

3.4.1 Synthesis of the Analogues and Receptor Segments

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

3.4.2 Characterisation of the Sera

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

3.4.3 Binding Potency of Full Lenght NPY Analogues

The affinity to the receptors Y_1 and Y_2 of all 36 Ala-substituted analogues of NPY has been reported previously [40]. In the present work, we have considered sixteen selected Ala-substituted NPY analogues and investigated their binding affinity additionally at the human receptors Y_4 and Y_5. Y_1- and Y_2-receptor binding data are listed for comparison (Table 2). The substitution of Pro at the positions 2, 5, 8 and 13 led in general to a decreased affinity. In particular, the substitution of the residues 2 and 5 reduced the hY_1-receptor affinity about 500- and 1000-fold, respectively; the substitution of Pro 5 decreased the hY_2-receptor affinity 600-fold,

and the replacement of Pro 8 led to a 11- and 69-fold lower affinity at the hY_4- and hY_5-receptor subtypes, respectively. By single exchange of any of the aromatic side chains of the Tyr residues, the positions 27 and 36 resulted to be the most important for the binding at the hY_1-receptor, with a loss of affinity of 1000- and 4200-fold after substitution, respectively, whereas the presence of Ala^{20} was characterised by a 300-fold reduced affinity at this receptor. The binding at the hY_2-receptor was drastically decreased (1200-fold) only by the substitution of Tyr^{36}. The hY_4-receptor binding showed to be only slightly influenced by the Tyr/Ala replacement (up to 62-fold lower affinity), while the binding at the hY_5-receptor was reduced > 400-fold by replacing Tyr^{27}. By any single removal of the basic side chain of either Arg^{33} or Arg^{35}, we observed a complete loss of affinity for the hY_1-receptor (> 4000-fold); by exchange of Arg^{19} and Arg^{25} the binding was 1200- and 48-fold less potent, respectively. The hY_2-receptor binding was totally lost by substitution of Arg^{35} and was reduced > 1300-fold by Arg^{33} exchange. These two C-terminal Arg residues proved to be very important also for the binding at the hY_4- and hY_5-receptor subtypes, especially Arg^{35} is required for affinity to the hY_5-receptor (> 1200-fold lower affinity after replacement with Ala). Interestingly, the [Ala^{19}]-pNPY analogue was almost as potent as the native ligand at the hY_5-receptor and even slightly better at the hY_4-receptor. By removing the negative charge of Asp^{11}, we could obtain a slightly more potent ligand at the receptors hY_4 and hY_5, whereas the potency at the other two subtypes decreased slightly (35- and 5-fold at the hY_1- and hY_2-receptor subtypes, respectively). The decrease in hydrophylicity at the position 32 by substitution of Thr with Ala also led to a decreased affinity, especially at the hY_1-receptor (> 3000-fold) and hY_2-receptor (> 1000-fold). These two receptors were also the most sensitive to the substitution of Gln^{34}, while the binding at the receptors hY_4 and hY_5 remained almost as good as for the native ligand. Moreover, the analogue [Leu^{34}]-pNPY could bind the hY_4-receptor 18-fold more potently than pNPY itself. Instead, the presence of D-Pro^{34} considerably reduced the affinity at all receptor subtypes, whereas it was previously reported that L-Pro at the same position was perfectly tolerated at the receptors hY_1, hY_4 and hY_5, but not at the hY_2-receptor [25]. IC_{50} values of the full length NPY analogues are summarised in table 2.

3.4.4 Binding Potency of Centrally Truncated NPY Analogues

The central segment of pNPY was suggested to be important for the peptide conformation and to adopt a turn-like structure that induces the molecule to fold like a hairpin [41]. This was investigated by the preparation of pNPY analogues, where the central region was removed and substituded by a spacer like 6-amino hexanoic acid (Ahx) [42]. By varying the cutting position either at the N- or the C-terminus, it was found that the analogue [Ahx^{5-24}]-pNPY could selectively bind to the hY_2-receptor with 1.4 nM affinity (Table 2). Furthermore, analogues with longer N- and C-terminal fragments showed similar affinity at the hY_1-receptor but also significant affinity at the receptors hY_4 and hY_5. Among these analogues, the one with the highest number of residues at the N- and C-termina, [Ahx^{9-17}]-pNPY, was the most potent ligand (13 nM at the hY_1, 45 nM at the hY_4 and 11 nM at the hY_5). Accordingly, the residues 9 to 18 are not of major importance for receptor affinity.

Three centrally truncated analogues of hPP were also investigated for binding to the Y-receptors. The ligands containing the Ahx moiety instead of the middle segments 5-24 and 5-20 did not show any affinity at the hY1- and hY5-receptor subtypes, and their affinity to the hY4-receptor was considerably reduced. Interestingly, the analogue [Tyr5-20]-hPP was found to selectively bind the hY4-receptor, however with a 670-fold reduced affinity. IC50 values of the centrally truncated NPY analogues are summarised in the Table 2.

3.4.5 Immunofluorescence on Cells expressing Y-Receptor Subtypes

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

Antibody Y_1 E2/2 showed selective binding in the fluorescence microscope for the Y_1-receptor (Figure 2 A-C). Selective binding to the Y_2-receptor was detected for antibody Y_2

E2/1 (Figure 2 D-F). Very strong binding was found for the antibodies Y_5 E2/2 and Y_5 E3 to the receptors Y_2 and Y_5 (Figure 3 A-F). Results of the fluorescence microscope assay are summarised in the Table 3.

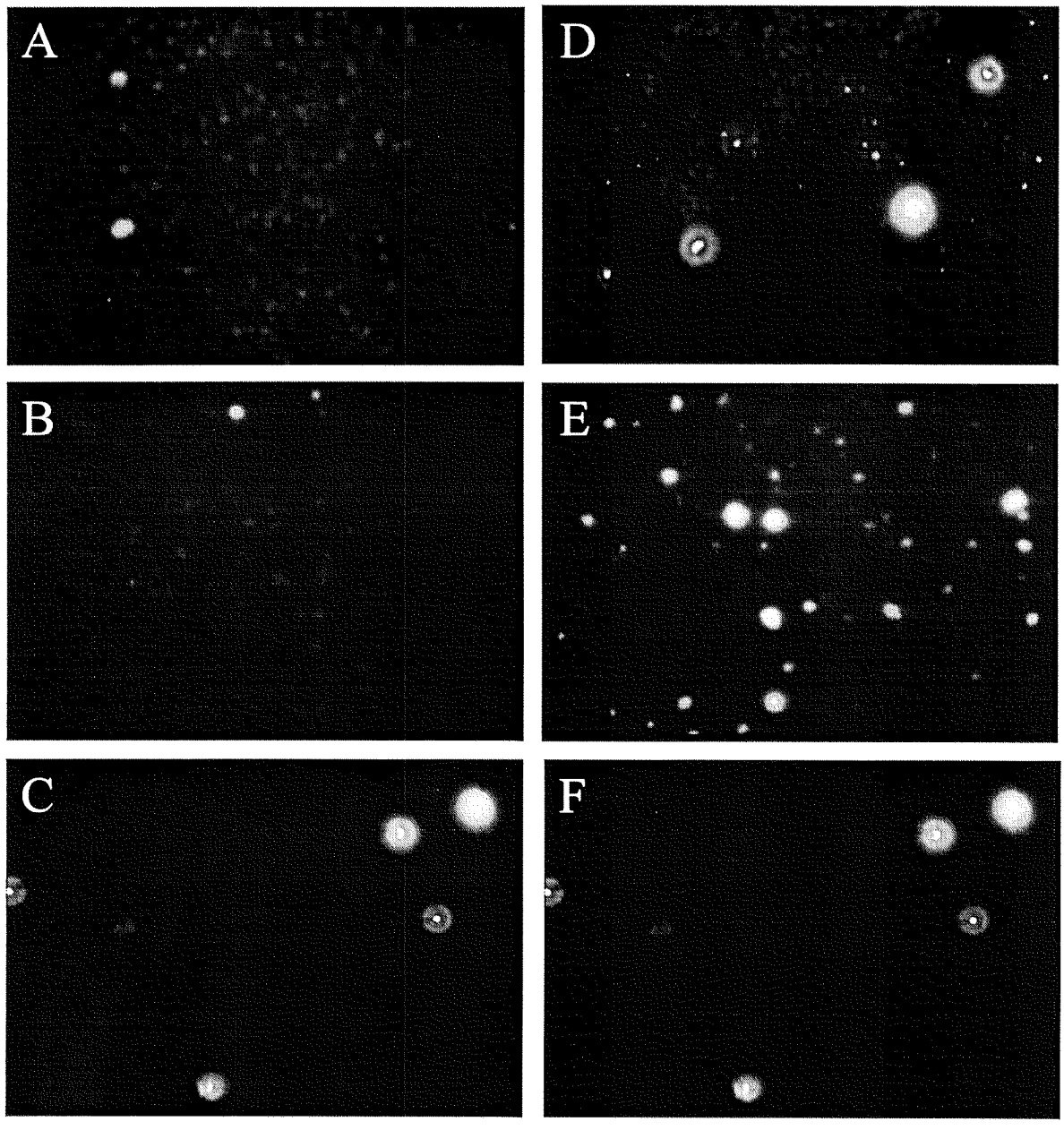

Figure 1: *Binding of antibody Y_1 E2/2 to: A) SK-N-MC cells (Y_1), B) SMS-KAN cells (Y_2), C) BHK cells (Y_5); Binding of antibody Y_2 E2/1 to D) SK-N-MC cells (Y_1), E) SMS-KAN cells (Y_2), F) BHK cells (Y_5).*

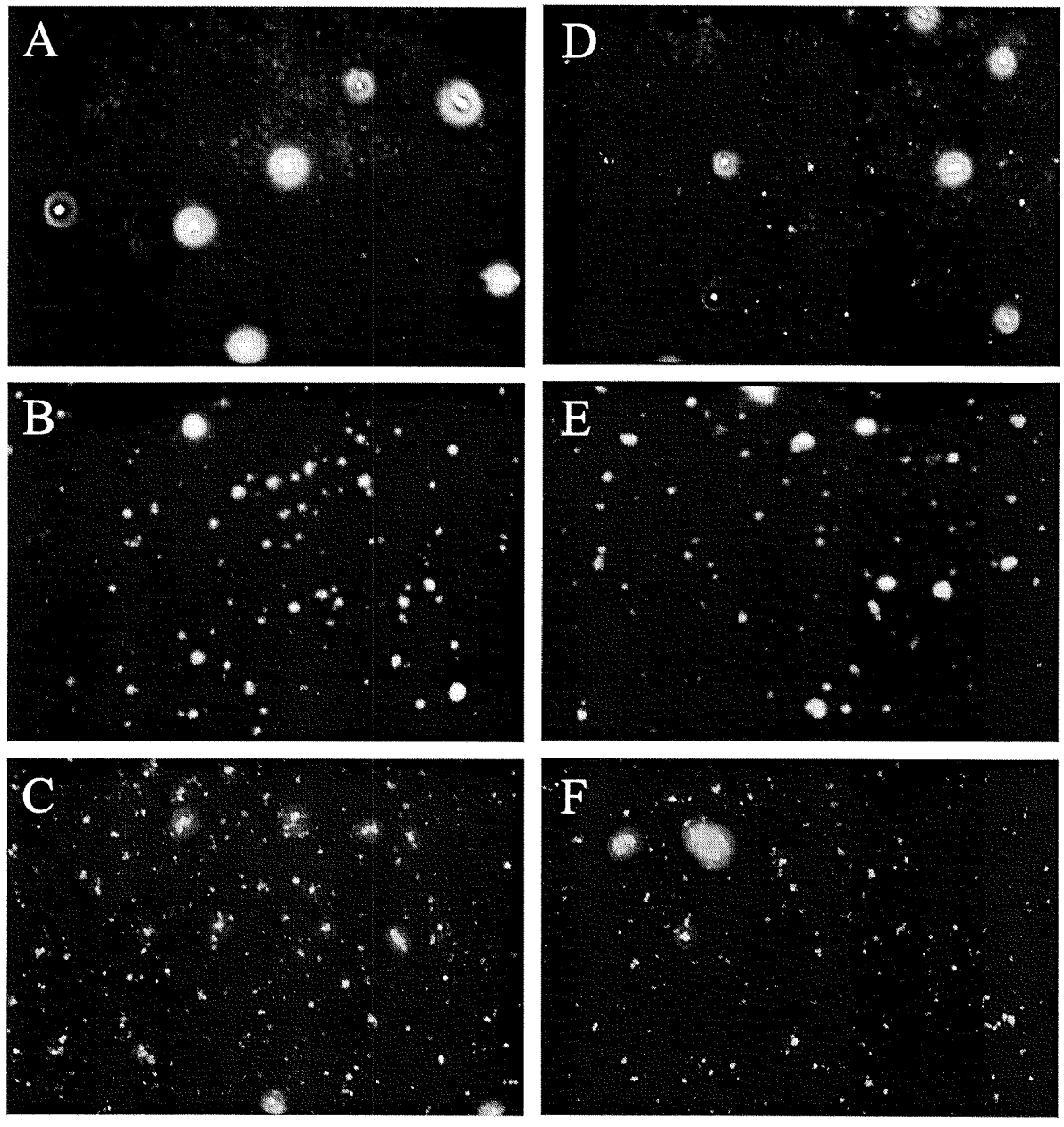

Figure 2: *Binding of antibody Y_5 E2/2 to: A) SK-N-MC cells (Y_1), B) SMS-KAN cells (Y_2), C) BHK cells (Y_5); Binding of antibody Y_5 E3 to D) SK-N-MC cells (Y_1), E) SMS-KAN cells (Y_2), F) BHK cells (Y_5).*

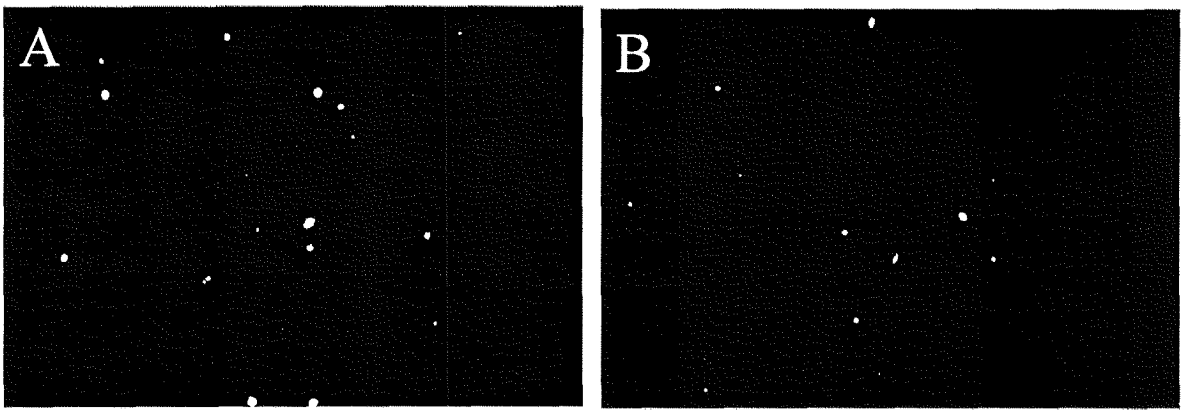

Figure 3: *Binding of antibody Y_5 E2/2 to BHK cells (Y_5) A) Normal conditions, B) Blocking of antibody Y_1 E2/2 with a peptide concentration of 10^{-4} M*

