GPC Receptors and Not Ligands Decide the Binding Mode in Neuropeptide Y Multireceptor/Multiligand System[†]

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ABSTRACT: Many G protein-coupled receptors belong to families of different receptor subtypes, which are recognized by a variety of distinct ligands. To study such a multireceptor/multiligand system, we investigated the Y-receptor family. This family consists of four G protein-coupled Y receptors in humans (hY_1R, hY_2R, hY_4R, and hY_5R) and is activated by the so-called NPY hormone family, which itself consists of three native peptide ligands named neuropeptide Y (NPY), pancreatic polypeptide (PP), and peptide YY (PYY). The hY_5R shows high affinity for all ligands, although for PP binding, the affinity is slightly decreased. As a rational explanation, we suggest that Tyr^{27} is lost as a contact point between PP and the hY_5R in contrast to NPY or PYY. Furthermore, several important residues for ligand binding were identified by the first extensive mutagenesis study of the hY_5R. Using a complementary mutagenesis approach, we were able to discover a novel interaction point between hY_5R and NPY. The interaction between NPY(Arg^{25}) and hY_5R($Asp^{2.68}$) as well as between NPY(Arg^{33}) and hY_5R($Asp^{6.59}$) is maintained in the binding of PYY and PP to hY_5R but different to the PP-hY_4R and NPY-hY_1R contact points. Therefore, we provide evidence that the receptor subtype and not the pre-orientated conformation of the ligand at the membrane decides the binding mode. Furthermore, the first hY_5R model was set up on the basis of the crystal structure of bovine rhodopsin. We can show that most of the residues identified to be critical for ligand binding are located within the now postulated binding pocket.

G protein-coupled receptors (GPCRs)[1] represent the largest family of cell-surface receptors. They are activated by a variety of structurally diverse ligands that range from bioamines and peptides to large polypeptide hormones (*1*). Small ligands bind within the transmembrane regions, whereas peptide ligands recognize their target receptors by specific amino acids on the top of the transmembrane (TM) regions or within the extracellular loops (*1*). Many of the more than 1000 human GPCRs represent important pharmaceutical targets (*2*), and nearly 50% of drugs in clinical use today act on GPCRs (*3*). Because many peptide ligands are recognized by a GPCR family with several receptor subtypes, drugs can act differently on these subtypes and subsequently cause cross-reactivities. Furthermore, multireceptor systems are frequently complicated by the existence of several ligands. Up to now, numerous multireceptor/multiligand systems have been discovered, for example, the orexin receptors OxR_1 and OxR_2 with their ligands orexin A and B (*4, 5*). Similarly, NPFF, NPSF, and NPAF are all ligands for the NPFF receptors $NPFFR_1$ and $NPFFR_2$ (*6–8*), and substance P as well as neurokinin A and B bind to NK1−NK3 receptor subtypes (*9–12*). Accordingly, knowledge in selective binding and efforts to understand receptor subtype selectivity are urgently required. BIBP3226 for example is an antagonist for the Y_1 receptor, but it is also described to bind to the $NPFFR_2$ receptor (*13, 14*), probably because of the similar C terminus of NPY and NPFF, which is mimicked by BIBP3226 (*15*).

	10	20	30
pNPY	YPSKPDNPGE	DAPAEDLARY	YSALRHYINL ITRQRY-NH_2
hPYY	YPIKPEAPGE	DASPEELNRY	YASLRHYLNL VTRQRY-NH_2
hPP	APLEPVYPGD	NATPEQMAQY	AADLRRYINM LTRPRY-NH_2

FIGURE 1: Comparison of the sequences of pNPY, hPYY, and hPP. The positions shown in bold were examined for the influence upon binding to the hY_5 receptor.

