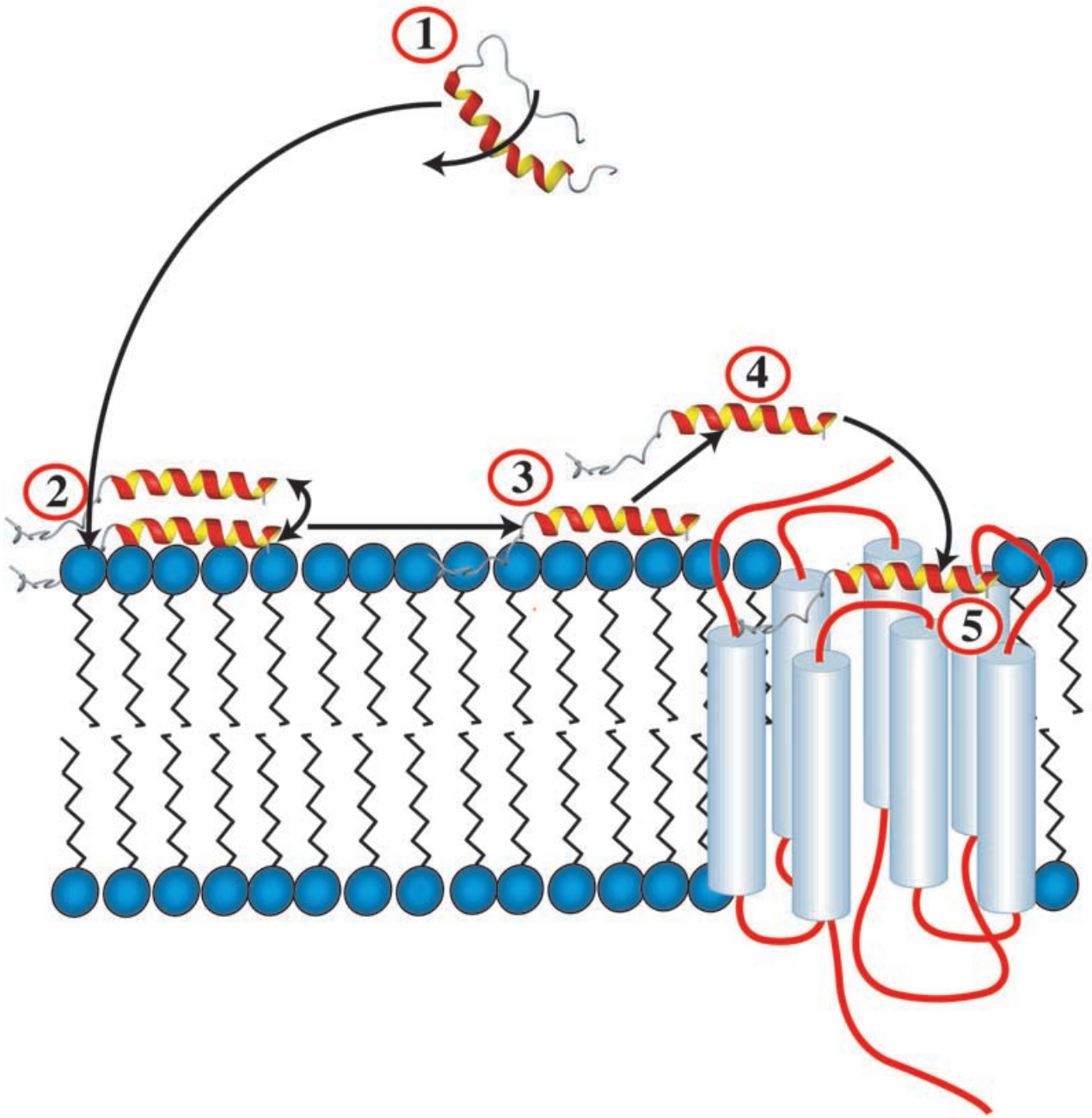
A pathway for receptor recognition by the hormones of the NPY family



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Are Hormones from the Neuropeptide Y Family Recognized by Their Receptors from the Membrane-Bound State?

Reto Bader[b] and Oliver Zerbe*[a]

Hormones and many other neurotransmitters, growth factors, odorant molecules, and light all present stimuli for a class of membrane-anchored receptors called G protein-coupled receptors (GPCRs). The GPCRs are the largest family of cell-surface receptors involved in signal transduction. About 1% of all known genes of Drosophila *and more than 5% of the genes of* Caenorhabditis elegans *encode GPCRs. In addition, more than 50% of current therapeutic agents on the market target these receptors. When the enormous biological and pharmaceutical importance of these receptors is considered, it is surprising how little is known about the mechanism with which these receptors recognize their natural ligands. In this review we present a structural approach, utilizing techniques of high-resolution NMR spectroscopy, to address the question of whether peptides from the neuropeptide Y family of neurohormones are recognized directly from solution or from the membrane-bound state. In our studies we discovered that the structures of the membrane-bound species are better correlated to the pharmacological properties of these peptides than the solution structures are. These findings are supported by the observation that many biophysical properties of these peptides seem to be optimized for membrane binding. We finally present a scenario of possible events during receptor recognition.*

1. Introduction

Almost twenty years ago Kaiser and Kezdy developed the concept that ligands that bind to membrane-embedded receptors recognize their targets from the membrane-bound state.[1] In their seminal paper they recognized that the active site of the receptor usually cannot accommodate more than five residues and that binding is mediated by only a few stereospecific interactions between the ligand and the receptor. Most hormones, however, comprise longer sequences than would be necessary to achieve these interactions. In fact, a lot of hormones and toxins contain more than 30 amino acids and many of those are unfolded in aqueous solution. Interestingly, these ligands contain amino acid sequences that fold into amphiphilic helices in a membrane-mimicking environment.[2] From this observation, Kaiser and Kezdy concluded that these amphiphilic stretches serve to anchor the hormones onto the membrane, from where they are subsequently recognized by their receptors. To verify their concept, Kaiser and Kezdy engineered a variant of apolipoprotein in which the amphiphilic sequence was changed to contain only Glu, Leu, and Lys and in which the residues that were thought to be involved in receptor binding were conserved. Despite little sequence homology to the wild type, this artificially designed peptide bound with high affinity to the receptor.[1]

Sargent and Schwyzer developed the ideas of Kaiser and Kezdy into the membrane-compartment concept.[3–7] Their model proposes that, for many ligands which target membrane-embedded receptors, membrane binding is an important event preceding receptor binding (Figure 1). Therefore, it is the membrane-bound conformation that is recognized initially by the receptor. Through membrane binding, the spatial search by the ligand for its receptor is restricted to (lateral) two-dimensional diffusion on the membrane surface. Moreover, conformations more closely related to the bioactive form

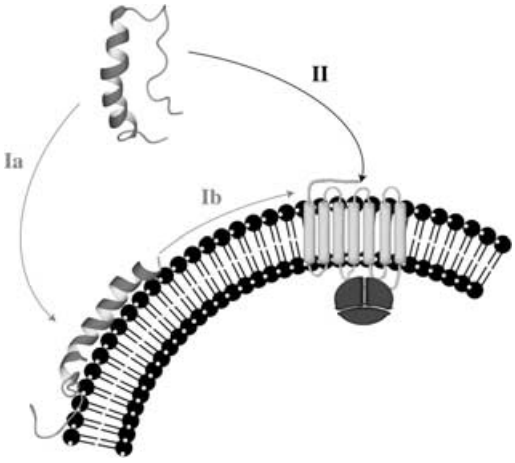

Figure 1. Schematic presentation of the two possible models for receptor recognition by membrane-embedded receptors: direct recognition of the peptides from solution (pathway II) or membrane association (Ia) followed by lateral diffusion to the receptor (Ib).

[a] *Priv.-Doz. Dr. O. Zerbe*
Institute of Organic Chemistry, University of Zürich
Winterthurerstrasse 190, 8057 Zürich (Switzerland)
Fax: (+41) 1-635-6833
E-mail: oliver.zerbe@oci.unizh.ch

[b] *Dr. R. Bader*
Present address:
Department of Chemistry, University of Cambridge
Lensfield Road, Cambridge CB2 1EW (UK)

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may be induced upon membrane binding which, in turn, may lower the energy barrier that needs to be overcome before the ligand can bind to the receptor. According to this idea, part of the peptide sequence is determined by membrane requirements and optimized for affinity to lipid bilayers. Moreover, this part also directs the ligand into the correct compartment, for example, to the membrane interface or into the membrane interior, and thereby influences the binding topology, which may be either surface-associated, integral, or a combination of the two. The topology-determining part of the molecule is called the *address*, whereas the part that contains residues directly involved in forming contacts to the receptor is called the *message*.

Schwyzer validated this concept through investigations of the receptor-subtype selectivity of ligands for neurokinin and opioid receptors.[4] He observed that the compartment of the membrane in which the ligands are positioned depends on the charge distribution in both the ligand and the extracellular domains of the receptors. Rather than providing exact shapes for lock-and-key fits, the purpose of the address region is to provide the ligands with the necessary charge and amphiphilicity required for the interaction with the membrane. This is consistent with the observation that conservative substitutions of amino acids in the address region do not have a major impact on receptor-binding affinity.