Table 3: *Fluorescence microscope detection of Y-receptors on SK-N-MC cells (neuroblastoma, hY1), SMS-KAN cells (neuroblastoma, hY2) and BHK cells (baby hamster kidney cells, rY5).*

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

antibody	Y_1-receptor SK-N-MC	Y_2-receptor SMS-KAN	Y_5-receptor BHK
Y_1 E2/2	+	-	-
Y_2 E2/1	-	+ +	-
Y_5 E2/2	-	+ + +	+ + +
Y_5 E3	-	+ + +	+ + +

3.5 Discussion

The major aim of this work was to compare analogues and anti-receptor antibodies with respect to selectivity and characterisation of the ligand binding site. The NPY/PP system is an unique set that consists of two peptides which bind to five receptors: NPY to Y_1, Y_2, Y_5 and y_6, PP to Y_4, but [Pro^{34}]-NPY to Y_1, Y_4, Y_5 and y_6. Accordingly, selectivity is a major problem. Furthermore, because of the different effects and the distribution of the receptor, it is of major importance to develop tools that allow the characterisation of the receptors on the protein level. Selective ligands, for example radiolabelled-, fluorescent-labelled- and anti-receptor-antibodies are approaches to address this issue. Furthermore, we were interested in the molecular characterisation of the binding site. Again, ligands with specific replacements [43] and anti-receptor antibodies [44] have been used to map ligand binding. Accordingly, we used both approaches and applied them to the NPY/PP system: on the one hand, full length and centrally truncated NPY analogues were synthesised and their binding potency at the NPY receptor subtypes was tested. On the other hand, polyclonal antibodies raised against synthetic receptor fragments on the second (E2) and third (E3) extracellular loop of the receptor subtypes were tested on intact cells expressing NPY receptors.

The receptor binding properties of the pNPY and hPP analogues presented in this work showed how the affinity of the native ligand at its receptor subtypes can be modulated by modifying its primary structure that is likely to change, consequently, its spatial conformation. It has been supposed that the members of the PP-family adopt a hairpin-like structure which is stabilised by the interdigitation of the tyrosine side chains on the surface of the C-terminal amphipatic α-helix with the proline residues of the N-terminus. Accordingly, all NPY receptors have been shown to be sensitive to the positions of Pro and Tyr, however each one in a different way. For example, on the base of the Ala-scan results, Pro2, Pro5, Tyr27 and Tyr36 proved to be the most important positions for the hY1-receptor, Pro5 and Tyr36 for the hY2-receptor, Pro8 and Tyr27 for the hY4- and hY5-receptor. These differences might be explained by assuming that the proline rich N-terminal region and the C-terminal helix face each other in a way that depends on the structural features of the specific receptor binding site.

The Ala-substitution of the arginine residues also led to different receptor affinities: Arg^{19} is important for the receptors hY_1 and hY_2, but not for the receptors hY_4 and hY_5, whereas Arg^{25} replacement resulted still in nanomolar affinity at the hY_2-receptor only. Instead, Arg^{33} and Arg^{35} are essential for the binding at all four subtypes, however it should be emphasised that Arg^{33} side chain is less important than Tyr^{27} at the hY_5-receptor. Furthermore, Thr^{32} and Gln^{34} replacements did not significantly reduce the affinity at the hY_5-receptor as they did in the case of the other subtypes. It has been well documented that the C-terminal pentapeptide of NPY represents the receptor binding site for the receptors Y_1 and Y_2 [29]. The results presented in this work have shown that this is also the case for the receptors Y_4 and Y_5, however with different properties. While the Ala-substitution at the positions 32-36 led to a dramatic loss of affinity at the receptors hY_1 and hY_2 compared to NPY, in the case of hY_5-receptor only the exchange of Arg^{35} led to a significant decrease in the binding potency. At the hY_4-receptor, both arginine residues were found to be crucial for high affinity. This observation suggests that the Y-receptor-ligand interaction is unique for each subtype and it involves different side chains which are required to adopt different spatial orientations, as shown, for example, by the low affinity of the analogue [D-Pro^{34}]-pNPY at all recepetor subtypes. Obviously, D-Pro at the position 34 changes the orientation in an unfavourable way. Accordingly, the amino acid substitution of natural ligands is extremely useful to characterise the ligand binding sites. For NPY it is clear, that it binds to the receptors Y_1, Y_2 and Y_5 in a very different way with different contact sites (Figure 4). To gain more insight into the mechanism of interaction between the ligand and each receptor, selective compounds are required. Among the Ala-substituted full lenght NPY analogues, [Ala^8]-pNPY, [Ala^{13}]-pNPY and [Ala^{27}]-pNPY can be considered hY_2-receptor selective ligands; in particular, the hY_2-receptor affinity of [Ala^{27}]-pNPY is > 100-fold higher than that at the other receptors.

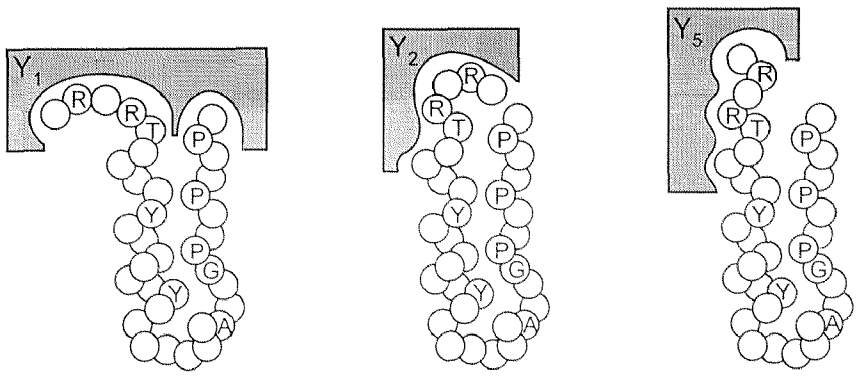

Figure 4: Scheme of different contact sites of NPY at its receptor subtypes.

Among the centrally truncated NPY analogues, only [Ahx$^{5-24}$]-pNPY has been shown to bind selectively to the hY$_2$-receptor. The other longer analogues are characterised by similar affinity values at various subtypes. Interestingly, in some cases comparable affinity could be obtained either by replacement of one position or by removal of a central segment that contains that position. For example, the analogues [Ala^{13}]-pNPY and [Ahx$^{8-19}$]-pNPY bind to the hY$_5$-receptor with an affinity of 17 nM and 19 nM, respectively.

To investigate the receptor binding site, also anti-receptor antibodies are useful tools. If the antibodies are obtained against receptor fragments, then the localisation of the functional groups will be possible by competition studies with the ligand. For the Y$_1$-receptor this method has been described by Wieland et al. [44]. In the present work, only a few sera for every receptor subtype were generated. To map the binding site with competition experiments, more sera for every receptor subtype are necessary.

To obtain antibodies which can be used to characterise intact cells, we prepared peptides based on the extracellular loops of the receptors for immunisation. The extracellular loops of the NPY receptors show some similarity, which makes it difficult to achieve full subtype selectivity, which furthermore is difficult to predict. Nevertheless, the generated sera have been shown to be precious tools. For the sera Y$_1$ E2/2 and Y$_5$ E2/2, subtype selectivity for the Y$_1$- and Y$_5$-receptor subtypes was achieved. The sera Y$_5$ E2/2 and Y$_5$ E3 bound equally well to the Y$_5$- and the Y$_2$-receptor subtypes. In combination, these sera are able to distinguish between the receptors Y$_1$, Y$_2$ and Y$_5$. Detection of binding is easy and reliable with a second fluorescent-labelled antibody that is directed

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

For both NPY analogues and anti NPY receptor antibodies, a rational approach to achieve subtype selectivity is difficult. Ligand-receptor interaction is usually not fully understood for every receptor subtype, which makes prediction of selectivity very hard. Because of sequence similarity amongst receptor subtypes, it is not easy to choose receptor fragments for immunisation that will not lead to any cross-reactivity. Furthermore, the peptides for immunisation may not be too small, in order to obtain the same secondary structure of the segment as in the receptor.

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

For both, NPY analogues and anti NPY receptor antibodies, a rational approach to achieve subtype selectivity is difficult. Ligand receptor interaction is usually not fully understood for every receptor subtypes which makes prediction of selectivity very hard. Because of sequence homologies amongst receptor subtypes, it is not easy to chose receptor fragments for immunisation that produce no crossreactivity. Furthermore, the peptides for immunisation may not be too small, because small peptides have often not the same secondary structure as the segment in the receptor and might be too small to trigger proper immune reaction.

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

3.6 Conclusions

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

In a next step, the analogues and the antibodies have to be tested on tissue because knowledge of receptor distribution is important for the understanding of the physiological and biological role of a receptor subtype. Furthermore, localisation studies should be performed also on the protein level, since mRNA localisation does not fit necessarily with the localisation of the mature protein, as suggested by the observation of the rapid axonal transport for the Y1-receptor [46].

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CHAPTER 4

Probing of the Neuropeptide Y - Y_1-Receptors Interaction with anti-Receptor Antibodies

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Abbreviations:

ABTS, 2,2'-azino-bis-(3-ethylbenzothiozolin-6-sulfonic acid)

Boc, tert-butyloxycarbonyl

CT, C-terminal segment

DMEM, Dulbecco's modified Eagle's medium

E1-3, extracellular loops 1-3

EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-hydrochloride

EMEM, Eagle's minimal essential medium

HEK, human embryonic kidney cells

NaCl/P_i, phosphate buffered saline

NaCl/Tris, Tris buffered saline

NK, neurokinine

NPY, neuropeptide Y

NT, N-terminal segment

Pmc, pentamethylchromanesufonyl

tBu, *tert*-butyl; (Tmd)Phe, 4'-(3-trifluoromethyl)-3-diazirine-3-yl-phenylalanine

Trt, trityl;

4.1 Abstract

The Y_1-receptor, which belongs to the family of rhodopsin-like G-protein coupled, 7 transmembrane helix spanning receptors, binds the 36-mer neuromodulator NPY (neuropeptide Y) with nanomolar affinity. Synthetic fragments of the N-terminus, extracellular loops and C-terminus of the Y_1-receptor, were used to generate 18 anti-receptor antibodies. Ten of them recognise the receptor expressed on intact cells as well as on membranes that have been prepared (with exception of one antibody raised against the intracellular C-terminus) as investigated by ELISA. SDS-PAGE of solubilized membranes, subsequent Western blotting and staining with the antibodies revealed two proteins of 73 and 51 kDa for the rat and the human receptor.

Competition with neuropeptide Y showed that the binding of seven antibodies is strongly inhibited in the presence of the native ligand. Using photoactivatible analogues, it could be demonstrated that the competition efficiency strongly depends on the position of the crosslinker within the ligand. Based on these studies a model for the ligand/receptor interaction is suggested. These antibodies represent novel tools for the structural characterization of the Y_1 receptor and its interaction with NPY and antagonists as well as for localization studies.

4.2 Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide that is widely distributed both peripherally as well as centrally. Similar to many other neurotransmitters NPY elicits diverse physiological effects, e. g. induction of food intake and potent vasoconstriction (for reviews, see [1-5]). Therefore, NPY is a neuronal and endocrine messenger that exerts its effects via several receptor subtypes of which the so called Y_1-, Y_2-, Y_4-, Y_5- and y_6-receptor are cloned [6-7]. The first receptor that has been cloned by several groups independently, sequenced and identified as G-protein coupled receptor is the Y_1-receptor subtype [8-10].

Various studies have shown that antibodies produced against hormone receptors are valuable tools [11-18]. Anti-receptor antisera can be raised by immunisation with purified, enriched receptors [18] or by immunisation with receptor fragments [10-17]. They are used for molecular mass determinations by SDS-PAGE and subsequent Western blotting, receptor purification by affinity chromatography on antibody-columns [17] and investigations of the receptor localisation [19,20], regulation [21] and desensitisation [22] are only a few applications of anti-receptor-antibodies [23]. Antisera targeted against defined regions of the receptor can be used to detect functional groups that are involved in ligand/receptor interaction [10-13, 22, 24].

Antibodies specifically recognising the NPY receptor subtypes can be used to determine the localisation pattern and the quantity of receptor protein expression as well as to detect the topology and function of a receptor subtype. Due to the lack of specific antibodies the localisation of NPY receptor subtypes has been restricted to the mRNA level so far. Since rapid axonal transport has been suggested for the Y_1-receptor [25], mRNA localisation must not necessarily fit with the localisation of the mature protein thus making antibody mediated localisation studies more advantageous. We describe here the production of polyclonal antibodies raised against Y_1-receptor segments, their characterisation and their use for the identification of ligand-receptor interactions. These data are included in a structural model of the receptor and its interaction with NPY.

4.3 Materials and Methods

4.3.1 Synthesis and Characterisation of the Segments of Y1-Receptor

Synthesis of the rY_1-receptor segments was achieved by Fmoc-strategy on an alkoxy-benzyl alcohol linker attached to polystyrene-1%-divinylbenzene (200 mg, 0.1 mM) as described previously [26]. The following side-chain protecting groups were used: Asp(tBu), Glu(tBu), Ser (tBu), Thr(tBu), Tyr(tBu), His(Trt), Asn(Trt), Gln(Trt), Cys(Trt), Lys(Boc), Trp(Boc) and Arg(Pmc) (tBu, tert. Butyl; Boc, tert-butyloxycarbonyl; Pmc, pentamethylchromanesufonyl; Trt, trityl). The peptides were characterized by RP-HPLC (gradient 15% acetonitrile to 60% acetonitrile within 30 min), electrospray mass spectrometry and amino acid analysis. The chemical characterization of the peptides is summarised in Table 1.