Our system to study a multireceptor/multiligand system is the neuropeptide Y family. The three peptides neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) (cf. Figure 1) bind to four G protein-coupled Y receptors in humans (hY_1, hY_2, hY_4, and hY_5) (*16*). All three peptides consist of 36 amino acids and are C-terminally amidated (*17*). They are involved in multiple physiological processes, especially in the regulation of food intake. These effects make NPY an attractive target for the potential treatment of human

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[1] Abbreviations: NPY, neuropeptide Y; PYY, polypeptide YY; PP, prancreatic polypeptide; YR, Y receptor; Y_1R, Y_1 receptor; Y_2R, Y_2 receptor; Y_4R, Y_4 receptor; Y_5R, Y_5 receptor; GPCR, G protein-coupled receptor; OxR, Orexin receptor; NPFF, neuropeptide FF; NPSF, neuropeptide SF; NPAF, neuropeptide AF; ELISA, enzyme-linked immunosorbent assay; EC_{50}, concentration producing half-maximal response; TM, transmembrane; ECL, extracellular loop.

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diseases, such as obesity and metabolic disorder (*18*). However, this multireceptor/multiligand system belongs to one of the most complex peptide systems, because three peptides share four receptors with different affinity and selectivity. NPY and PYY bind with high affinity to the hY_1, hY_2, and hY_5 receptor subtypes, whereas PP preferentially binds to the hY_4 receptor but is still able to activate the hY_5 receptor with high affinity (*19*). Many studies aimed to identify the Y receptor subtype that is involved in the orexigenic effect of NPY. The Y_1 and Y_5 receptors appear to be the most likely candidates for the orexigenic effects of NPY (*20*), but Y_2 and Y_4 receptors have also been reported to be possible targets (*21, 22*).

Recently, we were able to identify an acidic residue on top of TM helix 6 that is critical for ligand binding in all four Y receptor subtypes. This $Asp^{6.59}$ (nomenclature according to ref *23*) forms ionic interactions with one of the two Arg residues in the ligands NPY or PP (*15*). $Asp^{6.59}$ of the hY_2 and hY_5 receptors interacts with Arg^{33} of NPY. In contrast, Arg^{35} of the ligands NPY and PP revealed to be the counterpart for $Asp^{6.59}$ in the hY_1 and hY_4 receptors, respectively (*15*).

Accordingly, NPY binds to hY_1 and hY_5 receptors in a completely different manner, and the binding mode of PP to the hY_4 receptor is more related to the NPY−Y_1R interaction than to the NPY−Y_5R binding mode. Because all three ligands are recognized by the hY_5 receptor, although with different affinities (*19*), the question came up whether the ligand or the receptor decides the binding mode and the selected contact points. Therefore, ligands were modified at specific positions, and site-directed mutagenesis studies of the hY_5 receptor were performed to reveal critical residues in all three ligands as well as in the hY_5 receptor. Using a complementary mutagenesis approach, we identified a second interaction point between the NPY and the hY_5 receptor. Moreover, because Arg^{35} of PP interacts with $Asp^{6.59}$ in the hY_4 receptor, we demonstrate here that the two identified contact points Arg^{25}−$Asp^{2.68}$ and Arg^{33}−$Asp^{6.59}$ between NPY and the hY_5 receptor are not distinct from the contact points between PYY or PP and the hY_5 receptor. Furthermore, our data explain the reduced affinity of PP at the hY_5 receptor.

Docking studies of NPY were performed on the basis of the crystal structure of bovine rhodopsin as the template, considering the two experimentally identified contact points at the hY_5 receptor, which nicely explain the major interaction sites identified at the hY_5 receptor thus far.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. Peptides were synthesized by automated solid-phase peptide synthesis following the Fmoc/^{t}Bu (9-fluorenyl-methoxycarbonyl-tert-butyl) strategy (*17*). Rink amide resin was used to produce peptide amides, whereas Wang resin was used to obtain peptide acids. The peptides were purified by high-performance liquid chromatography (HPLC), and molecular masses were verified by matrix-assisted laser desorption ionization−mass spectrometry. Circular dichroism (CD) analysis of Ala-substituted porcine NPY (pNPY) peptides was performed (*17*) and showed no significant differences to wild-type pNPY.