Early work with NMR spectroscopy on the structural changes induced by binding of hormones to membranes was performed by Deber and Behnam, who looked at the association of enkephalins to phospholipid micelles.[8] Milon et al. have studied the membrane-bound conformation of enkephalin by transferred NOE experiments.[9] A conformationally restricted analogue similar to the latter membrane-bound conformation had been synthesized earlier[10] and was shown to possess μ activity.

The work of Schwyzer was followed by a number of similar investigations, for example, those by Moroder et al.,[11] who developed hydrophobic extensions for ligands in order to increase their membrane affinity. It should be emphasized, however, that the concept of the membrane-bound pathway of receptor recognition as developed by Schwyzer does not exclude the possibility of conformational changes both in ligand and the receptor resulting from intermolecular contacts that are established during the recognition event (induced fit). By using transferred NOEs, Inooka et al. managed to determine the conformation of a fragment of pituitary adenylate cyclase activating polypeptide, PACAP(1–21), bound to a PACAP-specific receptor.[12] Comparisons to the micelle-bound states revealed a highly similar C-terminal helix, whereas the seven N-terminal residues that are thought to be responsible for specific receptor–ligand interactions were only structured in the receptor-bound state. Moreover, technical difficulties in the purification, reconstitution, and structural characterization of large membrane proteins have led to the development of reduced ligand/receptor systems, in which receptor fragments and their interactions with a particular ligand have been characterized structurally by NMR spectroscopy and molecular modeling.[13,14]

In this review we report on recent structural studies of peptides from the neuropeptide Y family of neurohormones aimed at deriving experimental data in favor or disfavor of a membrane-bound receptor recognition pathway. In our studies we have pursued a structural approach, in which we determine the conformations of various peptides both in solution and when bound to membrane-mimicking phospholipid micelles. Pharmacological data are reviewed in order to decide whether the structural data obtained in solution or in the micelle-bound state are better correlated to trends observed in receptor-binding affinities. During the last few years we have developed two major strategies. The first strategy is based on the assumption that a pair of peptides that possesses similar binding profiles for the receptor subtypes should display similar structures and dynamics in the particular environment from which the peptides are recognized.[15,16] In the second approach we have attempted to rationalize receptor-subtype specificity from structural features of the membrane-bound state.[17–19]

Oliver Zerbe was born in 1963 and studied chemistry at the University of Hamburg, Germany, and in Southampton, UK. He received his PhD in 1994 in organic chemistry from the University in Zürich under the supervision of Wolfgang von Philipsborn. From 1994–1995 he was a postdoctoral researcher working with Kurt Wüthrich at the Federal Technical University of Switzerland (ETH) in Zürich. He received his Habilitation in medicinal chemistry from the Institute of Pharmaceutical Sciences, ETH Zürich, in 2004 under the guidance of Gerd Folkers. Oliver Zerbe is presently the head of the NMR facilities of the Institute of Organic Chemistry of the University of Zürich. His research interests are centered on structures of membrane-associated biomolecules, especially aspects of the recognition of hormones by their receptors. His group uses primarily recombinant methods and protein expression but also solid-phase peptide synthesis to produce the peptides of interest and high-resolution NMR techniques to determine their structures.

Reto Bader was born in 1971. He studied biochemistry at the University of Bern, Switzerland. After further studies in mathematics he moved on to ETH Zürich, Switzerland, and obtained his PhD under the supervision of Oliver Zerbe, Annette Beck-Sickinger, and Gerd Folkers. In 2002 he started a postdoctoral project with Chris Dobson at the University of Cambridge, UK, on the nucleation-controlled mechanism of protein self-assembly as observed in amyloid fibril formation. His general research interests cover any aspect of interactions of proteins with their environment and their link with protein function.

We have structured this review along the following lines: With consideration to the importance of peptide–membrane interactions for the subject, we start with a brief review of recent biophysical literature from that field in Section 2. After introducing the peptides from the neuropeptide Y family and their receptors in Section 3, we describe in Section 4 the spectroscopic tools (mainly NMR techniques) for the characterization of the structure and dynamics of these peptides free in solution, when bound to phospholipid micelles, and during the transition between the two states. Thereafter, we summarize our present understanding of the structures in the two environments in Section 5. Finally in Section 6, we present a specific case, the comparison of neuropeptide Y and peptide YY, to illustrate how we use structural arguments to decide from which state these peptides are initially recognized. We close this review in Section 7 by suggesting a cascade of events for the binding of these peptides to their receptors.

Table 1. Free energies for transferring whole amino acids from bulk solution to the water–membrane interface (ΔG_{wif}) or to a hydrophobic environment (ΔG_{oct}).[a]

	ΔG_{wif}[b] [kcal mol^{-1}]	ΔG_{oct}[c] [kcal mol^{-1}]		ΔG_{wif}[b] [kcal mol^{-1}]	ΔG_{oct}[c] [kcal mol^{-1}]
Ala	0.17	0.50	Ile	−0.31	−1.12
Arg+	0.81	1.81	Leu	−0.56	−1.25
Asn	0.42	0.85	Lys+	0.99	2.80
Asp−	1.23	3.64	Met	−0.23	−0.67
Asp	−0.07	0.43	Phe	−1.13	−1.71
Cys	−0.24	−0.02	Pro	0.45	0.14
Gln	0.58	0.77	Ser	0.13	0.46
Glu−	2.02	3.63	Thr	0.14	0.25
Glu	−0.01	0.11	Trp	−1.85	−2.09
Gly	0.01	1.15	Tyr	−0.94	−0.71
His+	0.96	2.33	Val	0.07	−0.46
His	0.17	0.11			

[a] Taken from ref. [22]. [b] Interface scale. [c] Octanol scale.

2. Peptide/Protein–Membrane Interactions

Binding of molecules to the membrane surface may be due to electrostatic or multiple hydrophobic interactions. The balance between electrostatic and hydrophobic interactions for membrane association and/or membrane insertion has been documented for many membrane-binding peptides and proteins. The total binding energy is the sum of the electrostatic energy (ΔG_{es}) and the hydrophobic energy ($\Delta G_{H\varphi}$), with ΔG_{es} calculated as given in Equation (1) from the effective charge (z_{eff}) of the protein and the membrane surface potential (Φ_0) multiplied by the Faraday constant (F; see, for example, ref. [20], and references cited therein).

$$\Delta G_{es} = z_{eff} F \Phi_0 \quad (1)$$

The second term, $\Delta G_{H\varphi}$, includes all other (nonpolar) interactions and is defined by Equation (2), in which σ_{NP} is the so-called solvation parameter and A_{NP} denotes the nonpolar accessible surface area.

$$\Delta G_{H\varphi} = -\sigma_{NP} A_{NP} \quad (2)$$

Whereas the total energy (ΔG_{tot}) is a quantity that can be derived experimentally, for example, from partition coefficients measured in equilibrium dialysis, separation into the electrostatic and nonelectrostatic components is far from trivial.

White and Wimley have measured thermodynamic data for the free energies required to transfer whole amino acid residues from the bulk water phase into the water–membrane interface or into the hydrophobic interior of membranes (Table 1).[21,22]

Interestingly, the side chains of the aromatic residues Trp and Tyr display the most favorable energies. Although Leu and Ile also partition favorably into the water–membrane interface, the free energies for insertion of these residues into the hydrophobic interior are more negative, an observation that is also true for Phe. Hence, Tyr and Trp seem to be particularly suitable for promoting association of peptides with membrane surfaces. This is supported by the empirical observation that these residues (but not Phe!) are frequently found at interfacial positions along membrane-spanning proteins.[23]

Killian and co-workers have reviewed how proteins adapt to the water–membrane interface.[24,25] Apart from the preference of Trp and Tyr for the interface, they have also observed a "snorkeling" behavior of Lys and Arg residues in transmembrane peptides,[26] in which the aliphatic side chains are oriented such that the charged end groups reach the aqueous phase. Therefore, Arg and Lys may be found at various positions in the membrane interior, provided that they are located not too far away from the membrane interface. The introduction of Asp or Glu residues is more likely to result in changed orientations of the peptide backbone relative to the membrane interface.[27] Although Arg/Lys and Trp residues are both found at the interface, Trp is mostly placed on the trans side of the membrane interface (in the aqueous phase) where it may act as a flexible anchor, whereas Lys is found at the cis side, an observation that is consistent with the "positive-inside" rule.[23] A decisive role for electrostatic interactions was also found by Bechinger for the case of a model peptide consisting of Leu, Ala, and His residues arranged in a periodic pattern.[28] Depending on the pH value, the His residues are charged, resulting in an amphiphatic helix that was shown by ^{15}N solid-state NMR spectroscopy to bind to the surface of a bilayer. Removal of the charge of the His residues by increasing the pH value changed the direction of the helix by 90°, thereby resulting in a transmembrane arrangement.

Ladokhin and White observed linear correlations between surface potential and the ΔG_{obs} value.[20] Their results from a set of peptides with an identical number of charged residues but with different hydrophobicities revealed that the effective charge of the peptide depends on the hydrophobicity of the peptide. As a rule of thumb they supposed that the effective charge of cationic peptides, z_{eff}, is reduced by about 20% for each increase of 3 kcal mol^{-1} in the hydrophobic energy term, $\Delta G_{H\varphi}$. They concluded that electrostatic and hydrophobic inter-

actions contribute in an *additive* manner to the overall interaction energy only in the *absence* of significant hydrophobicity.