4.3.2 Preparation of the Conjugates and Immunisation

For the immunisation of the 18 synthesised peptides, BSA and catalase conjugates were prepared. For each experiment 5 mg peptide was dissolved in 500 µL phosphate buffer (100 mM, pH 5.5) and mixed with 7.5 mg BSA or 7.5 mg *Aspergillus catalase* in 100 µL phosphate buffer while shaking intensively. Crosslinking was achieved by addition of 10 mg EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-hydrochloride) in 100 µl water and intensive shaking for 45 min. The reaction was stopped by overnight dialysis against 3 x 1 000 mL water in a membrane having an exclusion size of 20 000 Da. After lyophilization the loading was determined by amino acid analysis of the protein and protein-peptide to be 15-20 peptide molecules/BSA molecule. In order to obtain a specific antibody against the C-terminus of Y_1-receptor, the native sequences of peptides of the CT (C-terminal segment) were elongated by a Cys. These peptides were reacted with maleinimido-BSA (Pierce) according to the instruction of supplier, dialysed and lyophilised.

Table 1. *Sequence and analytical data of the peptides that were used to generate anti-receptor antibodies.*

Name	Sequence	mass (calc.) [Da]	mass (exp.) [Da]	HPLC [min]	loading of conjugate
NT1	1-20	2376.6	2375	24.3	18
NT2	10-30	2346.5	2344	26.2	14
NT3	20-38	2131.4	2130	25.1	19
NT4a	27-47	2175.5	2177	27.9	12
NT4b	27-47	2157.5	2155	27.8	13
E1/1	84-103	2278.8	2280	28.2	16
E1/2	97-118	2677.2	2678	26.9	17
E1/3	108-126	2056.8	2054	27.1	17
E2/1	166-183	2224.1	2225	28.6	18
E2/2	176-194	2164.5	2165	26.9	18
E2/3	185-203	2179.6	2178	27.6	13
E2/4	198-213	1914.1	1915	29.2	20
E2/5	202-221	2310.7	2312	27.2	14
E3/1	275-291	2145.0	2142	31.2	16
E3/2	282-299	2139.4	2138	30.6	15
E3/3	293-309	1915.4	1913	32.8	14
CT1	363-382	2364.9	2364	21.5	11
CT2	368-382	1837.2	1836	19.8	12

BSA conjugates were used for immunisation of chicken (NT/1, NT/4a, NT/4b, E1/2, E2/1 and E2/3) and rabbit (NT/3, E2/2, E2/4, E3/2) or with catalase conjugates in rabbit (NT/2, E1/1, E1/3, E2/5, E3/1, E3/3, CT/1 and CT/2 (NT, N-terminal segment; E1-3, extracellular loops 1-3, CT, C-terminal segment). For immunisation in chicken 50 µg BSA conjugated peptide, mixed 1:1 with Freund adjuvant, and intramuscular injections in breast muscles was performed. After 12 days it was boosted with the Freund incomplete adjuvant. For the isolation of IgY, 6 egg yolks were washed with destilled water. The egg yolk sac was removed and the remaining yolk were mixed. NaCl/Tris (Tris buffered saline, 140 mM NaCl, 10

mM Tris, pH 7.4; 20 ml) and $CHCl_3$ (20 ml) per egg were added and the mixture incubated 24 h at 4 °C until the phases were separated. After centrifugation (10 min, 300 x g) three phases appeared. The upper one was collected and precipitated with saturated $(NH_4)_2SO_4$ within 30 min without stirring, centrifuged for 10 min at 2000 x g. The pellet is resuspended in NaCl/Tris buffer and dialysed against NaCl/P_i (phosphate buffered saline, 140 mM NaCl, 10 mM sodium phosphate, pH 7.4) at 4 °C over night. Storage was performed at - 20 °C with 40 % glycerol or with 0.02 % NaN_3. Immunisation of rabbits was achieved with 500 µg BSA or catalase conjugated peptide, mixed 1:1 with Freund's complete adjuvant. It was boosted 4 times after 3-4 weeks, each. The sera were either directly used or the antibodies were precipitated with $(NH_4)_2SO_4$.

4.3.3 Titer Determination

In order to determine the titer of antibodies raised against peptide-BSA (or catalase) conjugate, the catalase conjugate (or BSA conjugate, respectively) was used to coat ELISA plates (5 µg in 100 µL/well) for titer determination. The peptides were dissolved in the coating buffer and incubated overnight at 37° C. Blocking was achieved with 2% skim milk in NaCl/P_i at 37° C for 1 h (200 µL/well). The antibody solutions were diluted 1:10, 1.100 and 1:1,000 with 0.2% BSA in NaCl/P_i and incubated at 37° C for 1 h (100 µL/well). The second antibody was diluted 1:5 000 (goat anti-rabbit POD) or 1:10,000 (goat anti-chicken POD) with 0.2% BSA in NaCl/P_i, incubated each for 1h at 37°C (100 µL/well). The plates were washed 4 times with NaCl/P_i-Tween by using an ELISA washer (SLT, Salzburg). Staining was carried out with ABTS (2,2'-azino-bis-(3-ethylbenzothiozolin-6-sulfonic acid) in citrate-phosphate buffer (1% ABTS, 0.003% H_2O_2, 100 mM citric acid, 100 mM K_2HPO_4, pH 4.1). The reagent (150 µL/well) was incubated in the darkness for 1h and the difference of absorption at 405 and 690 nm subsequently measured in an ELISA-reader 400 (SLT/Salzburg). Each value was determined at least 3 times and the average value $\pm$ S.E.M. was given. For the negative control, the preimmune serum was treated in parallel using the same dilution. Furthermore, in one series the plate was not coated with peptide but blocked with 2% skim milk in NaCl/P_i (=blank control). As positive control, one

series was directly coated with serum. The specific OD values were determined as follows: Average of the antibody values minus average values of preimmune sera (without sera = 0-controls). Since the blank values ranged below the values of preimmune sera, they were not considered for determination of the specific reaction. The determination of unspecific reaction resulted from the mean values of preimmune sera minus average of the blank values.

4.3.4 Investigation of Antibodies recognising the Y1-Receptor on prepared Membranes

Affinity of the produced antibodies for the target receptor was investigated at Y_1-receptors stably expressed in HEK (human embryonic kidney cells) 293-cell membranes (Eva et al., 1990) and Y_2-cells (SMS-KAN). The cells were grown as described by [27] and washed with NaCl/P_i, treated with 0.02 % EDTA in NaCl/P_i for 2 min. The EDTA solution was removed and the cells were suspended in Tris buffer, pH 7.5 with 0.1% bacitracin and 50 µM Pefabloc SC. The cell suspension was homogenised 15-times using a potter (Braun-Melsungen, Germany) at maximum speed. After centrifugation at 4°C for 10 min at 800 × g, the supernatant was decanted and centrifuged at 4°C for 30 min at 16500 × g. The supernatant was decanted and the pellet resuspended in 30 ml Hepes buffer (25 mM Hepes, 25 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4, 0.1% bacitracin and 50 µM Pefabloc SC), homogenised 15-times with a potter and recentrifuged at 4°C for 30 min at 16500 × g. The pellet was resuspended in 15 ml Hepes buffer without protease inhibitors. An aliquot was used to determine protein concentration and inhibitors were added accordingly. Membrane solutions (protein concentration 1 mg/mL) were diluted (1:20) with EMEM medium (Eagle's minimal essential medium), 25 mM Hepes and 3.75 mM $CaCl_2$ were added. The ELISA plates were incubated with the membrane suspension (100 µL) overnight at 4°C then carefully washed 4 times with NaCl/P_i. Incubation of the antibody solutions as well as detection and performance of the control experiments was performed by manual wash steps as described above. Each experiment was independently carried out twice at least in triplicate. Additional negative controls were performed using ELISA plates were coated with untransfected 293-cell membranes and SMS-KAN cell membranes (human Y_2).

Different concentrations of the antibody solutions were assayed (1:10, 1:30, 1:100, and 1:1000). In order to block the receptor-antibody reaction, the undiluted antibody solutions were incubated with peptide solutions, that were used to generate the antibody (1 mg/mL in NaCl/P_i, 1:1) for 1h at room temperature, diluted to a final dilution of 1:10, 1:30, 1:100 and 1:1000, and tested as described above.

4.3.5 Investigations of the antibodies recognising the Y1-receptor expressed on intact cells

Y_1-receptor expressing HEK 293 cells of the 13th passage and HEK 293 control cells were directly grown on ELISA plates to confluency. The supernatant was decanted and the antibody as well as preimmune sera were diluted (1:20, 1:100, 1:200 and 1:1000) in EMEM and DMEM (Dulbecco's modified Eagle's medium) when using for assays with transfected and untransfected HEK293 cells, respectively and incubated at 37° C for 1h. Blocking the antibody reaction by peptides was performed accordingly. EMEM was used for dilution (1:20, 1:100, 1:200 and 1:1000).

4.3.6 Binding Competition Experiments with NPY and photolabile Analogs on Membranes and intact Cells

Competition reaction of NPY and its analogs with antibodies were carried out in parallel to a reaction without competition. NPY (10 µM) was dissolved in incubation buffer (EMEM medium, 25 mM Hepes and 3.75 mM $CaCl_2$), incubated with membranes or intact cells (100 µL/well) and incubated at 37° C for 1 h. The solution was decanted and the plate washed once with NaCl/P_i. Incubation of the antibody solutions as well as detection and performance of the control experiments were performed as described above. The photoaffinity analogues of NPY containing (Tmd)Phe (4'-(3-trifluoromethyl)-3-diazirine-3-yl-phenylalanine) were dissolved in incubation buffer (10 µM, EMEM medium, 25 mM Hepes and 3.75 mM $CaCl_2$), 100 µL cells/well gently incubated at 37° C for 1 h in the dark. The solution was decanted and the plates were washed once with NaCl/P_i,

illuminated at 365 nm for 15 min and washed again. Incubation of the antibody solutions as well as detection and the control experiments were performed as described above.

4.3.7 Receptor Binding Assay

Rat Y_1 receptor expressing HEK 293 cells (Eva et al., 1990) were harvested with a mixture of 0.02% EDTA in NaCl/P_i and resuspended (ca. 40 million cells) in 10 mL incubation medium (EMEM/25 mM Hepes, 0.5% BSA, 50 µM PMSF, 0.1% bacitracin and 3.75 mm $CaCl_2$). After centrifugation (5 min, 150 x g) the pellet was washed once in 10 mL incubation buffer and resuspended again in the same volume of buffer, counted and diluted to 1.25 million cells/mL. To determine the affinity of (Tmd)Phe analogs of NPY, a binding assay according to [26,27] has been performed. IC_{50} values were 36 nM ([(Tmd)Phe1] NPY), 39 nM ([(Tmd)Phe20] NPY), 7 nM ([(Tmd)Phe20] NPY), 5 nM ([(Tmd)Phe27] NPY) or 250 nM ([(Tmd)Phe36] NPY. In order to investigate the competition between [125I]NPY and antibody for binding at the receptor, 10 µL of an antibody solution (undiluted, 1:10, 1:100 and 1:1 000) was incubated with 200 µL of the cell suspension for 90-120 min. Finally, 50 µL of binding buffer containing [^{125}I]-BH-NPY was added (final concentration 30 pM). Unspecific binding was determined with 1 µM NPY. Double measurements were carried out for each experiment.

4.3.8 Immunoblot

Y_1-receptor expressing BHK cells (baby hamster kidney cells), SK-N-MC cells (Y_1-receptor expressing human neuroblastoma cells), Y_1/HEK 293 cells (expressing the rat Y_1-receptor) and SMS-KAN cells (Y_2-receptor expressing human neuroblastoma cells) were solubilized by incubation in urea sample buffer (8 M urea, 63 mM Tris/HCl pH 6.8, 2 % SDS, 5 % mercaptoethanol, 0.01% bromophenol blue) at 95°C for 7 min and separated on a 12 % polyacrylamide gel according to Lämmli [28]. After 1 h blotting (350 mA/20 V) in a semi-dry electrophoretic transfer cell (BIO-RAD) onto nitrocellulose membrane in a transfer buffer containing 25 mM Tris/HCl pH 8.3, 150 mM glycine, 20 % methanol, the

blots were blocked over night with NaCl/Tris/Tween (0.5% Tween 20 in NaCl/Tris/HCl pH 7.4) containing 1 % BSA. The blots were incubated with antibodies for 90 min by gentle shaking, washed three times with NaCl/Tris/Tween incubated for 60 min with alkaline phosphatase-bound second antibodies and washed three more times. Immunoreactivity was detected with 5-bromo-4-chloro-indolyl-phosphate.

4.4 Results

4.4.1 Selection and Syntheses of Segments of Y1-Receptor

The primary sequence of rY_1-receptor consists out of 382 amino acids [8,30]. In order to map the binding sites of the Y_1-receptor, 18 peptides were selected. They contain all extracellular loops, the extracellular N-terminus, part of the transmembrane helices and two C-terminal, intracellular segments (Table 1). The peptides overlap each other by 3 to 6 amino acids. Size ranged between 16 and 20 amino acids thereby increasing the probability that the peptides selected fold into a secondary structure. The receptor sequence and the segments of the synthetic peptides are shown in Fig. 1.

4.4.2 Characterisation of the Antisera

A summary of the antibodies tested against the peptides is shown in Fig. 2 at a dilution of 1:100. The total absorption ranged between 3 to 6 absorbance units. The values of preimmune sera ranged between 0.5 and 1.5 absorbance units. They were subtracted from the total absorption to obtain specific binding (Fig. 2). The blank values were under 0.5 absorbance units, all antibodies hardly reacted with skim milk (< 0.8 absorbance units) or BSA (< 0.6 absorbance units) with exception of E2/4 (1.2 absorbance units) and CT/2 (1 absorbance units). The antisera yielding the highest titer were NT4 and E3/1, NT/1, NT/2, NT/4b, E2/2 and those with lower titers were NT/4a, E1/3, E2/1.