Generation of YR Mutants. The cDNAs of the human Y_1R, Y_2R, Y_4R, and Y_5R were C-terminally fused to the cDNA of the enhanced yellow fluorescent protein for fluorescence detection. The chimeric cDNAs of the Y_1R, Y_2R, and Y_5R were cloned into the eukaryotic expression vector pVitro2 (InvivoGen Europe, Toulouse, France). The cDNA of the Y_4R was cloned into the eukaryotic expression vector pEYFP-N1 (Clontech, Heidelberg, Germany). Mutations were introduced with the QuickChange site-directed mutagenesis method (Stratagene). Additionally to the C-terminally fused EYFP, an N-terminal nine amino acid residue epitope (YPYDVPDYA) derived from the influenza virus hemagglutinin protein (HA tag) was added to all YRs to quantify the cell-surface expression of the receptors in enzyme-linked immunosorbent assay (ELISA) studies. The residues are numbered according to the system of Ballesteros and Weinstein (*23*).

Cell Culture. Cell-culture material was supplied by PAA Laboratories GmbH (Pasching, Austria). COS-7 cells (African green monkey, kidney) were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were grown as monolayers at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Radioligand-Binding Studies. For radioligand-binding studies, 1.5×10^6 COS-7 cell were seeded into 25 cm^2 flasks. At 60−70% confluency, cells were transiently transfected using 4 μg of vector DNA and 15 μL of Metafectene (Biontex Laboratories GmbH, Martinsried/Planegg, Germany). Approximately 24 h after transfection, binding assays were performed on intact cells using *N*-[propionyl-^{3}H] pNPY (specific activity of 3.52 TBq/mmol; GE Healthcare Europe GmbH, Braunschweig, Germany). Binding was determined with 1 nM *N*-[propionyl-^{3}H] pNPY in the absence (total binding) and presence (nonspecific binding) of 1 μM unlabeled pNPY, hPP, hPYY, or the Ala-substituted peptides, respectively, as described previously (*24, 25*). Specific binding of each receptor mutant was compared to specific binding of the wild-type receptor, and the specific binding of each peptide analogue was compared to specific binding of the corresponding unmodified peptide. Binding assays were performed at least twice independently; each experiment was performed in triplicate.

Signal Transduction Assay. For signal transduction (inositol phosphate accumulation) assays, COS-7 cells were seeded into 24-well plates (1.0×10^5 cells/well) and transiently cotransfected with 0.2 μg of plasmid DNA encoding hY_5R C-terminally fused to EYFP and 0.05 μg of plasmid DNA coding for the chimeric G protein $G\alpha_{\Delta 6qi4myr}$ (kindly provided by E. Kostenis) using 0.75 μL of Metafectene (Biontex Laboratories GmbH, Martinsried/Planegg, Germany). The assay for ligand-stimulated production of inositol phosphate was described previously (*25*). Receptor stimulation was stopped by aspiration of the medium, and cell lysis was performed with 0.1 M NaOH (150 μL/well) for 5 min. After adding 0.2 M formic acid (50 μL/well) and sample dilution, intracellular IP levels were determined by anion-exchange chromatography as described previously (*26, 27*). Data were analyzed with GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). EC_{50} values were obtained from concentration−

response curves. All signal transduction assays were performed in duplicate and repeated at least twice independently.

ELISA. To quantify plasma-membrane localization, a cell-surface ELISA was performed with wild-type and mutant receptors that carried a N-terminal HA tag (*28*). COS-7 cells were seeded into 48-well plates (3.5×10^4 cells/well) and transiently transfected with 0.6 μL of Metafectene (Biontex Laboratories GmbH, Martinsried/Planegg, Germany) and 0.2 μg of vector DNA encoding the hY_5R C-terminally fused to EYFP and N-terminally fused to the HA tag. A total of 2 days after transfection, cells were fixed with 4% (m/v) paraformaldehyde in the absence of detergent and probed with a biotin-labeled anti-HA antibody (12CA5, Roche, Mannheim, Germany). Bound anti-HA antibody was detected by a peroxidase-labeled streptavidin conjugate (Sigma-Alrich, Taufkirchen, Germany). To quantify the bound peroxidase, the substrate TMB-soluble (Calbiochem, Darmstadt, Germany) was used. The enzyme reaction was stopped by the addition of 0.25 M HCl. The absorption was measured at 450 nm using an ELISA reader (Spectrafluor plus, Tecan, Crailsheim, Germany).