The relative importance of electrostatic and hydrophobic interactions in the binding of peptides and proteins to membrane bilayers has recently also been investigated by using surface plasmon resonance analysis (BiaCore)[29] and by affinity chromatography towards immobilized phospholipids[30] for the case of mellitin and for the C-terminal extracellular domain of a GPCR, the angiotensin II receptor. The data clearly show that the binding of peptides containing basic residues is stronger to negatively charged dimyritoylphosphatidyl glycerol (DMPG) phospholipids than binding to neutral dimyritoylphosphatidyl choline (DMPC). Moreover, two-step kinetics were observed, which were interpreted in terms of a rapid association with the membrane due to electrostatic interactions, followed by a slower event comprising reorientation of the peptide at the interface concomitant with insertion of hydrophobic side chains into the membrane core.

3. A Short Survey of Structural and Functional Aspects of Peptides from the Neuropeptide Y Family

3.1. The neuropeptide Y family of neuroendocrine peptides and their receptors

The neuropeptide Y (neuropeptide tyrosine, NPY) family includes NPY and the two peptide hormones peptide YY (PYY) and pancreatic polypeptide (PP). They are C-terminally amidated 36 amino acid peptides and their various physiological effects include vasoconstriction, stimulation of food intake, intestinal functions, regulation of circadian rhythms, and the release of pituitary sex hormones among others.[31] NPY and PYY are found in all vertebrates and their primary structures are highly conserved. NPY is widely distributed in the peripheral and central nervous systems and it is one of the most abundant neuropeptides in the brains of vertebrates. PYY displays predominantly gut endocrine expression in mammals, whereas lower vertebrates also have prominent neuronal expression.[32] PYY and NPY resemble each other more closely than they resemble PP. PP is found only in the pancreas of tetrapods and it has been suggested that it arose as a gene-duplication product of the PYY gene in early tetrapod evolution.[33] PP is one of the most rapidly evolving neuroendocrine peptides known.[32] Among all the sequences of peptides classified as members of the NPY family, the residues in only seven positions are constant among all species; these are Pro5, Pro8, Gly9, Ala12, Tyr27, Arg33, and Arg35 (Table 2). Residues Pro2, Tyr20, Thr32, and Tyr36 are also highly conserved.[34]

Five receptors that bind NPY-family peptides can be identified genetically and have been characterized pharmacologically. They are denoted Y1, Y2, Y4, Y5, and y6 and they belong to the rhodopsin-like superfamily (class 1) of G protein-coupled receptors. For a review of the physiological roles of NPY receptor subtypes, see ref. [35]. Briefly, most of the vascular effects of NPY and many of the psychological functions of NPY (for example, anxiolysis) are signaled mainly through the Y1 receptor.

Table 2. Sequences of porcine PYY (pPYY), porcine NPY (pNPY), and bovine PP (bPP).[a]

pPYY	YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY
pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY
bPP	APLEPEYPGDNATPEQMAQYAAELRRYINMLTRPRY

[a] Residues conserved among different species are underlined.

This receptor is distributed in various tissues including the brain, kidney, heart, and gastrointestinal tract. Although Y1 antagonists can inhibit NPY-induced feeding, the role of Y1 in the regulation of food intake is still controversial. Other than Y1, the Y5 receptor subtype also seems to play an important role in NPY-induced feeding, as suggested by studies involving antisense knock-down, knock-out animals, and Y5-selective agonists. The Y2 receptor is thought to be responsible for the presynaptic inhibition of neurotransmitter release by acting as an autoreceptor, which may explain the partial opposing relationship between Y1 and Y2 with regard to blood pressure, anxiety, and food intake. Accordingly, the Y2 receptor is expressed in various parts of the central nervous system, in the intestine, and in certain blood vessels. The Y4 receptor is also known as the PP-preferring receptor because it is the only receptor subtype at which PP binds with a higher affinity than NPY or PYY. Hence, it is mainly expressed in peripheral tissues including the colon, intestine, prostate, and pancreas and it is indeed likely that the Y4 receptor subtype may mediate many of the gastrointestinal effects produced by PP. The gene for the y6 receptor seems to have become nonfunctional during evolution due to a nonsense frameshift mutation in the third extracellular loop. Once the Y receptors had been cloned and functionally expressed in mammalian cell lines, it became possible to perform ligand-binding studies, which led to the development of potent and selective agonists and antagonists for the different receptor subtypes. The essential segments of NPY for receptor recognition were determined by using C- and N-terminal-truncated analogues.[36–38] The significance of each residue was systematically assessed by single exchange with L-Ala or the corresponding D isomer.[39,40] Moreover, single and multiple substitutions of important residues with both natural and unnatural amino acids and the introduction of conformational constraints by means of special amino acid units, spacer templates, or cyclizations followed by affinity measurements with these compounds resulted in a huge amount of affinity data for NPY analogues at the different receptor subtypes. Some of the key findings (as discussed more extensively by Cabrele and Beck-Sickinger[41]) shall be summarized briefly:

1. The C-terminal part of the ligand is of particular importance for high affinity and receptor activation in all four subtypes investigated. Firstly, complete loss of affinity of NPY at the Y1 receptor was reported when the C-terminal amide group at Tyr36 was substituted by the free carboxylic group. This indicated that a negatively charged C terminus is not tolerated at this and presumably all other Y receptors. Secondly, replacement of Arg35 or Arg33 by Ala

abolished binding of NPY at all Y receptors, a result suggesting that electrostatics may play a decisive role in Y receptor recognition.[38] Thirdly, the introduction of the dipeptide Ala–Aib (Aib = aminoisobutyric acid) at positions 31 and 32 (usually Ile31–Thr32) of NPY led to high selectivity towards the Y5 receptor subtype.[42]

2. The Y2 receptor is the only Y receptor that binds N-terminally truncated fragments of NPY and PYY with wild-type-like affinity. Even short fragments (13–36, 18–36, and 22–36) bind to the Y2 receptor with subnanomolar affinity. By contrast, N-terminal segments in the absence of the C-terminal part are completely inactive at all receptor subtypes.
3. Single substitutions of central positions of NPY by Ala are generally not associated with dramatic changes in the binding affinities at the Y receptors. Multiple substitutions introduced by swapping of the porcine NPY (pNPY) segment 19–23 (RYYSA) and the corresponding human PP (hPP) sequence (QYAAD), however, modulated the binding profiles at the various receptor subtypes remarkably.[43]

Table 3 summarizes some IC_{50} values at receptor subtypes Y1, Y2, Y4, and Y5 for the peptides that are most relevant to the discussion of the structural results presented in this review.

Table 3. Inhibitory constants [nM] of peptides of the NPY family at Y receptor subtypes Y1, Y2, Y4, and Y5.

Peptide	Y1	Y2	Y4	Y5	Type[a]	Ref.
human NPY	0.81	0.016	1.9	0.19	K_i	[81]
human PP	>1000	>2000	0.04	58	IC_{50}	[43]
human PYY	1.1	0.012	1.06	0.62	K_i	[81]
[Ala31, Aib32]-pNPY	>700	>500	>1000	6.0	IC_{50}	[41]
[Ala31, Pro32]-pNPY	>1000	666	>1000	118	IC_{50}	[41]
PYY(3–36)	760	0.03	15	17	IC_{50}	[41]

[a] Type of inhibitory constant quoted. K_i = inhibition constant, IC_{50} = concentration at which 50% inhibition occurs.

We believe that this class of peptides is particularly attractive for the examinination of structure–activity relationships and for the investigation of potential correlations with the membrane-bound state, because these peptides show significantly different affinity profiles at the various Y receptor subtypes.

Complementary ligand-binding studies were carried out by using a series of mutants of the human Y1 receptor, in which Asp and Glu residues located in putative extracellular domains were systematically replaced by Ala residues. In contrast to mutations in the N-terminal domain, substitution of acidic residues present in the three extracellular loops resulted in proteins unable to bind NPY.[44] In an effort to describe the interaction of NPY with the human Y1 receptor more generally, Sautel et al. found a cluster of residues that could not be mutated to Ala without significantly reducing the binding of NPY.[45] A molecular model of the interaction between NPY and elements of a hydrophobic pocket of the Y1 receptor formed by the receptor residues F286, H298 and Y100 surrounding the C-terminal pentapeptide of NPY was proposed.[46] A more complete model of the full human Y1 receptor, with the orientation of the transmembrane helices derived from a projection map of bovine rhodopsin, was later used to dock various NPY-family peptides such that best accordance with the site-directed mutagenesis studies was achieved. It was proposed that ligand binding is initiated by electrostatic interactions between highly positive regions in the N- and C-terminal parts of the peptides and a negative region in the extracellular receptor domains.[47]

3.2. The solution structures of peptides from the NPY family

Structural studies of NPY and NPY analogues that may be related to receptor-subtype specificity are important for the rational design of potent agonists or antagonists and a deeper understanding of the mechanisms underlying receptor recognition and possibly activation. Although the receptor-bound conformations of the ligand are expected to vary from one receptor subtype to another (due to induced fit), it is nevertheless likely that residual structure, present either in aqueous solution or when bound to a membrane, will reveal structural features that are relevant in the initial receptor recognition process, as outlined below.