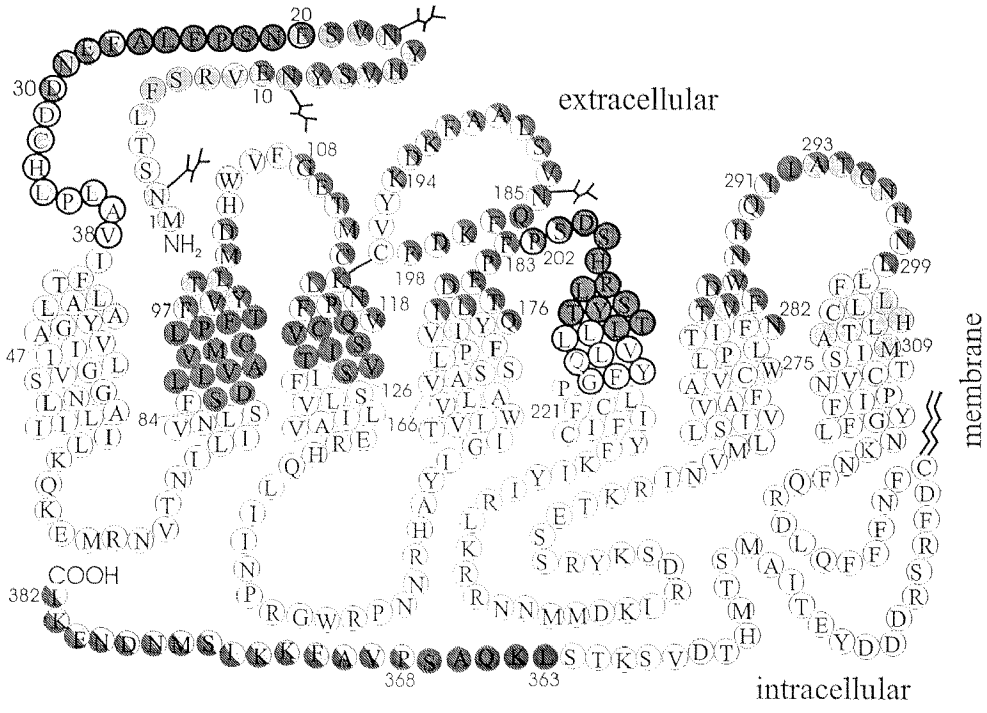

Figure. 1. *Sequence of the rY_1 receptor. Transmembrane segments are drawn according to hydropathy plots. Receptor fragments that were used to generate anti-receptor antibodies are marked in different level of grey and named according to the receptor segment (NT, E1, E2, E3 or CT).*

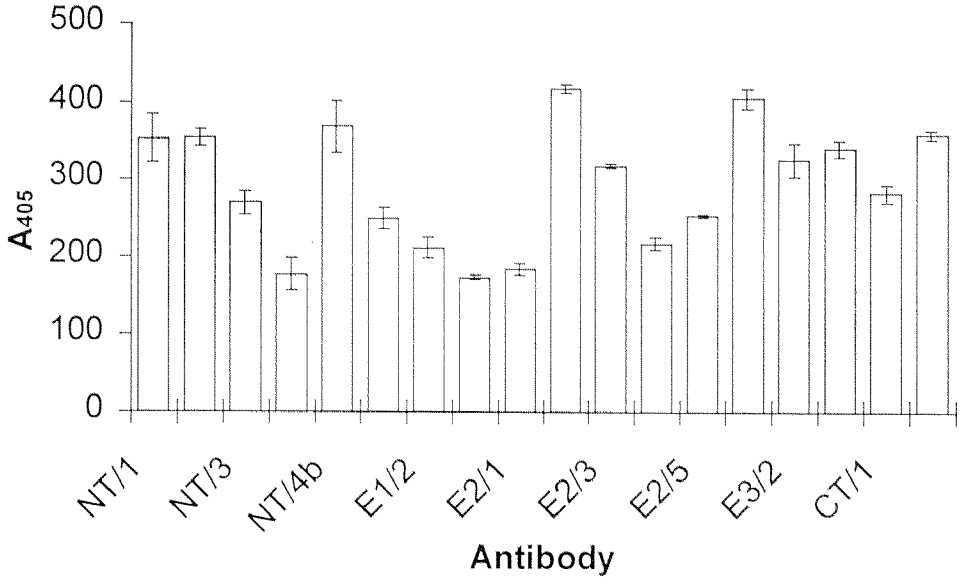

Figure 2. *Binding of the antibodies to the peptides that were used for immunisation. Each bar represents the specific activity (total binding - binding of the preimmune serum) tested in the anti-peptide ELISA.*

4.4.3 ELISA test on Membranes containing Y_1-Receptors

In order to determine those antipeptide-antibodies that recognise not only the peptide derived from the receptor, but the receptor itself as well, ELISA plates were coated with membranes. The selectivity of the antibodies for the Y_1-receptor was determined using rat Y_1-receptor expressing 293 cell membranes, SMS-KAN cell membranes (endogenously expressing human Y_2-receptors). Untransfected HEK293 cell membranes were used as negative controls. Absorption of the ELISA plate containing SMS-KAN cells (human Y_2 receptor) was generally less than 0.8 absorbance units with exception of the antibodies E1/3 (3 absorbance units) and E3/3 (1.5 absorbance units), that indicated some crossreactivity with the human Y_2-receptor. Using untransfected 293 cell membranes, all antibodies showed an absorption of less than 0.8 absorbance units with the exception of E1/3 (1.5 absorbance units) and E1/2 (1.8 absorbance units). This indicated, with exception of the latter two antisera, that very low unspecific binding was detected. The specific interaction was determined in dilutions of 1:10, 1:30, 1:100 and 1:1000. The best ratio of total binding versus preimmune serum binding (rabbit) or the negative controls (chicken) was found for an antibody dilution of 1:100 (Fig. 3A).

Highest specific absorptions were found for antibodies NT/1, E1/1, E1/3, E2/2, E2/3, and CT/1 at rat Y_1-receptor expressing HEK 293 cell membranes. Lower but specific binding was observed for antibodies NT/2, NT/3, NT/4b, E2/1, E2/4, E2/5, E3/2 and E3/3. The binding of these antibodies to the receptor could be blocked by preincubation with the peptide against it was raised. Antibodies NT/4a, E1/2, E3/1, and CT/2 hardly recognised rat Y_1-receptors in HEK 293 cell membranes.

4.4.4 ELISA test in stably Rat Y_1-Receptor expressing intact 293 Cells

In order to characterise the antisera and their interaction with the receptor on living cells, Y_1-receptor expressing 293 cells and untransfected 293 cells were directly grown on ELISA plates. High affinity for Y_1/HEK 293 cells was observed for antibodies NT/1, NT/3, E1/1, E1/3, E2/1, E2/2, E2/3, E2/4 and E3/2 (Fig. 3B). This binding was at least three fold higher than values of preimmune sera and could be suppressed by preincubation with the corresponding peptide. The antibodies

examined showed no increased affinity to untransfected HEK 293 cells (beside antibody E3/3 (1.5 absorbance units)). All antibodies that have not recognised the membranes (NT/4a, E1/2, E3/1 and CT/2), did not recognise the receptors in intact cells either. The antibodies NT/2, NT/4b and E3/3 while showing a significant affinity to membranes prepared from rat Y_1-receptor expressing HEK 293 membranes did no longer recognise the receptor in intact cells. CT/1, which was made against an internal receptor segment, exhibited high affinity to the Y_1/293 membranes, but no affinity to the intact cells (Fig. 3A, B).

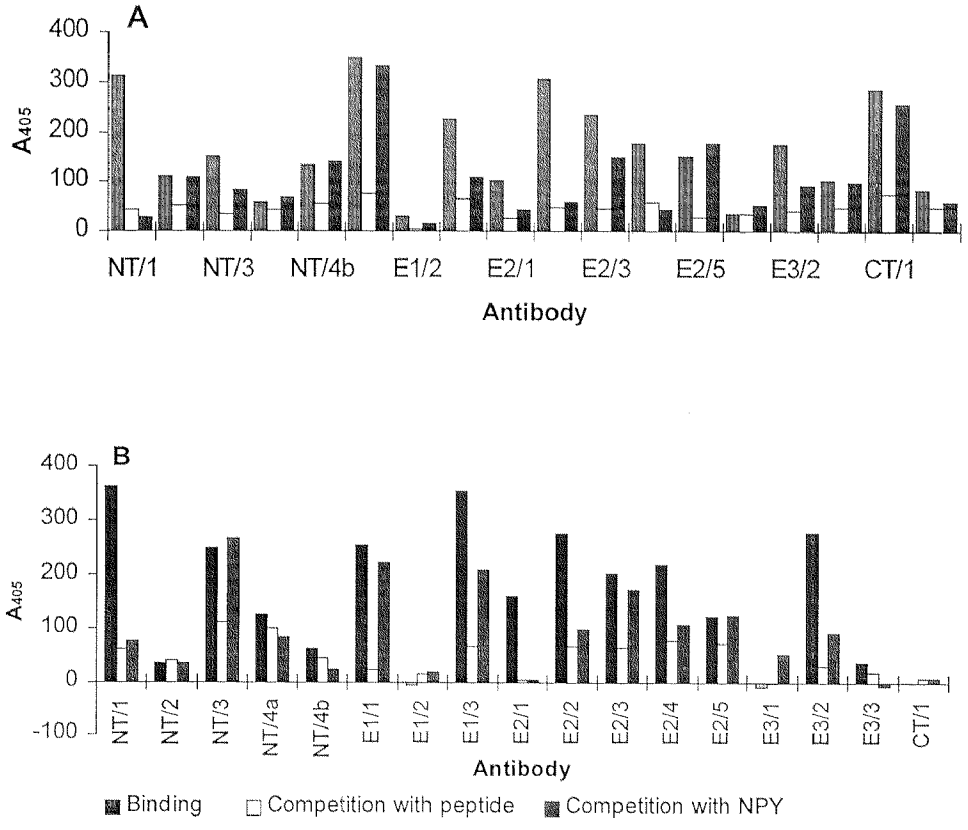

Figure 3. *Binding of the antibodies to the rat Y_1-receptor. A) Membranes, prepared from Y_1-receptor expressing-293 cells, B) Intact stably transfected HEK 293 cells directly grown on ELISA plates (dilution of antisera 1:100). Each bar represents the specific activity (total binding - binding of the preimmune serum) of the antibodies alone as well as the antibodies after preincubation with the peptides that were used to generate the antibodies and of the antibodies after preincubation with NPY.*

4.4.5 Competition with NPY and [(Tmd)Phe]-NPY Analogs

Interference of the antibody binding epitope with the NPY binding site was tested. NT/1 and E2/2 binding to the receptor has been inhibited by NPY to a high extend (91 ± 2 % and 81 ± 2 %, Fig. 3A). Lower, but significant inhibition of binding of the antibodies E1/3 (51 ± 4 %), E2/1 (57 ± 10 %), E2/3 (37 ± 4 %), E2/4 (75 ± 3 %) and E3/2 (47 ± 3 %) was detected. All antibodies showed the same competition effects in intact cells as with membranes (Fig. 3B), although differences in the extent have been observed.

The binding epitopes of the antibodies have been further characterized by competition experiments in intact cells that have been carried out using photoaffinity analogs of NPY. Five photolabile peptides have been synthesized in order to irreversibly block the NPY binding site [29]. The photolabile peptides differ with respect to the position of the photolabile amino acid (Tmd)Phe (Fig. 4). The position of the crosslinker had impact on the extend to which it blocked the access of the antibodies to the binding site (Fig. 4). Antibodies E1/3 and E2/3 could most effectively be blocked by [(Tmd)Phe1] NPY (75% and 50%, respectively). In contrast, covalent binding of [(Tmd)Phe36] NPY inhibited binding of antibody NT/1 to the receptor by about 60%. Antibody E2/1 exhibited the lowest inhibition, antibodies E2/4 and E3/2 were most significantly inhibited by covalent binding of [(Tmd)Phe20] NPY and [(Tmd)Phe21] NPY, and, in the case of E3/2, of [(Tmd)Phe1] NPY as well. E2/2 binding was inhibited by NPY independent from the position of photolabelling group. As in Y_1-receptor expressing HEK 293 cells initial competition data showed, that specific inhibition of ^{125}I-BH-NPY was obtained with undiluted antibodies E2/2 (50-100%) and NT/1 (40%) this is in agreement with the crosslinking data.

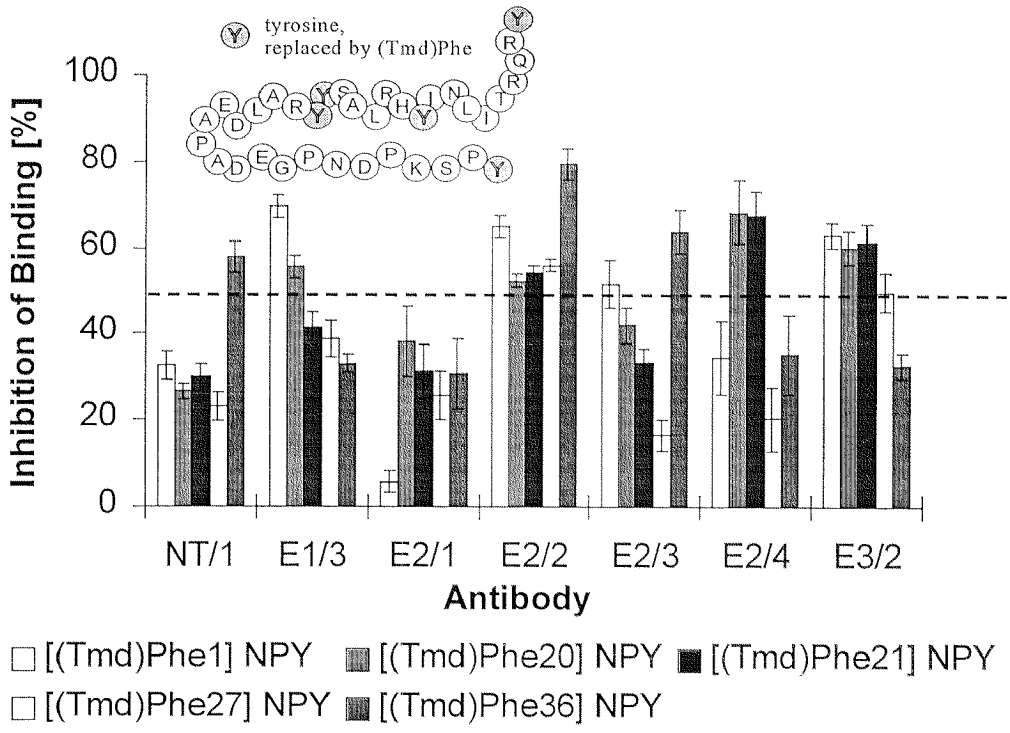

Figure. 4. *Inhibition of the binding of antibodies after covalently crosslinking of five different photoactivatable analogs of NPY. Positions of the replacement of tyrosine by (Tmd)Phe are shown in the lower panel.*

4.4.6 Immunoblot

Western blots have been performed in order to determine the size of the receptor proteins that are recognised by the antibodies. Membranes prepared from Y_1/HEK 293 cells (rat Y_1-receptor) or SK-N-MC cells (human Y_1-receptor) have been solubilized and proteins separated on a gel (Fig. 5). Two major bands of an apparent molecular weight of 73 and 51 kDa were recognised by all antibodies, except of NT 2, E3/1 and CT1, however with different intensity. E3/2, NT1, E2/3 additionally stained proteins of 82 kDa and/or 32 kDa. Antibody E1/3 labelled many more bands, indicating that it does not recognise a specific epitope. The size and the shape of the bands was similar for the rat and the human sequence, which suggests that both are expressed with a similar glycosylation pattern. Weak bands were found for NT/1, E2/1 and E2/2 despite their above described strong reaction with proteins prepared from intact cells, whereas E3/2 (Fig. 5*A*), E2/3 (Fig. 5*B*) and E1/2 (Fig. 5*C*) showed the most significant bands.