Receptor Modeling and Ligand Docking. The transmembrane helices were modeled on bovine rhodopsin using Research Collaboratory for Structural Bioinformatics (RCSB) entry 1F88 as a template (*29*). Five rounds of PSI-Blast (*30*) were performed with the sequence of hY_5R (UNIProt entry Q15761) as a query. The top 100 sequences from this search were then aligned to the sequence of bovine rhodopsin (UNIProt entry P02699) employing ClustalW (*31*), with the parameters set to their default values. The final alignment of the hY_5R sequence with the bovine rhodopsin sequence formed the basis of the subsequent modeling of the TM helices. The comparative models were created with the Homology Modeling module of the program package MOE 2005.06 (Chemical Computing Group, Inc., Montreal, Canada) neglecting the extracellular loops.

The three extracellular loops (ECLs) were modeled employing the CHARMM (*32*) based CLOOP protocol (*33*). The loops were constructed one after the other in all possible combinations. Thus, modeling the loops in the order ECL1 → ECL2 → ECL3 resulted in the model $hY_5R(123)$, the order ECL2 → ECL3 → ECL1 in the model $hY_5R(231)$, etc. The highly conserved disulfide bridge between $Cys^{3.25}$ and $Cys^{5.25}$ in the ECL2 was considered as a constraint in the CLOOP runs. For each construction run, 1000 trials per loop were performed. From these runs, the models with the lowest CHARMM energy were selected for the next run. Finally, the models were energy-minimized, employing the ABNR minimization protocol implemented in CHARMM. The N terminus has been neglected in the computations.

For the docking studies, the protein−protein docking program RosettaDock (*34*) was employed. Because of the open structure of ECL2, the receptor model $hY_5(132)$ was used. In this model, the residues $Asp^{2.68}$ and $Asp^{6.59}$, which are important for ligand binding, are accessible from the extracellular space. The NMR structure of NPY [RCSB entry 1RON (*35*)] was selected for docking. The RosettaDock perturbation docking runs were performed as described in ref *36*. Loose distance constraints of 10 Å between Arg^{33} of NPY and $Asp^{6.59}$ and Arg_{25} of NPY and $Asp^{2.68}$, respectively, were applied to ensure a docking of the ligand at the extracellular space. A total of 1000 docking runs were performed for each model. The resulting complexes were clustered on the pairwise root-mean-square deviations (rmsd values) with 2.5 Å as a cutoff.

Table 1: Influence of Mono- and Bivalent Ions on Binding and Potency

ion concentration		specific binding[a] (%)	EC_{50}[b] (nM)
without salt		100	2.2 ± 0.8
	Monovalent Ions		
NaCl	0.5 M		7.3 ± 4.0
	0.25 M	94 ± 3	5.0 ± 2.0
KCl	0.5 M		7.0 ± 3.6
	0.25 M	96 ± 4	4.5 ± 0.6
	Bivalent Ions		
$MgCl_2$	0.25 M		19.6 ± 2.1
	0.1 M	60 ± 1	18.7 ± 3.4
	0.01 M	96 ± 8	
	0.001 M	108 ± 4	
$CaCl_2$	0.25 M		117 ± 2
	0.1 M	29 ± 6	8.3 ± 0.9
	0.01 M	80 ± 10	
	0.001 M	93 ± 9	

[a] The specific binding in the presence of different concentrations of mono- and bivalent ions was compared to the specific binding without additional ions. [b] The potency was determined via the signal transduction assay.

RESULTS

Electrostatic Interactions Are Fundamental in the Human Y_5 Receptor System. First, we raised the question whether the major interaction between the hY_5 receptor and NPY is due to electrostatic attraction. Binding and signal transduction assays were performed in the presence of different concentrations of monovalent ions (Na^+ and K^+) or bivalent ions (Mg^{2+} and Ca^{2+}). Up to a concentration of 0.25 M Na^+ or K^+ ions, the specific binding was not significantly reduced (cf. Table 1). Even in the presence of 0.5 M monovalent ions, the EC_{50} values did not show significant differences compared to the EC_{50} value without additional ions. In contrast, the specific binding was dramatically reduced in the presence of 0.1 M bivalent ions as shown in Table 1. The addition of 0.1 M Mg^{2+} decreased the specific binding to 60 ± 1%, and in the presence of 0.1 M Ca^{2+}, the specific binding was barely detected (29 ± 6%). Furthermore, the EC_{50} values in the presence of 0.25 M bivalent ions are increased (Mg^{2+}, 19.6 ± 2.1 nM; Ca^{2+}, 117 ± 2 nM) suggesting a loss of affinity. The different influence of mono- and bivalent ions on the ligand−receptor affinity confirms the fundamental role of electrostatic attractions in the hY_5 receptor system.