The molecular conformation of NPY, either in pure water or in the presence of organic solvents like trifluoroethanol (TFE), hexafluoroisopropanol, and even dimethylsulfoxide (DMSO), has been discussed with controversy in the literature for decades. The presented tertiary structures can essentially be grouped into two classes of structural models. The first class is composed of models that closely resemble the tertiary fold derived from the crystal structure of dimeric avian PP (aPP).[48] It is characterized by an α helix involving residues 14–31 connected through a β turn to an N-terminal polyproline II helix and is referred to as the PP fold. The PP fold was later confirmed by the NMR-derived structure of bovine PP to exist also in solution.[49] Interestingly, the solution structure of monomeric PYY as determined by NMR spectroscopy reveals a PP-fold-type structure too.[50] NMR data that resulted in PP-fold models for NPY and NPY analogues were collected in water[51] and in DMSO.[52,53]

The second class comprises structures based on NMR data of NPY in water and in TFE. In this type of model, the N-terminal tail is fully flexible, whereas residues 11–36 in water[54–56] and residues 19–34 in TFE are in an α-helical conformation.[57] Figure 2 displays the conformer bundles derived from the solution structures of PYY and NPY as computed from NMR data. PYY adopts a typical PP-fold-type structure, whereas the N terminus of NPY is fully disordered.

Moreover, the data obtained in TFE were indicative of an NPY monomer. On the other hand, Cowley et al.[55] and Monks et al.[56] observed intermolecular NOEs for NPY in aqueous solution whose origin could only be explained by a dimeric model in which the two NPY molecules interact through side chains of their α helices and are aligned in the antiparallel orientation. However, the two authors reported different sets of intermo-

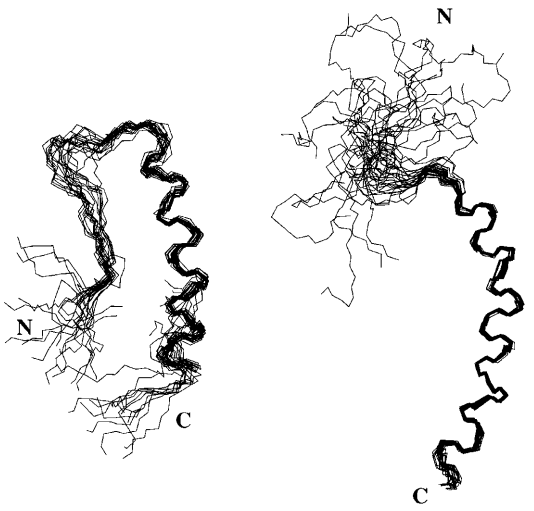

Figure 2. Bundle of the NMR-derived conformers of PYY (left; PDB code: 1RU5)[16] and NPY (right; PDB code: 1RON)[56] superimposed for best fit of the backbone atoms of residues 17–31 (NPY and PYY) and 4–8 (PYY).

lecular NOEs and therefore also proposed different models to fulfil the derived distance restraints. Based on investigations by CD spectroscopy, Nordmann et al.[58] suggested that the PP-fold conformation might exist at physiological concentrations whereas the dimer is the most abundant form at the concentrations needed for NMR studies. This is in agreement with the dissociation constant (K_d), which was determined to be 1.6 μM.[55] Nordmann postulated that dimerization is accompanied by the unfolding of the polyproline helix. Recently, Bettio et al. synthesized fluorescence-labeled analogues of NPY and showed by fluorescence-energy transfer measurements that there is no evidence for the presence of a hairpin structure at lower protein concentrations (10 μM), where the monomeric species should be more highly populated than in NMR studies at high protein concentration.[59]

Several authors dealt with the conformation of NPY fragments. N-terminal segments comprising the proline-rich region are unstructured and biologically inactive.[60] By contrast, the Y2-subtype-specific C-terminal NPY fragment NPY(13–36) is completely α-helical and monomeric in 30% hexafluoroisopropanol.[61] Other authors have investigated mutants of truncated or cyclized versions of NPY.[62–66]

Keire et al. also estimated from CD and NMR spectra the helical content of PYY and the highly Y2-subtype-specific PYY fragment PYY(3–36).[67] The calculated helicity for PYY is 42% and for PYY(3–36) is only 23%, a result showing that the removal of two N-terminal amino acids resulted in major conformational alterations in solution; this observation was confirmed by our own dynamics data on PYY(3–36) (see below). Interestingly, nearly the same value of helicity was reported for the much shorter PYY fragment PYY(13–36).[68]

4. A Toolbox for Structural Studies

4.1. Solution structures

Solution structures of peptides from the NPY family are determined by the methodology developed by Wüthrich and coworkers based on the sequential resonance-assignment method[69] and this method will not be described in more detail. Contacts between protons of the interface in the back-folded form are due to (hydrophobic) contacts of side-chain protons. However, this method requires complete resonance assignment and refinement up to a stage at which no more doubt remains about the presence of these long-range contacts. Another complication during spectral analysis arises from the fact that these molecules mostly form dimers (see above) and, hence, intra- and intermolecular NOEs need to be distinguished. While this is feasible in most cases, it is a much more elaborate process requiring a substantial amount of time when a larger number of mutants could be screened. Last but not least, the spectral regions in which these side-chain NOEs are found are often very crowded and unambiguous peak assignment or proper integration may be difficult. In our studies we have therefore pursued a different approach, in which we use internal-backbone dynamics to distinguish between back-folded and non-back-folded forms (Figure 3). While this method requires peptides that are uniformly ^{15}N-labeled, it additionally allows us to quantify how *rigidly* a peptide is back-folded. We have often conducted a more detailed Lipari–Szabo type analysis[70,71] of ^{15}N-*R*1,*R*2 and ^{15}N{^{1}H}-NOE data in order to derive the generalized order parameter S^2,[16–19] but we found that the ^{15}N{^{1}H}-NOE itself is usually sufficient to quantify the extent to which the peptide chain is back-folded. We would like to emphasize here that the ^{15}N{^{1}H}-NOE does not provide sufficient information to determine tertiary structure in general, but in our specific case, where the type of tertiary structures to be expected is evident (and supported by a large number of structures that we and others determined in the usual way), it is a reliable and fast method to define the extent of back-folding.

The peptides of the NPY family mostly exist in dimeric form at the concentrations used for NMR spectroscopy. It is difficult to establish the topology of dimerization, firstly because intra- and intermolecular NOEs need to be distinguished but also because the lifetime of the complex is short and hence interpretation of spectra from differential labeling is complicated. We have introduced a spin label into the molecule to resolve this problem.[17] Gln34 in NPY was replaced by the spin-label-containing amino acid 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl-4-carboxyl (TOAC), which was developed by Toniolo et al.[72] (It is essentially the spin label 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) derivatized in the form of an amino acid.) We mixed a large excess of nonlabeled TOAC-NPY with uniformly ^{15}N-labeled but otherwise nonmodified NPY so that ^{15}N-NPY exists to large extent as a heterodimeric complex with the spin-label-bearing TOAC-NPY. Signal reductions in the ^{15}N,^{1}H-correlation map due to the distance-dependent paramagnetic-relaxation-promoting effect of the spin label in the dimer were observed

at specific sites along the peptide sequence (Figure 4). It should be noted that the probe (the ^{15}N nuclei) and the spin label are parts of different molecules and that all signal attenuation effects stem from *intermolecular* effects within the heterodimeric complex, irrespectively of whether the dimer and the monomer are in equilibrium; data interpretation is therefore straightforward.

Attenuation of signal intensities is most pronounced around residues Ala14 and Ile28/Thr32 (Figure 4). The simultaneous proximity of the spin label to both sites is not compatible with the models for dimer arrangements published in the past[55,56] and we concluded from the data that both arrangements, the parallel and the antiparallel-helix alignment, are present.

4.2. Characterization of the peptides in their micelle-bound form

Structures of peptides in the micelle-bound form are also determined by using established methods[69] based on the use of distance restraints derived from proton–proton NOEs. In particular, we have utilized an NOE-relayed [^{15}N,^{1}H]-HSQC experiment to assign the ^{15}N,^{1}H-correlation map in the helical segment. Again, the $^{15}N\{^{1}H\}$-NOE was very useful for monitoring the changes in internal-backbone dynamics that occur upon binding of these peptides to the micelles. A convenient feature of the spectra of these peptides when bound to dodecylphosphocholine (DPC) micelles over those recorded in the absence of micelles is that signal dispersion is better; NOEs are comparably strong and minor peaks due to conformational heterogeneity resulting from the presence of Xxx-Pro cis forms are observed to a much lesser extent.