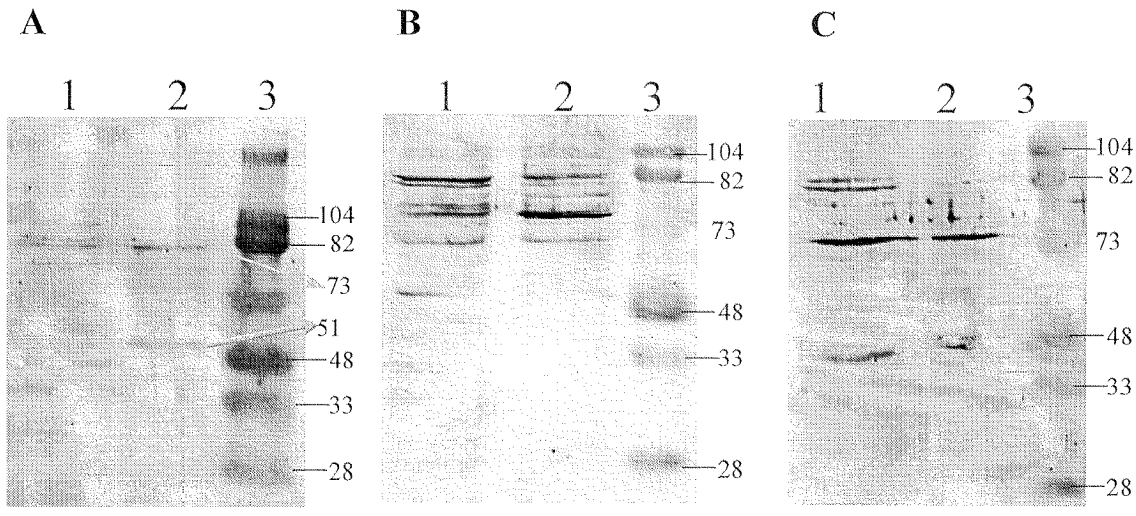

Figure. 5. *Western blots using anti-receptor antibodies recognising the Y_1-receptor on solubilized membranes. In the first lane (1) SK-N-MC membranes, that express human Y_1-receptors and in the second lane (2) rat Y_1-receptors (HEK 293 membranes) are used; prestained marker (3). The following antibodies were used: A: E3/2, B: E2/3, C: E1/2.*

4.5 Discussion

The major aim of our work was to study the ligand-receptor interaction of neuropeptide Y with the Y_1-receptor using polyclonal antibodies, which have been raised against synthetic receptor fragments. Therefore, all the exterior segments including the transmembrane regions as well as two peptides from the interior C-terminal region were used to generate the antisera. The binding of neuropeptide Y to its receptor must be achieved from the extracellular side [31] as the size and charge of the hormone make a passage through the membrane impossible. Antibodies against the peptide segments of intracellular regions are consequently unsuitable for hormone-receptor interactions studies. With the exception of two control peptides, all segments originated from the extracellular region. Peptides of this size can already exhibit secondary structures [32,33], so that antibodies can be expected to recognise the secondary structures and therefore the intact receptor, too. The antibodies recognised solubilized proteins of 73 and 51 kDa corresponding to the size of 70 kDa found by Sheikh and Williams [34] using Y_1-

receptor expressing cells, together with a 45 kDa proteolytic fragment of the latter. The faint 30 kDa band possibly represents a further degraded product. The 82 kDa band seen with 3 antisera might be differently glycosylated or represent an unprocessed translation product.

The polyclonal antibody NT/1, which is directed against the very first 20 amino acids of the N-terminus of the receptor, recognises Y_1-receptor containing membranes as well as intact rat Y_1-receptor expressing HEK 293 cells. This binding is blocked by the peptide as well as by NPY or (Tmd)Phe containing analogs. In a binding assay, NT/1 inhibits the affinity of ^{125}I-BH-NPY to Y_1/293 membranes. Since the region, against which NT/1 is directed, contains three possible glycosylation sites, it is striking that the antibody which was raised against an unglycosylated peptide recognises the intact receptor. Either not all the possible sites are glycosylated, or the antibody mainly recognises the segment that is localized between the glycosylation sites. Considering the strong interaction of these anti-receptor-antibodies raised against the N-terminus, it can either be assumed that the N-terminus belongs in fact to the receptor binding region, or otherwise it contributes decisively to the stabilisation of active receptor conformation, i.e., it opens the binding pocket. Participation of the N-terminal, extracellular segment for binding of neuropeptides to G protein-bound receptors is not entirely new. Fong et al. [35] identified by receptor mutation, two amino acids of the N-terminal segment of neurokinine-1 receptor (NK1) and two others at the first extracellular loop as essential for the binding of substance P.

Antibodies NT/3, NT/4a and NT/4b cover the region between N-terminus and transmembrane region 1. The difference between NT/4a and NT/4b is mainly based on the fact that the native sequence of NT/4a peptide conjugate contains Cys, whereas this is replaced by γ-aminobutyric acid in the NT/4b peptide, thus it can no longer build a disulfide bridge. The binding of NT/4a,b is neither competed by NPY nor these antibodies bind to intact cells. Accordingly, in a model the N-terminus could be fold back to the binding region. Two proline residues (rY_1-Pro23, Pro35) are found in this segment of the N-terminus, which is in agreement with a turn structure.

The binding of antibody E1/3 to its epitope can be inhibited by preincubation with NPY. Irreversible blockade with [(Tmd)Phe1] is achieved after covalent

coupling to the receptor. Therefore, the N-terminus of NPY is possibly localised near the regions recognised by E1/3, since the antibody shows a decreased affinity after covalent binding of NPY via position 1. The second extracellular loop is covered by antibodies E2/1, E2/2, E2/3, E2/4 and E2/5. The overlapping section consists of 6-10 amino acids. With the exception of E2/5 which partially contains the transmembrane region V, all antibodies bind membranes and intact cells with E2/2 showing the highest affinity. This binding can be suppressed by NPY and its analogs; in particular E2/2 seems to inhibit also the binding of ^{125}I-BH-NPY to rat Y_1-receptor in the competition assay. From these results it can be concluded that, among others, the second extracellular loop participates at the binding of NPY and that the amino acids are possibly involved in direct interactions. This is in agreement with side-directed mutagenesis studies that identified Asp194 and Asp200 in the second extracellular loop to be important for binding of Tyr36 of NPY. According to the prediction, the secondary structure of E2 can be described as the follows: At the end of TM4 follows a segment with undefined secondary structure, Pro182 orients the protein chain in the direction of E1 loop. Amino acids 185-200 are predicted to be helical. Cys197 terminates the helical conformation and builds a disulfide bridge with Cys112 inside the E1 loop which is essential for the receptor affinity. Antibody E1/3 which recognises the region surrounding position 112, is also spatially close to the binding region. By a further turn at Pro202, E2 reaches the transmembrane V region.

Out of the three antibodies produced against the third extracellular region E3 (E3/1, E3/2 and E3/3) either yielded only low titer (E3/1) or exhibited very high cross reactivity and unspecificity (E3/3). Only antibody E3/2 unambiguously recognised the Y_1-receptor expressed on membranes, intact cells and on the blot. After crosslinking the binding of E3/2 to the receptor is inhibited, which suggests an involvement of the third extracellular loop in ligand binding. This region was identified in other neurohormone receptors as well, such as NK1 or NK3 receptors, to bind to nonpeptide antagonists [36-38]. Furthermore, an overlapping binding site of NPY and the NPY derived antagonist BIBP 3226 was identified to include the E3-loop [39]. To what extent an antibody which is bound to this region also displays antagonistic properties, remains to be shown.

A schematic model of the NPY-Y_1-receptor interaction as suggested from the antibody interaction studies is shown in Fig. 6. The helices are arranged according to the electron density map obtained by Schertler et al. [40] and the interpretation of Baldwin [41]. The orientations seen from outside is counterclockwise, as proven by Schwartz et al. for the NK1 receptor by generation of Zn^{2+} binding sites [42-44];. With respect to the ligand, we suggest, that, the N-terminus of NPY is very flexible as binding of several antibodies (E1/3, E2/2 and E3/2) raised against different parts of the receptor, is more than 50 % inhibited after crosslinking via position 1. Covalent binding via the helical part (position 20, 21) is resulting in a loss of affinity of antibodies E2/4 and E3/2, and to minor extent of antibodies E2/2 and E1/3. Position 27 of NPY could possibly be close to E2/2 as the C-terminal segment of NPY, that is known to be important for high affinity, most efficiently blocks the interaction of the antibodies E2/2 and E2/3.

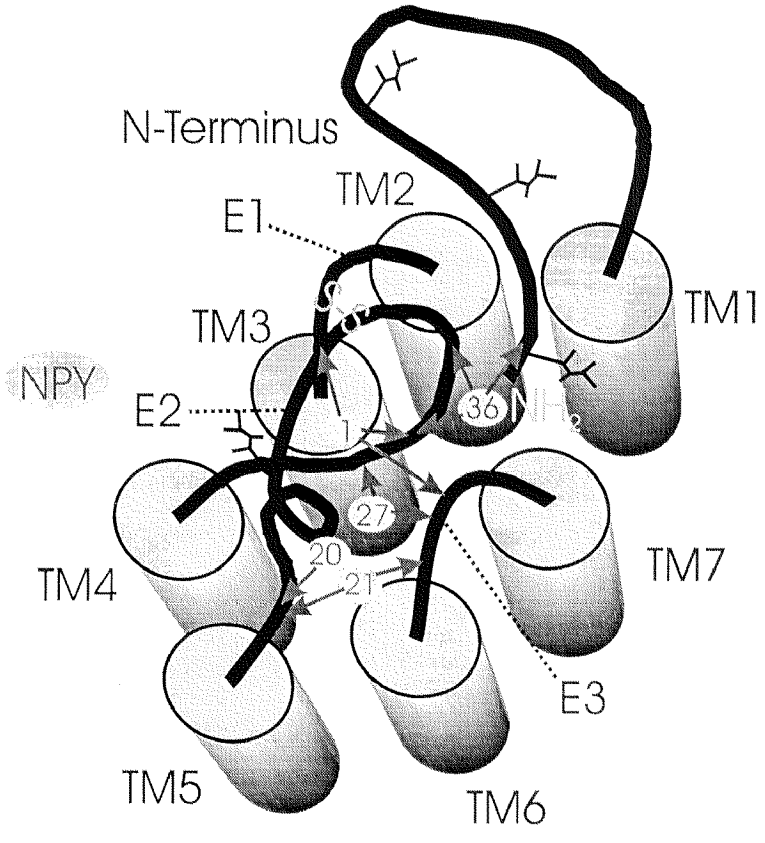

Figure 6. *Schematic model of the ligand-receptor interaction of neuropeptide Y as derived from the anti-receptor antibody studies.*

Since antibodies CT/1 and CT/2 are made against peptides from the interior segments, no binding to intact cells was expected for these antibodies. This confirms the model of 7 transmembrane helices with the C-terminus being localised inside. Antibodies NT/4a, NT/4b and E2/3 contain partial sequences of the transmembrane regions. These are perhaps covered by the receptor or by associated proteins, thus they are no longer accessible for antibodies in intact cells. The reason why this is not the case for antibodies E1/1, E1/3 and E2/1, which are also produced against membrane adjacent regions, is not clear. Possibly, these antibodies recognise mostly protein regions which are accessible from outside.

Convincingly, all antibodies whose binding properties can become competed with NPY are targeted against peptides from a few segments: NT/1 recognises the N-terminus, E1/3 is against the E1 loop, E3/2 against the E3 loop, all others cover the receptor E2 loop. Since especially antibodies E2/2 and NT/1, and to a less extend also E2/3, E2/4 and E3/2, block the binding of ^{125}I-BH-NPY to Y_1-receptor, corresponding regions have been identified using two independent methods.

4.6 Conclusion

For the first time, we use a combination of anti-receptor antibodies and photoaffinity labelling to characterise the binding site of neuropeptides. The data, we obtained are in full agreement with previously reported mutation work and therefore further support the importance of the second and third extracellular loop for ligand binding. Thus, our approach is a new method, which leads to data complementary to the data obtained by side directed mutagenesis, and might help to separate sensitive mutations for binding from those that completely destroy the overall receptor topology. Furthermore, the described antisera represent valuable tools for the isolation, purification and immunostaining of this receptor subtype. Additionally, we recently could show that the sera are valuable tools to study receptor localisation in the nucleus accumbens [45].

Acknowledgments

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CHAPTER 5

Molecular Characterisation of the Human Neuropeptide Y Y_2-Receptor

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Abbreviations:

Abbreviations: Ahx, 6-aminohexanoic acid

DATD, *N,N'*-diallyltartardiamide

DMF, dimethylformamide

Fmoc, 9-fluorenylmethoxycarbonyl-

^{3}H-Tmd27, [N_{α}-biotinyl-Ahx_2

^{3}H-propionyl-Lys^{4}, Ahx$^{5-24}$

(Tmd)Phe^{27}] NPY

^{3}H-Tmd36, [N_{α}-biotinyl-Ahx_2

^{3}H-propionyl-Lys^{4}, (Tmd)Phe^{36}] NPY

KLH, keyhole limpet hemocyanin

NPY, neuropeptide Y

(Tmd)Phe, 4-(3-trifluoromethyl)-3*H*-diazirin-3-yl-phenylalanine

OSu, N-hydroxysuccinimide

PAGE, polyacrylamide gel electrophoresis

SDS, sodium dodecyl sulphate

PBS, phosphate buffered saline

TBS, Tris buffered saline

Tmd27, [N_{α}-biotinyl-Ahx_2, Ahx$^{5-24}$(Tmd)Phe^{27}] NPY

Tmd36, [N_{α}-biotinyl-Ahx_2, (Tmd)Phe^{36}] NPY

Tris, tris(hydroxymethyl)amino-methane

Tween 20, polyoxyethylene-sorbitan monolaurate.