Identification of Critical Residues of the Ligands for Binding. Next, we compared the important residues for receptor binding of the three endogenous ligands NPY, PYY, and PP. Thus, we substituted the three Arg residues Arg^{25}, Arg^{33}, and Arg^{35} individually by Ala in all three native ligands. As shown in Table 2, the EC_{50} ratios of analogue/wild-type peptides, in which one of the three Arg residues was replaced by Ala, are significantly increased. These data indicate that these three Arg residues are important for binding to the hY_5 receptor in all three ligands. A further similarity between these ligands is that Arg^{35} is the most important residue in all cases, because replacement of Arg^{35} by Ala resulted in a more than 100-fold increase of the EC_{50} values (cf. Figure 2A). Contrary to this, substitution of Arg^{25}

Table 2: Effect of the Single Amino Acid Replacement upon Binding and Potency of NPY, PYY, and PP at the hY_5 Receptor Wild Type

number	peptide	sequence	mass $[M + H]^+$ (*m/z*) calcd.	mass $[M + H]^+$ (*m/z*) exp.	t_{ret} (min)	binding[b] (%)	EC_{50}[a] (nM)	EC_{50}[a] *x*-fold over wild type
1	pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2	4254.7	4254.5	22.3	100	4.9 ± 1.6	1
2	[Ala^{25}]-pNPY	YPSKPDNPGEDAPAEDLARYYSAL**A**HYINLITRQRY-NH_2	4169.6	4169.2	23.4	68 ± 5	50 ± 21	10
3	[Ala^{27}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRH**A**INLITRQRY-NH_2	4162.6	4162.9	22.2	27 ± 9	290 ± 57	59
4	[Ala^{33}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLIT**A**QRY-NH_2	4169.6	4169.6	22.6	58 ± 10	185 ± 49	38
5	[Ala^{35}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQ**A**Y-NH_2	4169.6	4169.0	23.6	−3 ± 6	1592 ± 209	324
6	[Ala^{36}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQR**A**-NH_2	4162.6	4162.5	21.0	9 ± 4	1709 ± 134	348
7	pNPY free acid	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-**OH**	4255.7	4255.5	21.6	1 ± 14	2913 ± 714	593
8	hPYY	YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2	4310.9	4310.2	19.6	100	5.2 ± 2.5	1
9	[Ala^{25}]-hPYY	YPIKPEAPGEDASPEELNRYYASL**A**HYLNLVTRQRY-NH_2	4225.7	4225.5	20.4	51 ± 11	167 ± 46	32
10	[Ala^{27}]-hPYY	YPIKPEAPGEDASPEELNRYYASLRH**A**LNLVTRQRY-NH_2	4218.8	4219.3	18.9	19 ± 18	474 ± 107	92
11	[Ala^{33}]-hPYY	YPIKPEAPGEDASPEELNRYYASLRHYLNLVT**A**QRY-NH_2	4225.7	4225.5	19.7	88 ± 1	63 ± 41	12
12	[Ala^{35}]-hPYY	YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQ**A**Y-NH_2	4225.7	4225.9	20.1	−10 ± 13	4527 ± 71	874
13	[Ala^{36}]-hPYY	YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQR**A**-NH_2	4218.8	4219.0	18.5	23 ± 9	2137 ± 342	413
14	hPYY free acid	YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-**OH**	4311.8	4311.5	19.0	2 ± 10	9046 ± 2664	1746
15	hPP	APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-NH_2	4182.8	4182.4	22.0	100	26 ± 10	1
16	[Ala^{25}]-hPP	APLEPVYPGDNATPEQMAQYAADL**A**RYINMLTRPRY-NH_2	4097.7	4098.3	26.1	31 ± 8	211 ± 45	8
17	[Ala^{27}]-hPP	APLEPVYPGDNATPEQMAQYAADLRR**A**INMLTRPRY-NH_2	4090.7	4090.3	21.8	78 ± 8	36 ± 9	1
18	[Ala^{33}]-hPP	APLEPVYPGDNATPEQMAQYAADLRRYINMLT**A**PRY-NH_2	4097.7	4097.4	22.1	5 ± 5	671 ± 245	26
19	[Ala^{35}]-hPP	APLEPVYPGDNATPEQMAQYAADLRRYINMLTRP**A**Y-NH_2	4097.7	4097.2	22.3	−7 ± 7	3628 ± 621	141
20	[Ala^{36}]-hPP	APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPR**A**-NH_2	4090.7	4090.7	21.0	4 ± 13	8199 ± 1961	318
21	hPP free acid	APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-**OH**	4183.8	4184.0	21.2	31 ± 6	16590 ± 1910	643