The micelle-binding interface was determined based on [^{15}N,^{1}H]-HSQC spectra recorded in the presence of the micelle-integrating spin label 5-doxylstearate. Figure 5 displays data for the Y5-receptor-selective NPY derivative [Ala31,Pro32]-NPY as compared to those of wild-type NPY.[18]

Strong signal reductions are observed in the C-terminal half of the peptides. In this segment, attenuations follow a 3–4 residue periodicity, thereby indicating that the helical segment is positioned *parallel* to the micelle surface. In addition, attenuations steadily decrease towards the N terminus, a fact supporting the view that the N terminus in these peptides diffuses freely in solution. The positions that are part of secondary structure elements (as determined by NOEs) can also be identified readily by characteristic values of the $^{15}N\{^{1}H\}$-NOE. Comparison of the heteronuclear NOEs at the C termini of [Ala31,Pro32]-NPY and NPY indicates that the loop in the segment comprising residues Asn29–Tyr36 is more flexible in the mutant than in the wild-type peptide. Signal attenuations resulting from proximity to the membrane-integrated

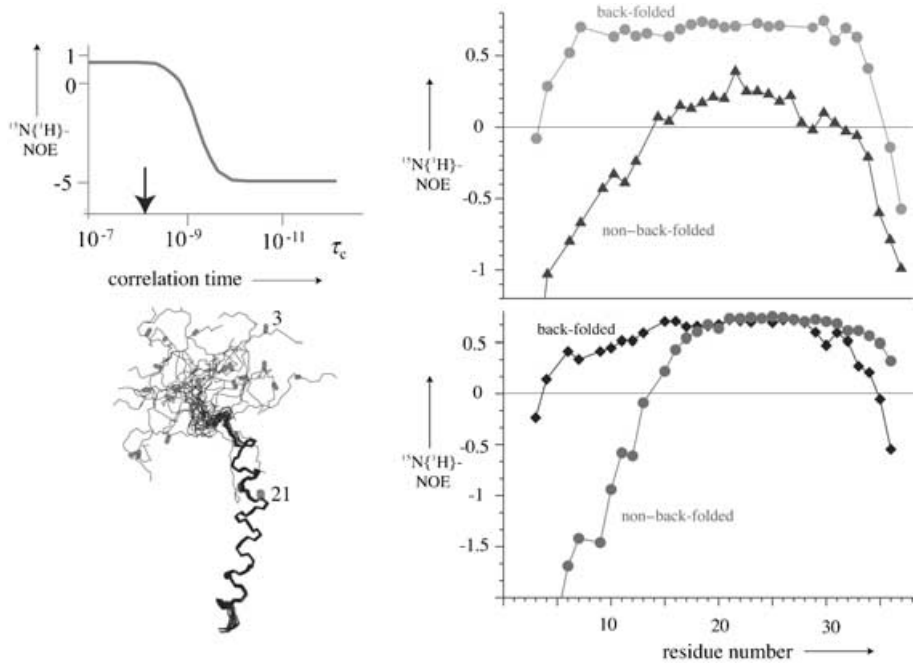

Figure 3. Dependence of the $^{15}N\{^{1}H\}$-NOEs on the correlation time of the N–H bond vector. A typical value for the overall correlation time, τ_c, for a peptide from the NPY family is indicated by an arrow (top left). Positions of N–H bond vectors of residue 21 ($S^2=0.89$) and residue 3 ($S^2=0.19$) are shown in bold in the superimposed conformer bundle of NPY (bottom left). Top right: the $^{15}N\{^{1}H\}$-NOE is displayed for PP (●) and NPY (▲). Bottom right: the $^{15}N\{^{1}H\}$-NOE is displayed for PYY in solution (◆) and when bound to DPC micelles (●).

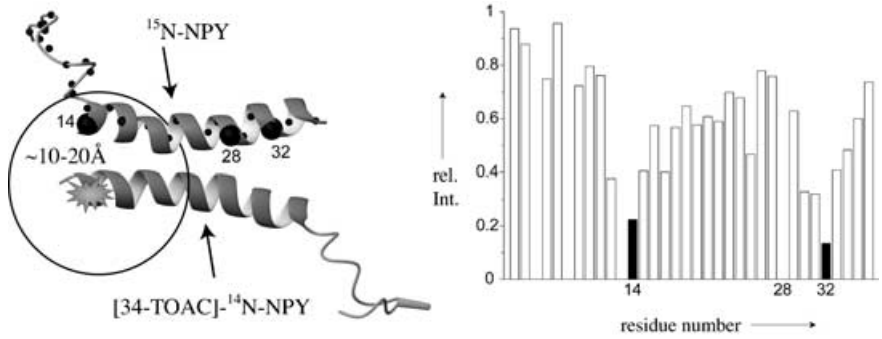

Figure 4. Left: schematic presentation of the complex formed by (^{14}N)-TOAC-NPY and unifomly ^{15}N-labeled NPY. Right: the relative signal intensities ($I_{spin\text{-}label}/I_{no\text{-}spin\text{-}label}$) as derived from the [$^{15}N$,$^{1}H$]-HSQC correlation experiment. Positions of strongest attenuations are marked in black.

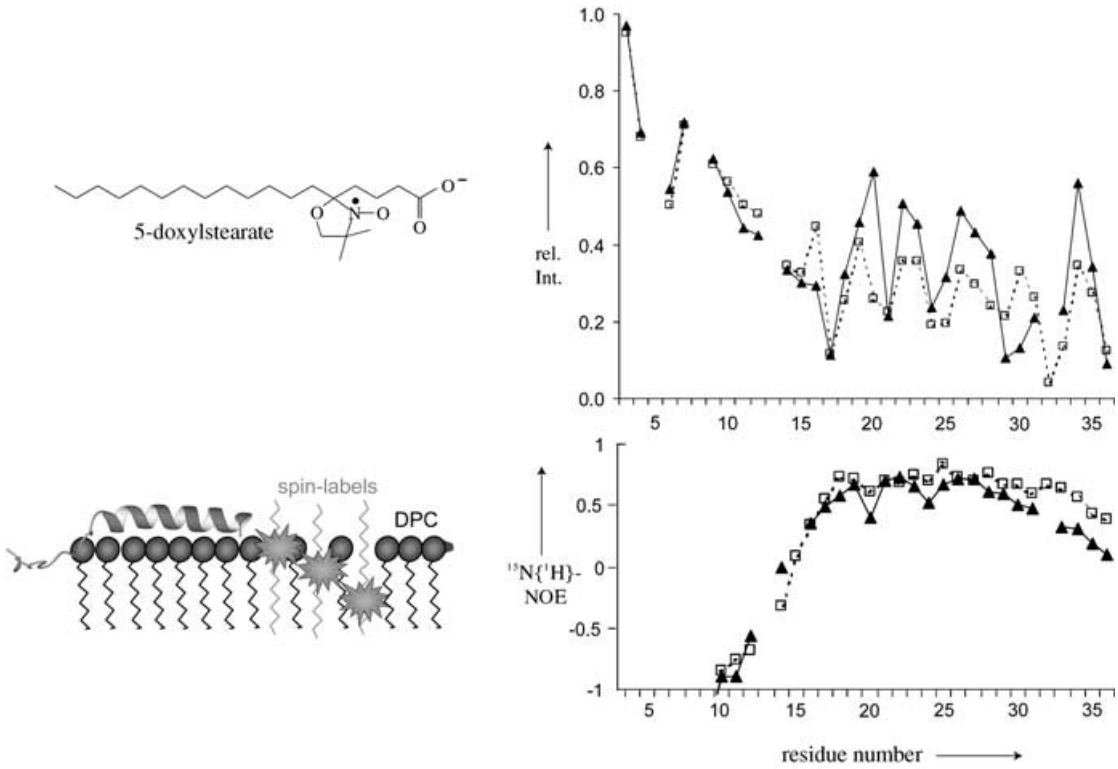

Figure 5. Top right: relative signal intensities ($I_{spin\text{-}label}/I_{no\text{-}spin\text{-}label}$) for NPY (□) and [Ala31,Pro32]-NPY (▲) as derived from the [^{15}N,^{1}H]-HSQC correlation experiment performed in the presence and absence of the spin label 5-doxylstearate. Bottom right: the values of the ^{15}N{^{1}H}-NOE for NPY (□) and [Ala31,Pro32]-NPY (▲). The chemical structure of 5-doxylstearate and its interaction with membrane-associated NPY are depicted on the left.

spin label are strongest at residues 29 and 36 and at residues 32 and 36 in the mutant and wild-type NPY, respectively. Taken together, the data suggest that [Ala31,Pro32]-NPY and NPY are anchored differently on the membrane.

We have also used measurements of proton–deuterium exchange for amide protons to locate solvent-shielded protons, which are supposed to point towards the peptide–membrane interface. However, we realized that interpretation of these data is complicated by the fact that stable hydrogen bonds may also reduce amide-proton exchange rates. As the peptide is in equilibrium between the aqueous and micellar phases, hydrogen-exchange rates reflect structural features of both states, each to a different and unknown extent. We have additionally, in cooperation with the group of Aguilar from Monash University (Clayton, Victoria), determined the binding affinities for association of peptides from the NPY family to zwitterionic and negatively charged membranes by using SPR spectroscopy.[15] In these experiments, SPR chips are coated with phospholipids and binding of the peptides to these surfaces is monitored in real-time by using BiaCore technology. In principle, the technique allows us to independently calculate on and off rates for peptides binding to the phospholipids.[73]

4.3. Experiments to characterize the transition from the solution state to the membrane-bound state

Conceptually it is very important to know in which way the structures of the peptides change when they diffuse from bulk solution towards the membrane or vice versa. Accordingly, we have designed experiments to mimic this transition. The design of the experiments is based on the observation that the structure of PYY, which displays a back-fold in aqueous solution, is not back-folded in methanol (unpublished results, see inset in Figure 6) and therefore largely resembles the fold of the peptide when bound to DPC micelles. In fact, we have determined the structure of PYY in methanol and noticed that