5.1 Abstract

Five neuropeptide Y receptors, the Y_1-, Y_2-, Y_4-, Y_5- and y_6-subtypes have been cloned, which belong to the rhodopsin-like G-protein coupled, 7 transmembrane helix-spanning receptors and bind the 36-mer neuromodulator NPY (neuropeptide Y) with nanomolar affinity. In this study, the Y_2-receptor subtype expressed in a human neuroblastoma cell line (SMS-KAN) and in transfected Chinese hamster ovary cells (CHO-Y2) was characterized on the protein level by using photoaffinity labeling and anti-receptor antibodies. Two photoactivatable analogues of NPY were synthesized, in which a Tyr residue was substituted by the photoreactive amino acid 4-(3-trifluoromethyl)-3*H*-diazirin-3-yl-phenylalanine ((Tmd)Phe): [N_α-biotinyl-Ahx_2, (Tmd)Phe^{36}] NPY (Tmd36) and the Y_2-receptor subtype selective [N_α-biotinyl-Ahx_2, Ahx^{5-24}, (Tmd)Phe^{27}] NPY (Tmd27). Both analogues were labeled with ^{3}H-succinimidyl-propionate at Lys^4 and bind to the Y_2-receptor with affinity similar to the native ligand.

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

5.2 Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide that is widely distributed both, peripherally as well as centrally. Similar to many other neurotransmitters NPY elicits diverse physiological effects, e. g. induction of food intake and potent vasoconstriction (1-4). Accordingly, NPY is a neuronal and endocrine messenger that exerts its effects via several receptor subtypes of which the so called Y_1- (5-7), Y_2- (8-10), Y_4/PP_1- (11, 12), Y_5- (13) and y_6-receptor (14) have been cloned (15). Sequence comparisons show that the receptors Y_1, Y_4, and y_6 are more closely related to each other than to the receptors Y_2 and Y_5. The different

receptor subtypes are localised in various tissues, in the central nervous system as well as in the periphery. Tissues with high density of NPY receptors are blood vessels, kidney, adrenal glands, colon, heart, pancreas, intestine, nerve endings and brain. While their distribution appears to be species specific, all subtypes belong to the large superfamily of G-protein-coupled, heptahelical receptors (16). The Y_2-receptor is the predominant NPY receptor subtype in the brain and particularly abundant in the hippocampus (17). The cloned Y_2-receptor consists of 381 amino acids (10) and has the typical heptahelix receptor features including a potential glycosylation site in the amino-terminal part, two extracellular cysteins that may form a disulfide loop, and a single cysteine in the cytoplasmatic tail that probably serves as an attachment site for palmitate. Characterisation of the receptor subtypes has been restricted so far to pharmacological experiments and investigations at the mRNA level. Because of possible post-translational modifications like glycosylation and palmitoylation, characterization on the protein level is necessary. For example, a receptor subtype identified on the mRNA level may or may not be functionally expressed. Moreover, since rapid axonal transport has been suggested for the Y_1-receptor (18) mRNA localisation must not necessarily fit with the localisation of the mature protein. Characterisation and localisation of the protein can be achieved by photoaffinity labeling and immunodetection. The first method, photoaffinity labeling, has been widely used for the identification of binding sites in different receptor systems (19-21). The use of this method to determine molecular mass depends on the specific labeling of the receptor. The problem of unspecific labeling may be overcome by constructing a ligand with a built-in photoreactive amino acid that generates a highly reactive, short-lived species, for example a carbene (21, 22). To increase the chance of productive coupling reactions, the photoactivatable amino acid should be placed near or within the binding site. To reach maximal specificity, we compared the results of photoaffinity labeling using two different ^{3}H-propionylated analogues of NPY (Fig. 1) with built-in photoreactive amino acids: [N_α-biotinyl-Ahx_2, ^{3}H-propionyl-Lys^4, (Tmd)Phe^{36}] NPY (^{3}H-Tmd36) and [N_α-biotinyl-Ahx_2, ^{3}H-propionyl-Lys^4, Ahx^{5-24}, (Tmd)Phe^{27}] NPY (^{3}H-Tmd27). Centrally truncated NPY analogues like [Ahx^{5-24}] NPY and Tmd27 additionally have been shown to be selective for the Y_2-receptor subtype (23).

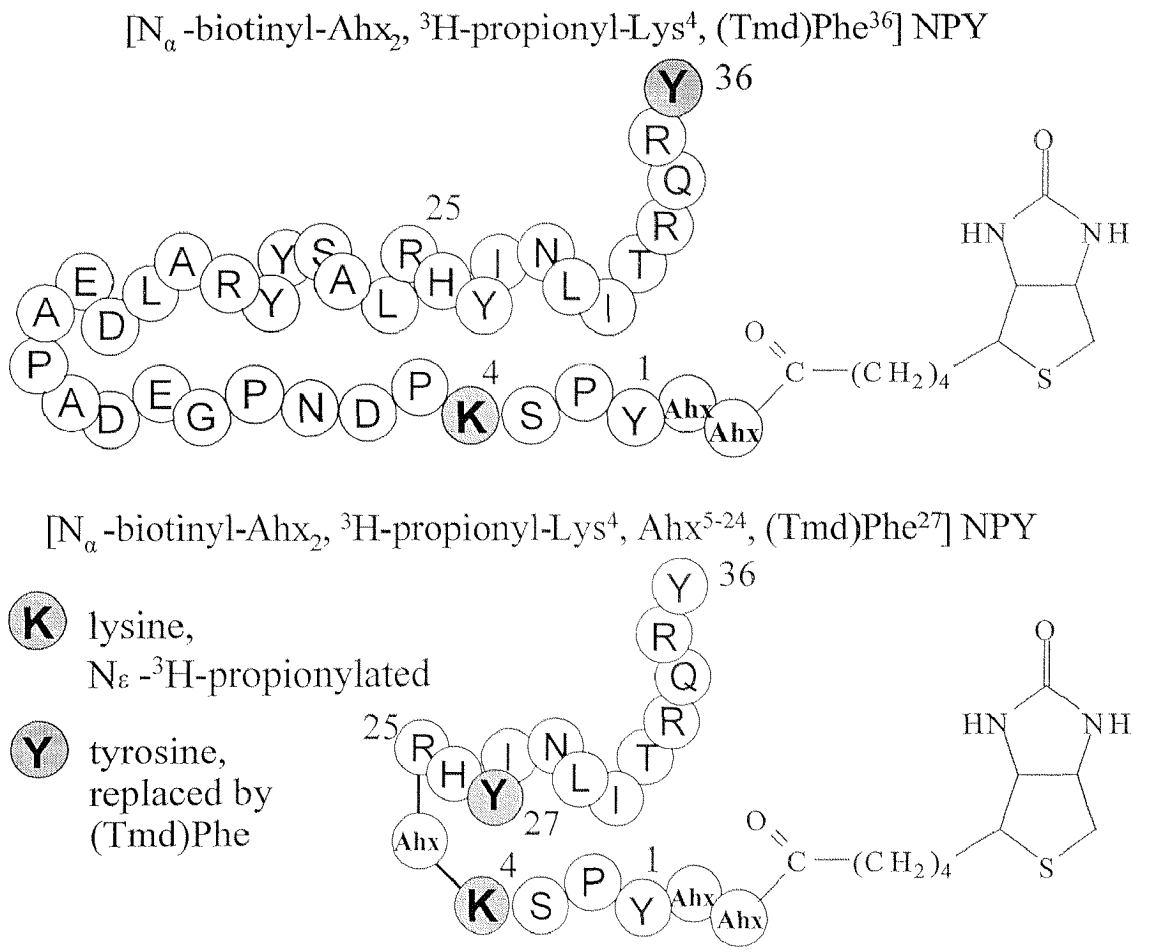

FIGURE 1: *Sequence and modified positions of the photoactivatable NPY analogues.*

Second, various studies have shown that antibodies produced against hormone receptors are valuable tools (24-27). Anti-receptor antisera can be raised by immunisation with purified, enriched receptors (24) or by immunisation with receptor fragments (25-27). Molecular mass determination by SDS-PAGE and subsequent Western blotting, receptor purification by affinity chromatography on antibody-columns (26) and investigations of the receptor localisation (28) are only a few applications of anti-receptor-antibodies (29). Antibodies specifically recognising the NPY receptor subtypes could be used to determine the localisation pattern and the quantity of the receptor protein expression as well as to determine the topology and function of a receptor subtype. In this study, we compared the molecular masses of human neuropeptide Y Y_2-receptors expressed endogenously in a human neuroblastoma cell line (SMS-KAN) and in Chinese hamster ovary cells (CHO-hY2) that have been transfected with human

Y_2-receptor cDNA (10). To increase the reliability of the results, molecular masses were determined by two independent methods, photoaffinity labeling and immunodetection.

5.3 Experimental Procedures

5.3.1 Peptide Synthesis

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

Tmd27: retention: 20,85 min; retention after irradiation: 18,11; $mass^{calc.}$: 2763,5 amu; $mass^{exp.}$: 2763,8 amu.
Tmd36: retention: 12,40 min; retention after irradiation: 11,29; $mass^{calc.}$: 4795,37 amu; $mass^{exp.}$: 4795,80 amu.
Synthetic fragment of second extracellular loop:

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

5.3.2 Peptide Modification

Tmd27 and Tmd36 were labeled with ^{3}H-succinimidyl-propionate (3,59 TBq/mmol; Amersham, Switzerland) at Lys^{4}. For each analogue 1 ml of ^{3}H-succinimidyl-propionate solution (37 MBq) in toluene was dried in a nitrogen stream and the analogue was added equimolar in 10 µl DMF. After addition of 0,1 % DIPEA in DMF the mixture was incubated for 20 h at darkness and room temperature. The complete reaction mixture was diluted in 1 ml 10 % acetonitrile/0.1 % trifluoroacetic acid and loaded onto a handpacked C-18-column (0,5 cm^3 bed volume), followed by washing with 5 ml 10 % acetonitrile/0.1 % trifluoroacetic acid. The peptides were eluted with 1 ml 60 % acetonitrile/0.1 % trifluoroacetic acid. Specific activity of ^{3}H-Tmd27 and ^{3}H-Tmd36 was 3,0 and 2,8 TBq/mmol, respectively.

5.3.3 Membrane Preparation and Receptor Binding

Expression cloning of the human Y_2-receptor was performed as described previously (10), cultivation of SMS-KAN cells and membrane preparation was performed according to (33). Displacement of ^{3}H-propionyl-NPY (3,18 TBq/mmol; Amersham, Switzerland) by NPY resulted in K_i = 0,67 nM for both cell lines (33). Binding of the tritiated photoactivatable analogues was assayed as follows. The membrane preparation was diluted in incubation buffer (MEM/25 mM Hepes, 1 % bovine serum albumin, 50 µM Pefabloc SC, 0.1 % bacitracin, 3.75 mM $CaCl_2$). 200 µl of the suspension containing 20 µg protein were incubated with 25 µl 8,3 nM solution of the analogues and 25 µl of solutions of NPY in increasing concentrations to give a total volume of 250 µl. After 1,5 h at room temperature, the incubation was terminated by centrifugation of the samples for 10 min at 3,000 × g and 4 °C. The pellets were washed with PBS, resuspended in PBS, mixed with scintillation cocktail and radioactivity was determined.

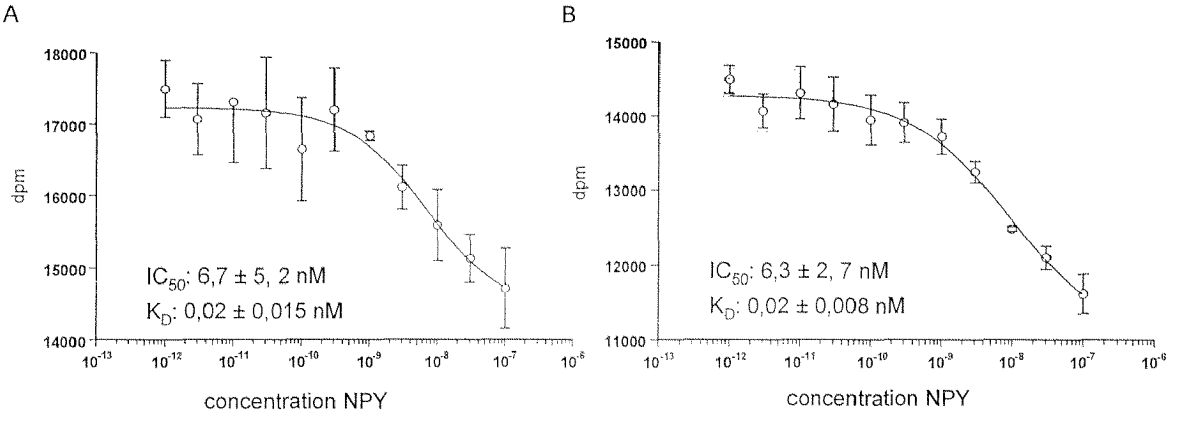

FIGURE 2: *Competition assay of [N_α-biotinyl-Ahx_2, ^{3}H-propionyl-Lys^4, Ahx$^{5-24}$, (Tmd)Phe^{27}] NPY (A) and [N_α-biotinyl-Ahx_2, ^{3}H-propionyl-Lys^4, (Tmd)Phe^{36}] NPY (B) against NPY at CHO-Y2 membranes. For details see Experimental Procedures.*

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

[N_α-biotinyl-Ahx_2, ^{3}H-propionyl-Lys^4, Ahx$^{5-24}$, (Tmd)Phe^{27}] NPY:

K_D = 0,02 ± 0,015 nM

[N_α-biotinyl-Ahx_2, ^{3}H-propionyl-Lys^4, (Tmd)Phe^{36}] NPY: K_D = 0,02 ± 0,008 nM

5.3.4 Photocrosslinking of Y_2-Receptor Containing Membranes

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

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

Specificity of photoaffinity labeling was shown by competition with different concentrations of NPY (Fig. 3) as follows. Membranes (50 µg) were incubated with the photoactivatable analogue (3 nM) and increasing concentrations of NPY (1 pM to 10 µM). After washing the probes were irradiated. A control set of probes was handled in parallel, but without irradiation. After irradiation and washing, all probes were incubated with NPY (10 µM) to remove receptor bound ligand which was not covalentely attached to the receptor. Bound radioactivity was determined after 3 washing steps. All determinations were performed in triplicate with mean values and errors given in Fig. 3.