[a] The potency of the peptides was determined via the signal transduction assay. [b] The specific binding of each modified ligand was compared to the specific binding of the corresponding unmodified ligand.

or Arg^{33} led to slightly different effects for PYY on the one side and NPY and PP on the other side. In NPY and PP, replacement of Arg^{33} by Ala resulted in a loss of potency by about 30-fold, whereas replacement of Arg^{25} led only to an 8−10-fold decreased potency. However, in PYY, [Ala^{25}]-PYY was more sensitive than [Ala^{33}]-PYY.

Then, Tyr^{27} and Tyr^{36} were replaced by Ala in NPY, PYY, and PP. The replacement of Tyr^{36} by Ala revealed a dramatic loss of potency reflected by a more than 300-fold increase of the EC_{50} values for all peptides. Tyr^{27} of NPY and PYY seems to play an important role in binding, too, suggested by the more than 50-fold increased EC_{50} values for the [Ala^{27}]-NPY/PYY peptides. Remarkably, replacement of Tyr^{27} in PP did not result in an increased EC_{50} value compared to unmodified PP, in contrast to NPY and PYY.

Replacement of the C-terminal amide with the free acid led to a dramatic loss of potency in all three ligand family members. The major difference between NPY, PYY, and PP was found for Tyr^{27}. Substitution of Tyr^{27} by Ala in PP does not significantly influence agonist potency at the hY_5 receptor wild type, whereas the replacement of Tyr^{27} by Ala in PYY ($EC_{50} = 474 \pm 107$ nM) and NPY ($EC_{50} = 290 \pm 57$ nM) resulted in a significantly decreased potency (cf. Figure 2A). This suggests that one contact point between PP and the hY_5 receptor is lost compared to NPY and PYY and may account for the reduced affinity of PP to hY_5R. All other residues of the ligands NPY, PYY, and PP, which are relevant for the hY_5 receptor, interact more or less in the same manner with the hY_5 receptor.

Influence of Substitutions of Conserved Amino Acid Residues of the hY_5 Receptor on Ligand Binding and Potency. To identify direct interaction points between the different ligands and the hY_5 receptor, highly conserved amino acid residues in the extracellular regions of the hY_5 receptor were exchanged by alanine. These receptor mutants were transiently expressed in COS-7 cells and functionally tested with wild-type NPY. The replaced positions are shown in Figure 3. No significant differences in binding, potency, and cell-surface expression were detected for the mutants indicated in white in comparison to the wild-type receptor (data not shown). This suggests that these amino acid residues do not participate directly or indirectly in the binding of NPY.

The replacement of those amino acids by Ala, which are highlighted in black (cf. Figure 3) resulted in a significant decrease of potency. As shown in Table 3, mutations of $Asp^{1.30}$, $Asp^{2.68}$, $Trp^{2.70}$, $Phe^{4.63}$, $Glu^{5.27}$, $Arg^{5.35}$, $Asp^{6.59}$, and $Tyr^{7.35}$ led to a significant loss of affinity. The specific binding of all of these mutants was reduced to only 5−20% compared to that of the wild type. To further characterize the mutant receptors, signal transduction assays were performed to determine the potency. A more than 100-fold reduction of potency was observed for $Asp^{2.68}$Ala, $Glu^{5.27}$Ala, and $Asp^{6.59}$Ala mutants. A slightly decreased potency of 6−50-fold was demonstrated for the $Asp^{1.30}$Ala, $Trp^{2.70}$Ala, $Phe^{4.63}$Ala, $Arg^{5.35}$Ala, and $Tyr^{7.35}$Ala mutants.