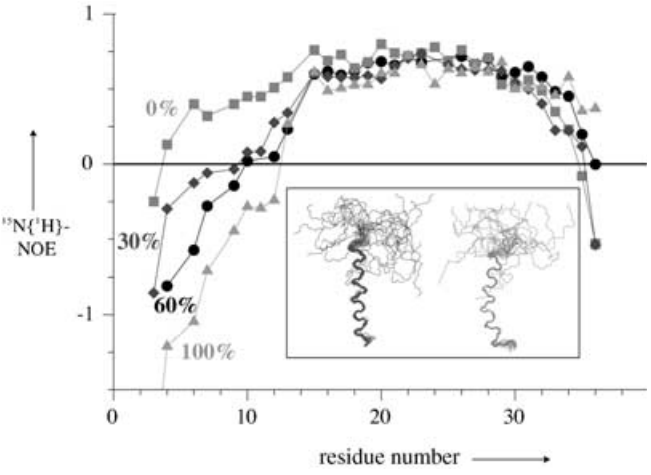

Figure 6. Values of the ^{15}N{^{1}H}-NOE for PYY in various water/methanol mixtures (percentage of methanol indicated). The insets display the NMR conformers of PYY in methanol (left) and when bound to DPC micelles (right).

the most substantial difference of the structure in methanol as compared to that of the DPC-micelle-bound form is the absence of the bend at the C-terminal helix. Considering that the surface of DPC micelles displays a substantial curvature, we suggest that the bent helix seen in micelle-bound NPY is most probably induced by accommodation of the hydrophobic side to the micellar surface. Otherwise, PYY is *not* back-folded in both methanol and DPC micelles, the C-terminal pentapeptide is conformationally rigidified, and the C-terminal helix extends over a similar region. We then prepared solutions of the peptide in various water/methanol mixtures and recorded the $^{15}N\{^1H\}$-NOEs to follow the changes in back-folding (Figure 6).

As a result, a smooth transition between the back-folded and the non-back-folded species is observed. The C terminus is apparently rigidified with increasing amounts of methanol whereas the N-terminal half of the molecule becomes more flexible. However, from these equilibrium data we cannot infer whether intermediate states are populated or whether the heteronuclear NOE as an ensemble-averaged quantity reflects two-state behavior between the back-folded and non-back-folded species.

Again, we would like to stress the use of this parameter for investigating the conformational transitions: The method is comparably quick and it would be difficult (impossible, we believe) to track the subtle conformational changes by using proton–proton NOEs. In fact, peaks measured in some of the water/methanol mixtures with intermediate ratios are rather broad due to conformational exchanges, a problem that would preclude the determination of structures of sufficient quality. The use of proton–proton NOEs is very useful in the case of rigid structures but special care must be taken in highly dynamic systems. The loss of back-folding is accompanied by both the loss of characteristic medium-range ^1H–^1H NOEs in the polyproline helix and by the loss of NOEs between residues of the N terminus and the C-terminal helix. The latter will decrease in intensity until they are invisible, the point of which depends partly on the sensitivity of the spectrometer and the concentration of the peptide. Thereby, an arbitrary cut-off is introduced. By contrast, the heteronuclear NOE is measurable over the full dynamic range with sufficient precision. Moreover, weaker NOEs are traditionally interpreted in terms of longer but still *well-defined* distances, which is certainly not true in our systems. We suggest that the heteronuclear NOE *in this particular case* is a reliable method to demonstrate changes in the extent of back-folding, whereas the use of ^1H–^1H NOEs would clearly require more sophisticated additional analysis such as molecular dynamics and ensemble-averaging methods.

5. Understanding Structural Features in Different Environments

5.1. Understanding structural features of the neuropeptides in solution

The presence or absence of the PP fold is certainly the most distinguishing structural feature of this class of peptides in solution. Out of the three principal members of the family, bPP and PYY display the back-fold, whereas a growing body of evidence suggests that NPY is not back-folded (see above). The difference between NPY and PYY is striking, since their sequence homology is larger than 80% and aromatic or charged residues are found at the same positions. Originally we were proposing that intramolecular, as well as intermolecular, aromatic-ring stacking interactions of Tyr7 and Tyr20 mutually stabilize both the PP fold and the dimer. We subsequently characterized the [Ala7]-bPP mutant and discovered that, in contrast to our expectations, the back-fold was preserved. Nevertheless, the back-folded segment around residues 7–12 was significantly destabilized with respect to wild-type bPP. PYY contains Ala at position 7 and the dynamics data display reasonable similarity to those of [Ala7]-bPP.

The major difference in sequence between NPY and PYY is Pro14 in PYY being shifted to position 13 in NPY. In fact, the position of Pro in that segment has been used to assign sequences to either the PYY or NPY family.[34] Since the segment 10–14 forms the N-terminal-helix capping turn we felt that the differences in back-folding propensities between NPY and PYY might be due to conformational restraints imposed by the Pro residues at the according positions. In order to better understand which residues are important for inducing the PP fold, we have therefore looked at a series of mutants by using the $^{15}N\{^1H\}$-NOE to quantify the extent of back-folding. Accordingly, we have expressed uniformly ^{15}N-labeled [Ala14]-PYY and [Pro13,Ala14]-PYY. As depicted in Figure 7, [Ala14]-PYY is still back-folded. However, the back-folded segment appears to be slightly more flexible.

Interestingly, the mutant [Pro13,Ala14]-PYY is no longer back-folded and we conclude from this fact that Pro14 in PYY does not provide conformational restraints enforcing the backbone to adopt the PP fold. The comparison of [Ala14]-PYY with [Pro13,Ala14]-PYY rather indicates that it is Pro at position 13 that is incompatible with the occurrence of the PP fold in NPY. We would like to add at this point that Pro is found at position 13 in aPP, which, however, has low sequence homology to NPY or PYY. We have also looked at PYY(3–36), a naturally occurring variant of PYY, for which a controversially debated role in the regulation of food uptake was proposed recently.[74,75] Keire et al. have stated that PYY(3–36) is no longer back-folded,[67] an observation that is confirmed by our dynamics data. Indeed, the values for the heteronuclear NOEs are very similar for [Pro13,Ala14]-PYY and PYY(3–36). Since no interactions between the first two residues and those from the C-terminal helix are observed in PYY, we believe that this change in tertiary structure is induced because the charge from the N terminus is moved closer to the hydrophobic contact formed between Pro5 and Tyr27.

We and others realized that both NPY[56] and PYY(3–36), which are both non-back-folded, form larger aggregates at concentrations higher than 1 mM. Our experiments with TOAC-NPY indicated the presence of both parallel and antiparallel arrangements. Whether a mixed type of arrangement is present in a single oligomer (for example, a three-helix bundle) or whether it represents rapidly interconverting dimers is unclear at this stage. It needs to be pointed out that the secondary

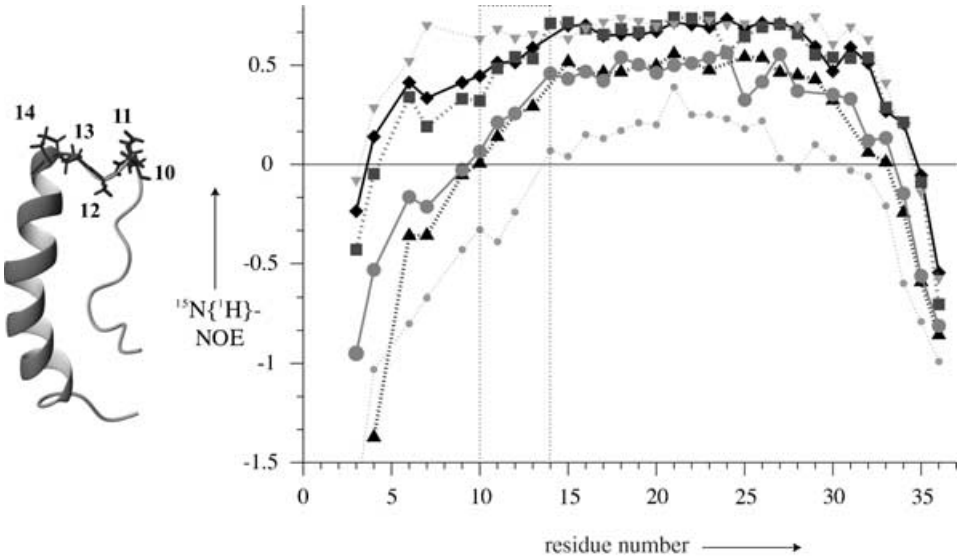

Figure 7. Values of the ^{15}N{^{1}H}-NOE for mutants of PYY. For reference the values of PP (▼) and NPY (●) are also presented. Data are given for PYY (◆), [Ala14]-PYY (■), [Pro13,Ala14]-PYY (●), and PYY(3–36) (▲). The secondary structure of PYY in solution is presented on the left with the side chains of the helix-capping residues 10–14 depicted and annotated.

structures of all non-back-folded peptides investigated by us so far are much less rigid than the back-folded peptides.