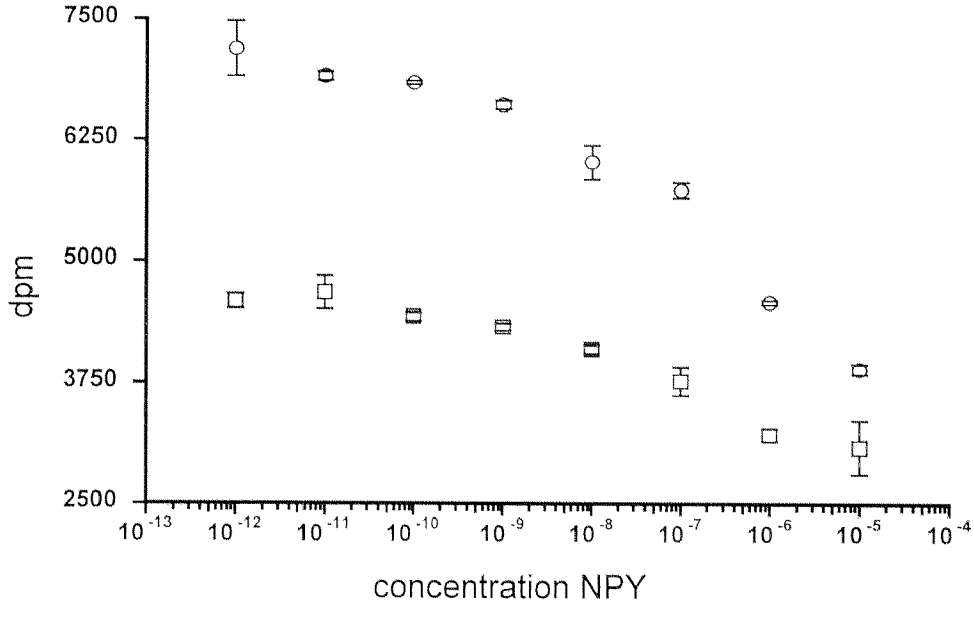

FIGURE 3. *Dose dependent inhibition of crosslinking at CHO-hY2 membranes with [N_{α}-biotinyl-Ahx_2, ^{3}H-propionyl-Lys^4, Ahx^{5-24}, (Tmd)Phe^{27}] NPY by NPY (circles). Control probes were handled in parallel without irradiation (squares). A second incubation with an*

excess of NPY after irradiation was used to remove receptor bound but not covalentely attached ligand. All determinations were performed in triplicate with mean values and errors given.

5.3.5 Preparation of the Conjugates and Immunisation

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

5.3.6 Immunoblot

Membranes from SMS-KAN- and CHO-hY2 cells were prepared by incubation at 95°C for 7 min in an urea sample buffer (8 M urea, 63 mM Tris/HCl pH 6.8, 2 % SDS, 5 % mercaptoethanol, 0.01 % bromophenol blue) and were separated on a 12 % polyacrylamide gel according to Lämmli (35). After blotting for 60 min (350 mA/20 V) in a semi-dry electrophoretic transfer cell (Biorad) onto nitrocellulose membrane in a transfer buffer containing 25 mM Tris/HCl pH 8.3, 150 mM glycine, 20 % methanol, the blots were blocked over night with TBS/Tween (0.5 % Tween 20 in TBS/HCl pH 7.4) containing 1 % BSA. The blots were incubated with antibodies for 90 min with gentle shaking, washed three times with TBS/Tween, incubated for 60 min with alkaline phosphatase-bound secondary antibodies, and washed three times again. Immunoreactivity was detected with 5-bromo-4-chloro-indolyl-phosphate (Sigma, Buchs, Switzerland). Control was performed with membranes from SMS-KAN-, CHO-hY2- and not transfected CHO-cells using anti-hY2-serum preincubated with the peptide against which the antibodies were raised and 0-sera (Fig. 5).

5.4 Results

5.4.1 Synthesis and Analysis of Photoactivatable NPY Analogues

Since NPY-analogues, containing Phe but not Trp are still recognised at the hY_2-receptor (38), we decided to use (Tmd)Phe instead of other photoactivatable amino acid of larger size as for example p-benzoylphenylalanine, which previously has been used for efficient photocrosslinking of other peptides (39, 40). Fmoc-(Tmd)Phe was coupled manually in twofold excess, all the subsequent cycles were performed using a peptide synthesiser. Preparation of Fmoc-(Tmd)Phe-OH as well as all synthesis, cleavage and purification steps were carried out in the absence of light. The peptide was characterised by means of analytical HPLC and electrospray mass spectrometry. In addition, a small sample of each peptide was dissolved in water and illuminated, which caused a change in the retention time as observed by HPLC. This result confirmed that the side chain of (Tmd)Phe remained intact during the remaining cycles of peptide synthesis after coupling of (Tmd)Phe. Tmd27 and Tmd36 were labeled successfully with ^{3}H-succinimidyl-propionate. Both peptides contained a single reactive amino group (sidechain of Lys^4) which facilitated the coupling of a single label per peptide. The labeled peptides, ^{3}H-Tmd27 and ^{3}H-Tmd36, showed a high specific activity of 3,0 and 2,8 TBq/mmol, respectively, which suggests a labeling efficiency of at least 90 %.

The binding of both NPY analogues to the Y_2-receptor was determined in a competition assay against NPY using the labeled analogues as radioactive tracer (Fig. 2). Although both peptides were modified at three positions (N-terminal spacering and biotinylation, propionylation at Lys^4 and exchange of Tyr against (Tmd)Phe in a single position), their binding properties at the Y_2-receptor were only slightly reduced compared to ^{3}H-propionyl-NPY used in the binding assay (^{3}H-propionyl-NPY: K_D = 0,018 ± 0,008 nM; ^{3}H-Tmd27: K_D = 0,02 ± 0,015 nM; ^{3}H-Tmd36: K_D = 0,02 ± 0,008 nM).

5.4.2 Photoaffinity Labeling

SMS-KAN- and CHO-hY2 membranes were crosslinked using the labeled photoactivatable NPY analogues ^{3}H-Tmd27 and ^{3}H-Tmd36. For each crosslinking experiment a probe was handled in parallel with the addition of 1 µM NPY as control. SDS-PAGE was performed subsequently, followed by cutting of the gels in 1,3 mm slices and determination of the radioactivity of each gel slice. Four sets of experiments have been performed. SMS-KAN membranes were crosslinked using ^{3}H-Tmd27 and ^{3}H-Tmd36, respectively, and the same experiments were processed with CHO-hY2 membranes. Two major radioactive protein bands (Fig. 4, Table 1) were found in all cases.

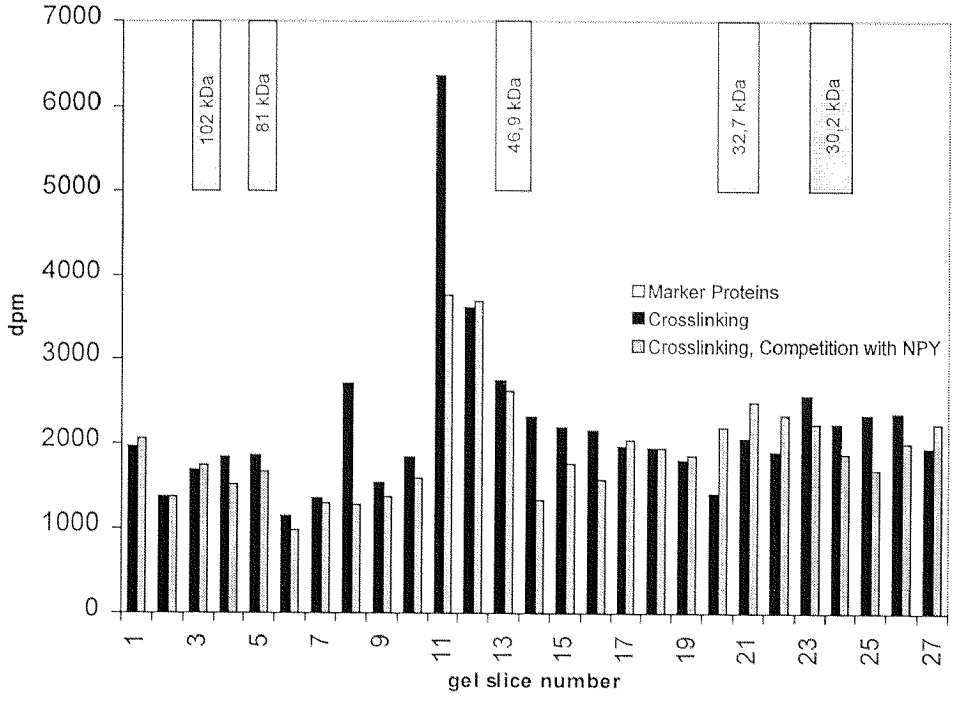

FIGURE 4: *Crosslinking experiment of [Nα-biotinyl-Ahx2, 3H-propionyl-Lys4, Ahx5-24, (Tmd)Phe27] NPY at CHO-Y2 membranes. Control was performed with an excess of NPY. After SDS-PAGE the gel was cut into 1,3 mm slices and radioactivity was measured. A set of marker proteins was run on the same gel. The numbers of the gel slices in which the marker proteins turned up are shown as dotted bars and were used to establish a calibration curve. The curve was used to determine the molecular mass range for the proteins covered by each gel slice.*

In parallel, a set of marker proteins always were run on the same gel. The numbers of the gel slices in which the marker proteins turned up were used to establish a calibration curve for each gel. The curves were used to determine the molecular mass range for the proteins covered by each gel slice. Depending on the calibration method the molecular masses obtained for the proteins varied up to 4 kDa. Molecular masses of the receptor proteins detected for SMS-KAN membranes were found to be 54 and 44 kDa using ^{3}H-Tmd27 for crosslinking and 53 and 44 kDa using ^{3}H-Tmd36. Photoaffinity labeling on CHO-hY2 membranes led to the identification of two proteins as well with molecular masses of 59 and 46 kDa with ^{3}H-Tmd27 and 58 and 46 kDa with ^{3}H-Tmd36. In some cases minor bands in the range of 30 kDa were observed, which we presume to represent partially degraded receptor proteins. Both sequence positions (Tyr^{27} and Tyr^{36}) exchanged against (Tmd)Phe allowed insertion of the photoactivatable group into the receptor protein upon irradiation at 366 nm. Specificity of photoaffinity labeling was shown by competition with different concentrations of NPY (Fig. 3). Using both analogues for crosslinking of SMS-KAN membranes, the molecular masses identified by SDS-PAGE (and subtracted by the mass of the photoactivatable NPY analogue used) correspond well in both cell lines. A slight difference was found between the two cell lines for the upper mass (54 versus 58 kDa) which most likely is due to difficulties in exact weight determination by SDS-PAGE.

5.4.3 Immunoblot

Membranes from SMS-KAN- and transfected CHO-hY2 cells were solubilised and proteins separated on a gel followed by Western blotting using the Y_2-receptor selective antibodies. Control was performed with membranes from SMS-KAN-, CHO-hY2- and not transfected CHO-cells using anti-hY2-serum preincubated with the peptide against which the antibodies were raised and 0-sera (Fig. 5). Again, two major protein bands were detected and the marker proteins were used to establish a calibration curve. The molecular masses observed for the Y_2-receptor expressed in SMS-KAN cells were approximately 58 kDa and 54 kDa. For CHO-hY2 membranes slightly lower masses of 57 kDa and 51 kDa were found (Fig. 5, Table 1). Depending on the calibration curve these masses varied up to 4 kDa. As

in the photoaffinity labeling experiments, weaker bands the range of 30-40 kDa were found, too, which are believed to represent degradation products of the receptor protein, and which are still recognised by the antibody.

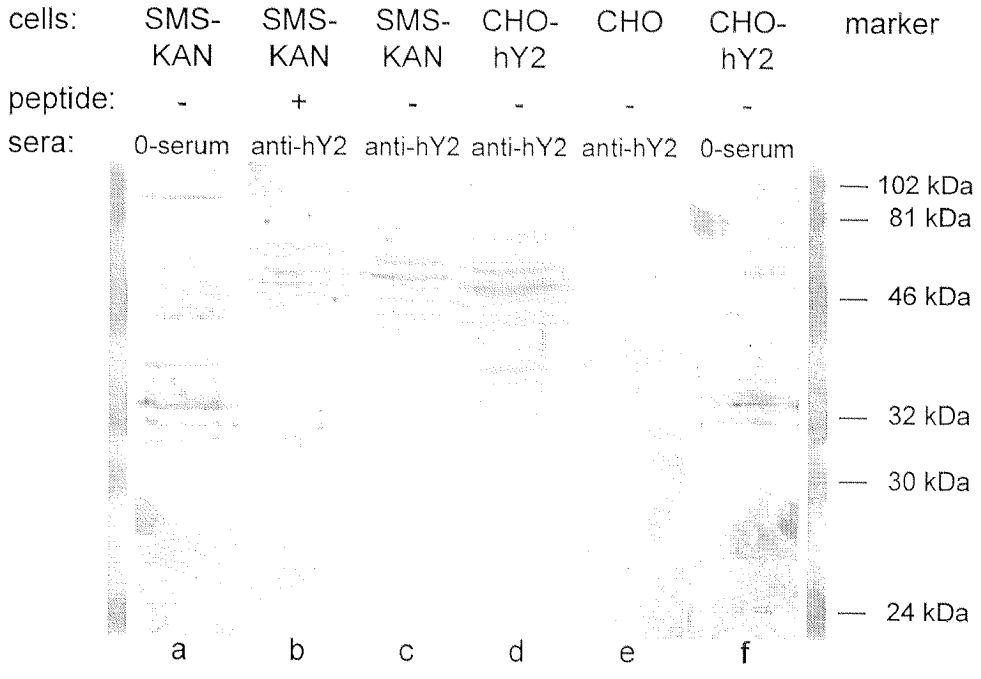

FIGURE. 5. *Western blot of solubilised SMS-KAN (lane c) and CHO-hY2 membranes (lane d). Specificity of the Y2-receptor recognition by the anti-Y2-receptor antibodies is shown by staining with 0-sera (lane a and f), anti-hY2-serum preincubated with the peptide against which the antibodies were raised (lane b) and not transfected CHO-cells (lane e). Staining was achieved using alkaline phosphatase conjugated secondary antibodies.*

5.5 Discussion

We successfully synthesised photoactivatable, tritium labeled analogues of NPY with high receptor affinity (^{3}H-Tmd27 and ^{3}H-Tmd36). Using these ligands, photoaffinity labeling of the NPY Y_2-receptor subtype expressed in SMS-KAN- and transfected CHO-hY2 cells identified two labeled proteins with molecular weights of 54/44 kDa and 59/46 kDa, respectively. Specificity of photoaffinity labeling was shown by competition with different concentrations of NPY (Fig. 3). Y_2-receptor subtype selective antibodies were raised by immunisation of rabbits with a synthetic fragment of the second extracellular loop of the Y_2-receptor and

subtype selectivity was shown by ELISA and Western blotting. These antibodies were used for Western blotting in order to confirm the molecular mass determination by photoaffinity labeling. Again, two proteins were identified with molecular weights of 58/54 kDa for SMS-KAN- and 57/51 kDa for transfected CHO-hY2 cells. The molecular weight determination is based on a mass calibration curve established for 5-6 marker proteins. The ratio of the migration of the proteins in the gel and their molecular masses is not linear. Thus a curve has to be fitted to the marker weights, which might cause differences in the molecular weights of the proteins detected, depending on the mathematical model used. In addition to the inherent inaccuracy of SDS-PAGE, the masses determined for the Y_2-receptor might vary up to 4 kDa. The masses identified by photoaffinity labeling with two different photoactivatable analogues are in good agreement for both cell lines. When compared to the mass determination via Western blotting, the upper masses identified are in good agreement for both cell lines as well, whereas the masses of the lower protein bands seem to differ slightly (Table 1).