$Asp^{2.68}$ of hY_5 Receptor Interacts with Arg^{25} of NPY. Next, we set out to identify counterparts of the amino acid residues in the hY_5 receptor and NPY by a complementary mutagenesis approach. This approach was performed with all hY_5 receptor mutants that revealed a reduced potency shown in black in Figure 3. For this purpose, the NPY analogues listed in Table 2 were tested in the signal transduction assay at

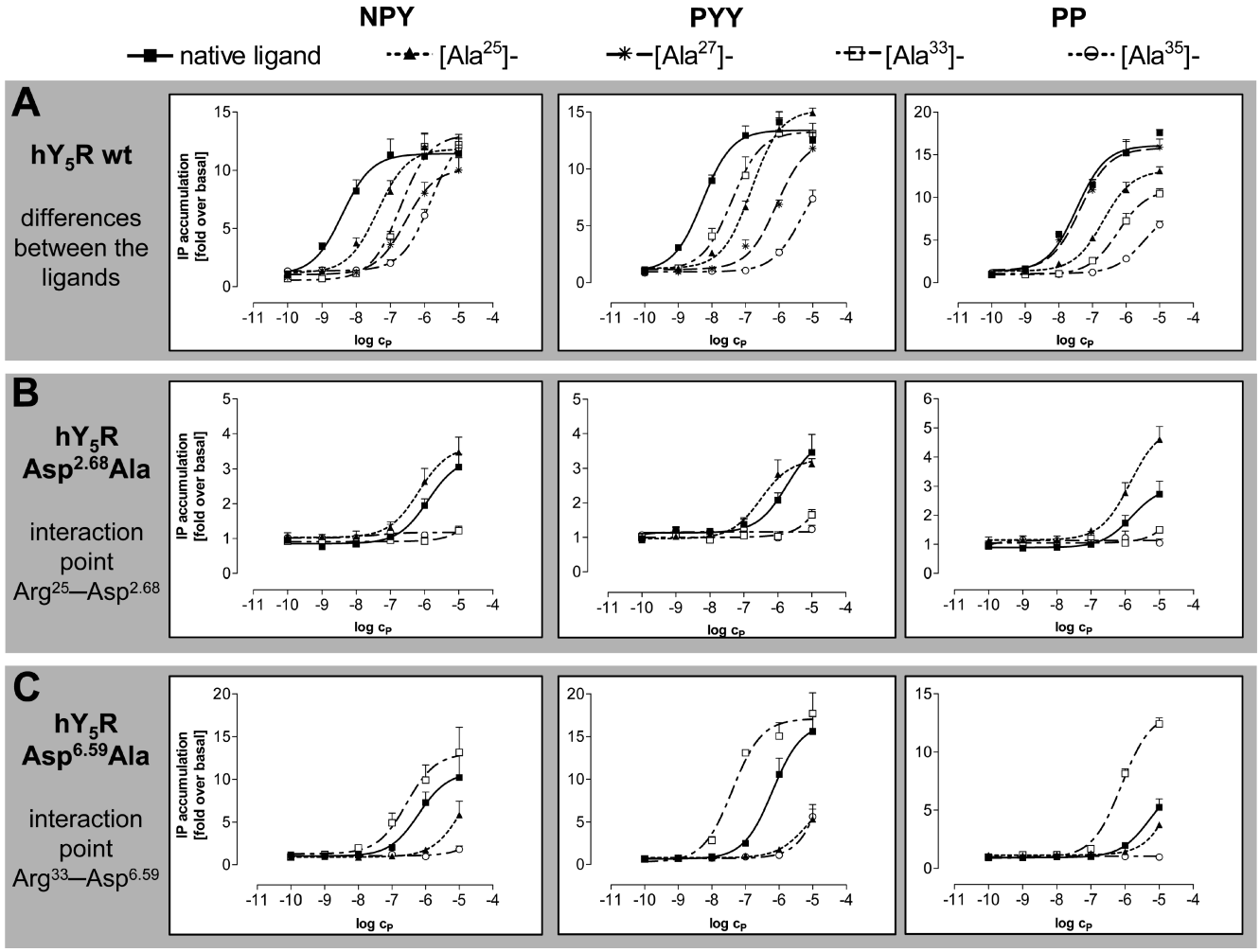

FIGURE 2: Differences and similarities of the NPY family members in binding to the hY_5 receptor. (A) Signal transduction assays were performed at the hY_5 receptor with NPY, PYY, and PP analogues, in which the Arg^{25}, Tyr^{27}, Arg^{33}, and Arg^{35} were individually substituted by Ala. The most significant difference was found in PP, in which the Tyr^{27} did not reveal a loss of affinity compared to NPY and PYY. (B) As the counterpart for the $Asp^{2.68}$ in the hY_5 receptor, the Arg^{25} of the ligands was identified using the complementary mutagenesis approach. (C) Interaction between Arg^{33} of NPY and $Asp^{6.59}$ of the hY_5 receptor was also confirmed for the Arg^{33} of PYY and PP. Concentrations are given in molars.

the hY_5 receptor mutants, and the potencies were compared to the potency of the unmodified NPY.

All NPY analogues shared decreased potencies at the hY_5 receptor mutants $Asp^{1.30}$Ala, $Trp^{2.70}$Ala, $Phe^{4.63}$Ala, $Glu^{5.27}$Ala, $Arg^{5.35}$Ala, and $Tyr^{7.35}$Ala. In such a complementary mutagenesis approach, one can assume that the decreased potency of a mutant receptor, in which an interacting residue was replaced, remains unchanged when tested with the NPY analogue, in which the counterpart of the replaced receptor residue is substituted as well. Accordingly, when the dominant peptide−receptor bond at that specific point is already disrupted by mutation of this residue in the receptor, the replacement of the interacting residue