5.2. Understanding structural features of the neuropeptides in the micelle-bound state

The membrane-compartment theory of receptor recognition suggests a significant role of the membrane in mediating the transfer of the peptide hormones from the aqueous phase to the lipid-rich environment of the membrane-embedded receptor.[7] Structure–activity relationship studies should therefore take into account several parameters of the membrane-associated state of a peptide hormone, including conformation, orientation, and partitioning in the membrane, as well as molecular motion. We have collected a complete set of estimates reflecting such properties for the principal members of the NPY family, including NPY, PP, PYY, and the Y5-subtype-preferring NPY analogue [Ala31,Pro32]-NPY.[16–19]

Overall, some common structural features emerge that are shared by all NPY family peptides in their micelle-associated states. The N terminus is relatively flexible, that is, released from its back-folded form, as observed in the solution state of PP and PYY. The hydrophobic side of the C-terminal α helix, which starts between residues 14 and 17, forms the membrane-binding interface with hydrophobic side chains inserting into the hydrophobic interior of the membrane. The C-terminal α helix is generally very well defined with the generalized order parameters, S^2, adopting values larger than 0.75 for the segments Ala18–Arg33 and Ser18–Thr32 in NPY and PYY, respectively, whereas the C-terminal tyrosine amides of NPY and PYY exhibit somewhat lower but similar S^2 values of 0.28 and 0.36, respectively. The S^2 value of the N-terminal residues steadily increase, but they are well below 0.2 up until residue 12. Here, the highly uncorrelated slow internal motions are interpreted as random diffusion of the N terminus in the aqueous environment of the micelle.

Membrane anchoring of all peptides investigated occurred through intercalation of hydrophobic or aromatic side chains such as Ile, Leu, Val, or Tyr/Phe/Trp into the hydrophobic interior. Signal reductions due to spatial proximity of the spin label 5-doxylstearate revealed a 3–4 residue periodicity, which suggests that the helix is oriented *parallel* to the micelle surface. In general, membrane anchoring may be very well predicted from the free energies of transferring whole amino acids from aqueous bulk solution into the membrane–water interface or the hydrophobic interior (Figure 8), as determined by White and co-workers.[21,22]

The plot reveals strikingly lower (energetically more favorable) energies for residues from the C-terminal half of the peptides compared to the values for those in the N-terminal half (Figure 8). This presents additional evidence that the C-terminal α helix has been evolutionarily optimized for membrane binding. The data nicely reflect the amphiphilicity of the helical segment. Moreover, residues displaying favorable energies have also been identified as membrane-anchoring residues. When Tyr7 of bPP is replaced by Ala, the N-terminal segment is no longer associated with the micelle surface (unpublished data), which is in perfect agreement with the presented view.

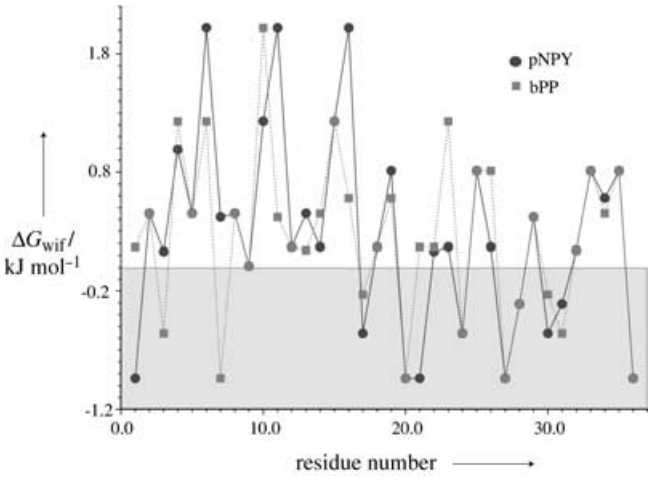

Figure 8. Free energies of transfer of whole amino acids from the aqueous bulk phase into the phospholipid interface, ΔG_{wif}, as derived by White and co-workers,[21,22] plotted for the sequences of pNPY (●) and bPP (■).

The C-terminal pentapeptide is believed to be important for receptor activation, presumably through electrostatic interactions involving Arg33 and/or Arg35,[38] and hence special attention shall be given to structural features of this particular segment. Interestingly, binding of NPY family peptides to the membrane surface is accompanied by a conformational stabilization and reorientation of the C-terminal pentapeptide. It is generally much more rigid in the micelle-bound species, as reflected by positive $^{15}N\{^1H\}$-NOEs (Figure 5, lower panel). By contrast, $^{15}N\{^1H\}$-NOEs of NPY in solution adopt generally lower values, which are even negative between residues 31–36. C-terminal amidation is essential for binding to the Y receptors and modification of NPY to yield the free carboxy terminus leads to almost complete loss of affinity at the human Y1 receptor.[76] Interestingly, our spin-label studies and the $^{15}N\{^1H\}$-NOE recorded with an ^{15}N-NPY mutant with a free carboxy terminus indicate that Tyr36 is not close to the membrane–water interface any longer and the C-terminus diffuses freely in solution.

The results for membrane-binding affinities as determined by SPR spectroscopy are depicted in Table 4 for pNPY, pPYY, and bPP. We noticed that the overall association rates are *moderate* and the spanned range of association constants is rather small. Surprisingly, binding to the zwitterionic bilayers is generally tighter than to the negatively charged bilayers. The overall association constants on the zwitterionic surfaces are correlated to the overall (positive) charge in the C-terminal helix. However, the data lack proof that general receptor binding or subtype specificity is correlated to the strength in membrane binding. From these data, we suspect that a general affinity for the membrane, inducing conformational rigidification and topological preorientation, is necessary but that the location of the peptide with respect to the membrane is best characterized by a probability distribution of distances between the peptide and the membrane surface. As it may be possible that the receptor binding pocket is only accessible by the peptide once it has come off the membrane to some extent (see below), moderate membrane-binding affinities may actually promote better receptor binding.

Table 4. Data for association of peptides to phospholipid membranes as determined by SPR spectroscopy.[15]

Peptide	Lipid[a]	Rate constants (two-state reaction model; 2.5–25 μM)[b]				
		k_{a1} [s^{-1}]	k_{d1} [s^{-1}]	k_{a2} [s^{-1}]	k_{d2} [s^{-1}]	K_{ass} [M^{-1}]
bPP	PC	1340	6.54×10^{-2}	3.64×10^{-3}	2.92×10^{-3}	4.58×10^{4}
	PC/PG[c]	971	4.01×10^{-2}	4.43×10^{-3}	5.91×10^{-3}	4.23×10^{4}
pPYY	PC	472	3.00×10^{-2}	4.12×10^{-3}	1.71×10^{-3}	5.36×10^{4}
	PC/PG[c]	180	1.82×10^{-2}	3.41×10^{-3}	2.04×10^{-3}	2.63×10^{4}
pNPY	PC	501	1.74×10^{-2}	2.48×10^{-3}	1.81×10^{-3}	6.83×10^{4}
	PC/PG[c]	385	4.78×10^{-2}	1.32×10^{-3}	2.57×10^{-3}	1.22×10^{4}

[a] PC = phosphatidyl choline; PG = phosphatidylglycerol. [b] k_{a1} and k_{a2} = rate of association for steps 1 and 2, respectively; k_{d1} and k_{d2} = rate of dissociation for steps 1 and 2, respectively; K_{ass} = association constant. [c] PC/PG (4:1, *w/w*).

6. Pharmacological Differences of Peptides from the NPY Family Are Better Reflected in the Micelle-Associated Structures

The principal members of the NPY family of neurohormones, NPY, PYY, and PP, display different affinities at the various Y receptor subtypes. Whereas NPY and PYY, which have an overall sequence homology of approximately 80%, possess nanomolar binding affinities at all receptor subtypes, PP binds very tightly to the Y4 receptor, to a lesser extent to the Y5 receptor, and very poorly to the Y1 and Y2 subtypes. We have determined the structures of these peptides when bound to DPC micelles[16,17,19] and redetermined the structure of PYY in solution.[16] A comparison of the structures of these peptides in the two environments is displayed in Figure 9.

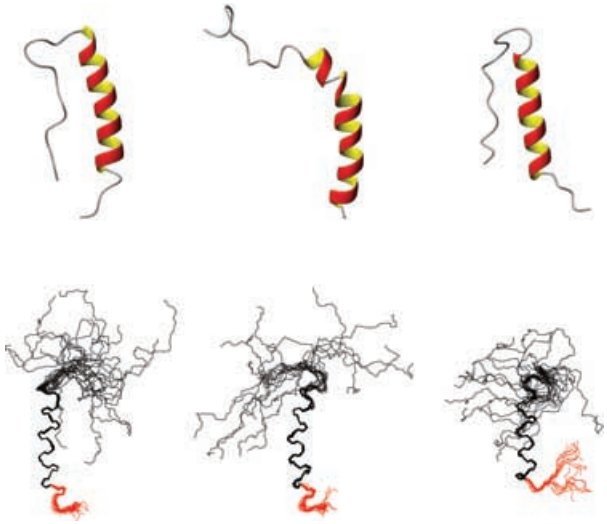

Figure 9. Comparison of structures of PYY (left), NPY (middle), and bPP (right) unligated in solution (top, single conformer) and when bound to DPC micelles (bottom, superposition of NMR ensemble). The C-terminal pentapeptide of the micelle-bound peptides is depicted in red. Reproduced with permission from ref. [16], copyright (**2004**), Elsevier.

Clearly, the *solution structures* of NPY and PYY are different in that NPY does not back-fold whereas PYY does. In this respect, PYY and PP, two peptides displaying very different pharmacological binding profiles, are much more similar. By contrast, the structures of *micelle-bound* NPY and PYY are almost identical whereas the conformation, especially of the C-terminal pentapeptide, is clearly different in bPP.