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

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

This might be explained by the error in mass determination or the different experimental efforts for photoaffinity labeling and Western blotting and the

different detection methods that have been applied. Thus, the results of the molecular mass determination of the Y_2-receptor suggest the same protein masses of 58 and 50 kDa in two different cell lines. SMS-KAN cells are human neuroblastoma cells which are endogenously expressing the Y_2-receptor subtype, whereas CHO-hY2 cells have been transfected with human Y_2-receptor cDNA. The corresponding molecular masses suggest similar posttranslational modifications in both cell lines. When compared to the mass of the Y_2-receptor calculated from the sequence (42 kDa), a difference of up to 16 kDa caused by posttranslational processing was observed. Glycosylation alters the migration behavior of proteins in SDS-PAGE in a nonlinear way (41). This means that the exact increase in the mass of the Y_2-receptor caused by glycosylation cannot be determined exactly by SDS-PAGE, but our results suggest a remarkable glycosylation of the Y_2-receptor at the consensus sequence of the N-terminus. Preliminary deglycosylation experiments showed a decrease of the molecular masses after application of endoglycosidase F and peptide-N-glycosidase F. Therefore, the two different masses of the proteins identified in each cell line are most likely due to different glycosylation of the Y_2-receptor.

Previous studies using chemical crosslinking with PYY and NPY analogues in different cells or tissues revealed huge differences in the molecular masses for the Y_2-receptor. Accordingly, in rat hippocampus and rabbit kidney membranes the Y_2-receptor was identified as a glycoprotein of 50 kDa (42), which was found also for bovine (43), human (44) and porcine hippocampal membranes (45). The same molecular weight was identified in a renal proximal tubule cell line of mice (46), whereas molecular masses from 39 kDa up to 70 kDa were found in other tissues and species (47-50). These results suggest that NPY Y_2-receptor subtypes in hippocampal membranes from different species are conserved in their size and different from those in other tissues (43). The existence of at least two different Y_2-receptor subtypes, located centrally and peripherally, is suggested by Northern hybridization (51). Our approach for molecular mass determination of the Y_2-receptor subtype identified two glycoproteins of 58 and 50 kDa in SMS-KAN cells as well as in transfected CHO-hY2 cells, which are believed to represent the same protein with two different amounts of glycosylation. This approach included two different methods for molecular mass determination, specific crosslinking by

photoaffinity labeling and Western blotting using Y_2-receptor selective antibodies, which provides a high reliability of the masses determined.

Both photoactivatable analogues have been shown to allow the formation of a covalent ligand-receptor complex. In the putative receptor binding region the ligands were only slightly modified, which suggests, in combination with the observed high receptor affinity, a receptor binding mode similar to the native ligand. The biotin label of the photoactivatable analogues, as well as the subtype selective anti-receptor antibodies, will facilitate the purification of the covalent ligand-receptor complex. This will be used to identify the crosslinked positions of the receptor after enzymatic cleavage. Thus, the photoactivatable NPY analogues and anti-receptor antibodies described are valuable tools for identification of the receptor regions involved in ligand binding. Because of the Y_2-receptor subtype selectivity of ^{3}H-Tmd27 and the used antibody, this will be possible even in tissues in which other Y-receptor subtypes are present.

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5.7 Appendix

5.7.1 Deglycosylation of the Y_2-Receptor

In order to investigate the proportion of glycosylation at the Y_2-receptor, membranes were treated with endoglycosidase F and peptide-N-glycosidase F. These enzymes release intact N-linked oligosaccharides from glycoproteins. Endoglycosidase F hydrolyses β 1-4 linked di-N-acetylchitobiosein high mannose and hybrid N-glycans. Peptide-N-glycosydase F hydrolyses the aspartyl-glycosamine bond between asparagine and the proximal N-acetyl glucosamine of many N-glycans (Fig.1).

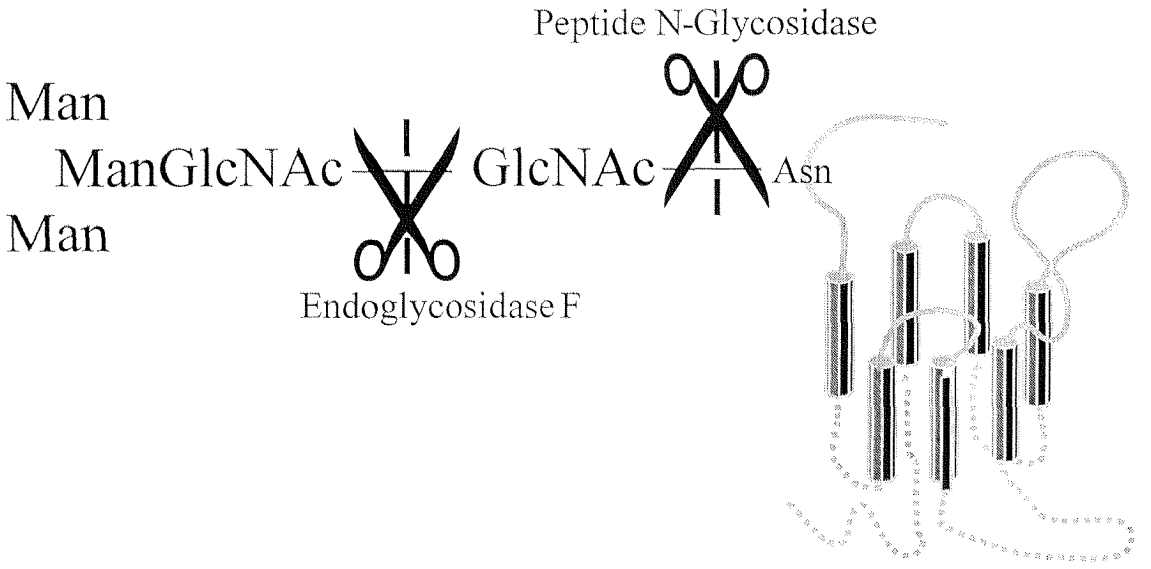

Figure 1: *Hydrolysation of glycosylated proteins by endoglycosidase F and peptide-N-glycosidase F.*

Membranes were centrifugated, washed twice with PBS and heated for two minutes to 95°C with 0.5 % SDS and 5 % β-mercaptoethanol in incubation buffer (100mM sodium phosphate, pH 7.5, 250 mM EDTA, 0.02 % sodium azide). To an aliquot of 10 µl membranes, 5 % Nonidet P-40 and 5 units peptide-N-glycosidase F were added and incubated for 20 h at 37 °C. Proteins were separated on a gel

followed by Western blotting. The receptor protein was stained using the Y_2-receptor selective antibodies (Fig. 2).

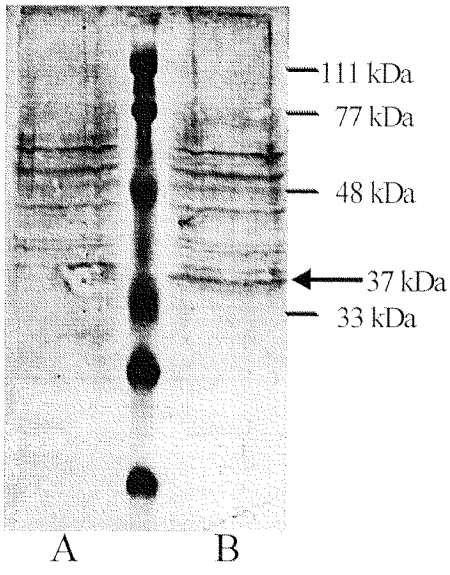

***Figure 2**: Deglycosylation of the Y_2-Receptor. A: CHO Y_2 (untreated) B: CHO Y_2 treated with peptide-N-glycosidase F. The blot was stained using the Y_2-receptor selective antibodies.*

The receptor protein bands at 57 kDa and 51 kDa appear weaker after deglycosylation . A new band appears at approximately 37 kDa (arrow). This is slightly below the mass of the Y_2-receptor calculated from the sequence (42 kDa). This might be explained by the inaccuracy of mass determination with gel electrophoresis or receptor degradation during the experimental procedure.

In order to reach complete deglycosylation, Y_2-receptor expressing CHO membranes were treated with a mixture of endoglycosidase F and peptide-N-glycosidase F (1:1). To purify the receptor, 325 µl membranes were seperated on a gel followed by Western blotting. The area between 48 kDa and 80 kDa of the blot was cut out and eluted with 40 % acetonitrile. The acetonitrile was removed and the sample was dialysed. For deglycosylation, the same experimental procedure as above was applied. A new band at approximately 37 kDa appeared whereas the receptor bands at 58 kDa and 50 kDa. disappeared completely (Fig. 3).

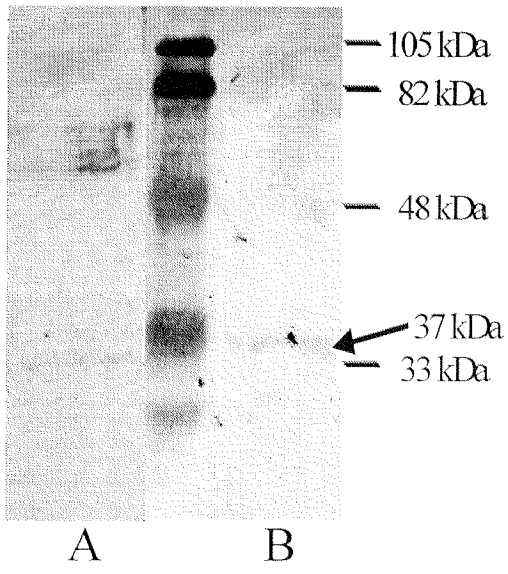

Figure 3: *Deglycosylation of the Y_2-Receptor. A: CHO Y_2 purified B: CHO Y_2 purified and treated with a mixture of endoglycosidase F and peptide-N-glycosidase F. The blot was stained using the Y_2-receptor selective antibodies.*

These results suggest that the Y_2-receptor sustains N-linked oligosaccharides. Peptide-N-glycosidase F is not able to completely remove the glycosylation which may be explained by the steric hinderance of the enzyme. Endoglycosidase F cuts the glycosylation distant to the receptor, which seems to be more efficient. Subsequently, the receptor becomes accessible to peptide N-glygosidase.

5.7.2 Solubilisation and Purification of the Y_2 receptor

To investigate the crosslinked positions of the receptor for identification of the receptor region that is involved in ligand binding, purification of the receptor or the covalent ligand-receptor complex is necessary. The Y_2-receptor selective antibodies allow affinity purification using columns with the antibodies covalently attached to a matrix or bound to a protein A column. Membrane proteins are not soluble, therefore the receptor must be solubilised with a suitable detergent before it can be applied to an immunoaffinity column.

Y_2-receptor containing CHO membranes were resuspended in HEPES-buffer (25 mM HEPES, 25 mM $CaCl_2$, 1mM $MgCl_2$, 0.1 % Bacitracin, 50 μM Pefabloc SC, pH 7.4), 1 % β-octyl glucoside was added. The samples were incubated for 1 h at

4 °C with shaking, centrifuged at 20,000 x g for 1 h at 4 °C. An aliquot of the supernatant was separated on a gel followed by Western blotting. The blot was stained using the Y_2-receptor selective antibodies (Fig. 4 A). The receptor bands at 57 kDa and 51 kDa were strongly stained, which means, that a large amount of receptor protein was solubilised.

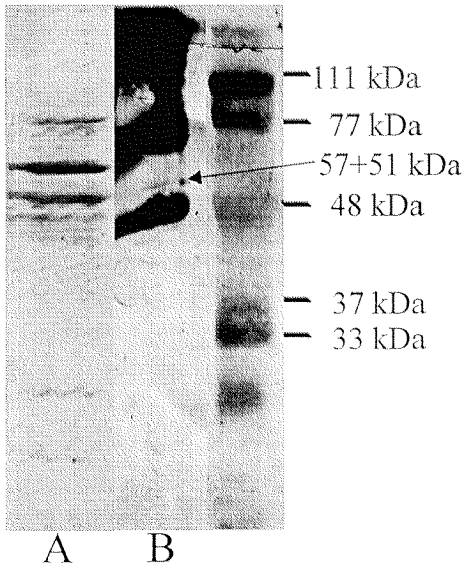

Figure 4: *Solubilisation and purification of the Y_2 receptor. A: Supernatant after treatment with β-octyl glycoside. B: Purification with protein A column.*

Y_2-receptor antisera was given to a protein A column. The column was flushed with Tris buffer (50 mM Tris, pH 7.0) until absorption at 280 nm was constant. Solubilised receptor was added and the column was flushed again until absorption at 280 nm was constant. The bound receptors were eluted with a glycine buffer (0.1 M glycine-HCl, pH 2.5). The fractions were collected in Tris buffer (1 M Tris-HCl, pH 8.0). Receptor containing fractions were pooled, concentrated and applied to a gel followed by Western blotting. The blot was stained using the Y_2-receptor selective antibodies (Fig. 4 B). The receptor bands at 57 kDa and 51 kDa are very weak between the intense stained band of the immunoglobulines (arrow). Since the antibodies are not covalentely attached to the protein A column, they are eluted with glycine buffer together with the receptor proteins. This mixture was separated by SDS-PAGE using reducing conditions (β-mercaptoethanol). On the subsequent blot the receptor proteins were detected with the Y_2-receptor selective

antibodies and stained with a secondary alkaline phosphatase linked anti IgG antibdody. This secondary antibody also stains the antibodies eluted from the column which causes the strong bands at approximately 50 kDa (heavy chain of the IgG molecule), at 75 kDa (partly reduced IgG molecule) and at 150 kDa (non reduced, whole IgG molecule). In a gel run under none reducing conditions the receptor bands would be better visible because only one IgG band at 150 kDa could appear. To avoid contamination with immunoglobulines, purification of the Y_2-receptor selective antibodies with ion exchange chromatography, followed by covalent immobilisation of the antibodies at cyanogen bromide-activated sepharose is recommended.

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Papers

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9/1995	diploma in chemistry, Swiss Federal Institute of Technology, Zürich, Switzerland
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