Moreover, identical residues of NPY and PYY form the membrane-binding interface, which is not surprising considering the high sequence identity in the C-terminal part. Major membrane-anchoring points are formed by the side chains of residues Leu17, Tyr20, Tyr21, Leu24, Ile28, Asn29, Ile31, Thr32, and Tyr36. The conformation, micelle-anchoring topology, and stability of the C-terminal pentapeptide are nearly identical in NPY and PYY. Residues Thr32 and Tyr36 are anchored through their side chains onto the membrane and the helix extends up

to residue 36. The C-terminal Tyr amide forms the main anchoring point of the C terminus. Interestingly, deamidated NPY is inactive and our spin-label data indicate that for that modification the C terminus is no longer held in the vicinity of the interface. The positively charged side chains of Arg33 and Arg35 point away from the membrane into the aqueous environment. By contrast, the root mean square deviation values of C-terminal residues of micelle-bound bPP are significantly higher than in NPY and PYY but nevertheless indicate the presence of residual structure. Spin-label data for bPP suggest that the backbone of residues 33–35 is close to the micelle surface or even partly buried. The helix ends at residue Thr32 and Pro34 presents the main anchor in the C-terminal pentapeptide. Figure 10 shows a comparison of the backbone conformations of NPY, bPP, and [Ala31,Pro32]-NPY for C-terminal residues.

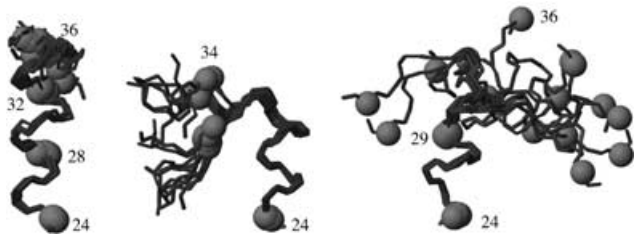

Figure 10. Comparison of NPY (left), bPP (middle), and [Ala31,Pro32]-NPY (right) bound to DPC micelles. The figure displays the superposition of NMR structures of the C-terminal segment comprising residues 24–36. Membrane-anchoring residues are annotated and indicated by spheres.

Whereas Pro in position 34 apparently anchors bPP on the membrane, the double mutation introduced in [Ala31,Pro32]-NPY disrupts the α-helical conformation of the C-terminal pentapeptide and results in a longer and more flexible loop between membrane anchors in position 29 and the C-terminal Tyr36 amide.

The positions of Arg33 and Arg35 are consequently less well defined in [Ala31,Pro32]-NPY than in bPP, particularly with respect to their orientation relative to the membrane–water interface. Moreover, both of these peptides display a stronger conformational heterogeneity at their C termini than NPY and PYY. We have speculated that the resulting difference in positioning of Arg33 or Arg35 with respect to the water–membrane interface may partially account for differences in the receptor-subtype specificity observed for these peptides.[16–19]

The most striking structural difference of bPP as compared to the structures of NPY and PYY when bound to DPC micelles is the extent to which the N-terminal half of the molecule interacts with the membrane. Membrane association by N-terminal residues is virtually absent in NPY and PYY, but spin-label and hydrogen-exchange data indicate proximity to the membrane–water interface for Leu3, Glu4, Glu6, Gly9, and Ala12 in bPP. The latter findings are further supported by significantly reduced hydrogen-exchange rates at Leu3, Glu6, Tyr7, and Ala12 and by the absence of peaks due to the cis conformer around the Asn7–Pro8 peptide bond. We speculated that the difference in membrane anchoring of the N-terminal halves of NPY/PYY compared to that of bPP may be due to the additional Tyr7 residue in bPP, which replaces Asn and Ala residues in NPY and PYY, respectively.

We believe that the comparison of structures of NPY and PYY in solution and when bound to DPC micelles indicates that the peptides from the NPY family are recognized from the membrane-bound state. The rationale behind this conclusion is that these ligands, which possess almost identical binding profiles at the Y receptor subtypes, are expected to display similar conformations *in the particular environment from which they are recognized*. Otherwise, the conformational rearrangements that would be necessary to allow binding should result in differences in entropy, which would propagate into changes in free energy of binding that would, in turn, translate into differences in binding affinities. Moreover, in case of an induced-fit binding mode, which cannot be excluded per se, it is highly unlikely that the necessary rearrangements *at all receptor subtypes* are similar if the structures of the two ligands are different. We would like to emphasize that the above-described approach uses *unmodified* peptides and, therefore, problems from modifications introduced to enhance membrane binding, which may result in unknown differences in the receptor-binding mode, are avoided.

7. A Scenario for Binding to the Receptor: Conclusions and Outlook

For binding of the hormones to their receptors, we propose a multistage scenario. Firstly, hormones are attracted by the membrane through electrostatic interactions. The extent of these attractions is regulated by the content of negatively charged phospholipids in the membrane composition and by the content of cationic ligand residues. In a second step, the peptide reorients such that hydrophobic residues penetrate the hydrophobic interior. The hormone subsequently diffuses laterally along the membrane and it is this particular state from which the peptide is recognized *initially* by the receptor. However, this state may be heterogeneous, thereby reflecting an equilibrium between the membrane-associated state and the unligated solution state. Once the membrane-associated and preoriented ligand has diffused into proximity of the receptor, it may then enter the binding pocket of the receptor. The initial conformation for this transition would be close to the membrane-bound state but may undergo further conformational changes following an induced-fit mechanism. We would like to especially emphasize that our model does not exclude such an induced-fit mechanism. However, in cases where *any* of the *sequential* events is unfavorable and associated with an unrealistically high energy barrier, binding will not take place. The membrane-bound pathway might therefore be a mechanism to cross an energetically unfavorable transition state more easily.

Binding to the membrane interface is promoted by both hydrophobic and electrostatic interactions (see above). Since electrostatic interactions depend on the inverse of the separating distance, in contrast with hydrophobic interactions which scale with the inverse of the sixth power, they are more effec-

tive over much larger distances and probably dominate *initial* contacts. BiaCore measurements by Aguilar and co-workers revealed that binding to membranes proceeds with a two-step mechanism.[30,73] Firstly, electrostatic interactions led to fast on rates, after which the orientation of the ligand is changed. The hydrophobic side comes to point towards the interface, thereby facilitating intercalation of the side chains of hydrophobic residues into the hydrophobic interior. According to the exact positioning of the peptide in the membrane, electrostatics may become even more important since the dielectric constant in that region is reduced (to about 3–10) so that Debye–Hückel screening of charges by the water dipole is much less efficient.

A number of studies have shown the importance of electrostatic interactions for binding of ligands to G protein-coupled receptors.[77–79] Presently, it is not clear whether electrostatic interactions or π-cation interactions between the Lys/Arg side chains and aromatic residues[80] dominate binding affinity. It is of interest to note that the negative charge is located in the receptor in most of the known cases and not vice versa! There is no obvious reason for that from an energetic point of view, unless one assumes an interaction between the membrane and the ligand that is promoted by cationic ligand residues. Again the importance of π-cation interactions may account for this surprising observation. The high occurrence of aromatic residues at the water–membrane interface also indicates that this type of interaction may be of relevance.

An interesting question asks how the neuropeptides enter the binding pocket once the membrane-bound species has laterally diffused along the membrane into the proximity of the receptor. Mutagenesis data indicate that the binding site involves some residues from the third extracellular loop (see above),[44] but clearly other interactions will also take place. With the assumption that this binding pocket is not easily accessible for the membrane-attached peptide, conformational rearrangements of the extracellular loops could possibly provide the necessary changes to accommodate the ligand. Although no structural data for Y receptors are presently available, it is likely that the seven-helix bundle provides a rather rigid scaffold that does not allow large rearrangements of the extracellular loops. It is therefore possible that the ligands actually need to come off the membrane in order to diffuse into the binding pocket. Our SPR data indicate that the membrane-binding affinity of these peptides is moderate and, hence, there is a substantial population of peptides in solution, albeit in closer proximity to the membrane. Biophysical investigations have shown that solvent properties such as the dielectric constant are very different in the interface region compared to those in bulk water. Our experiments with water/methanol mixtures indicate that the population of molecules possessing the overall structural features of the membrane-bound form is high even when the dielectric constant is considerably increased. Hence, overall structural features from the membrane-bound species are preserved unless the peptides diffuse back deeply into the bulk-water phase.

We believe that our data support the view that hormones from the neuropeptide Y family are indeed recognized from the membrane-bound state. In this review we have described techniques and concepts developed by us to relate the pharmacology to the structures of these peptides; we observed that the biological data are better correlated to the structures of the micelle-bound species. Our work, of course, only provides *indirect* evidence and is mainly restricted to the event of *initial* recognition. In fact, much more additional work is necessary to fully prove the membrane-compartment concept, including a thorough investigation of the binding kinetics. In addition, we presently have no structural data on binding of the peptides to the Y receptors or fragments thereof and the pathway for diffusion of the peptides from the membrane-bound state into the binding pockets needs to be investigated in more detail. We would also like to emphasize that we have performed our investigations exclusively for the full-length peptides. Many small-molecule antagonists have been developed and it is unlikely that recognition of all of them occurs from the membrane-bound state. Many of these molecules actually lack a specific membrane-binding interface. The controversy and debate about this model may have been stirred by the fact that the concept has been erroneously generalized to account for all ligands.

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