in the ligand has no further impact on the binding affinity at this receptor mutant. This implies that the residues $Asp^{1.30}$, $Trp^{2.70}$, $Phe^{4.63}$, $Glu^{5.27}$, $Arg^{5.35}$, and $Tyr^{7.35}$ in the hY_5 receptor are not the binding partners of the replaced residues in the Ala-substituted NPY, because the mutant receptor affinity is further shifted (data not shown).

Interestingly, this complementary mutagenesis approach revealed similar potencies for [Ala^{25}]-NPY and NPY at the $Asp^{2.68}$Ala mutant, whereas all other NPY analogues showed a right-shifted curve (cf. Figure 2B). The data of the complementary mutagenesis approach are shown in Table 4 and revealed that Arg^{25} of NPY interacts with $Asp^{2.68}$ of the hY_5 receptor. The evidence for this interaction is demonstrated by the ratio of the EC_{50} values of the NPY analogues and the wild-type NPY. The EC_{50} ratio of [Ala^{25}]-NPY at the hY_5 receptor wild type (cf. Table 4) as well as the EC_{50} ratio of the hY_5 receptor mutant $Asp^{2.68}$Ala with NPY (cf. Table 3) is higher than 1. This implies that both residues, the Arg^{25} of NPY and the $Asp^{2.68}$ of the hY_5 receptor, participate in binding. The EC_{50} ratio for the hY_5 receptor mutant $Asp^{2.68}$Ala tested with [Ala^{25}]-NPY is lower than 1, which is caused by the gain of function. Thus, a second contact point between NPY and the hY_5 receptor has been identified. When the EC_{50} ratios of the [Ala^{33}]-NPY were re-analyzed at the hY_5 receptor wild type and the two mutants $Asp^{2.68}$Ala and $Asp^{6.59}$Ala, it became clear that only the EC_{50} ratio of [Ala^{33}]-NPY at the hY_5 receptor mutant $Asp^{6.59}$Ala is lower than 1, which means that the already identified contact point Arg^{33}−$Asp^{6.59}$ could be confirmed by comparing the EC_{50} ratios.

To test for steric limitations and the importance of the negative charge, $Asp^{2.68}$ was also mutated to Glu, Asn, and Arg. As shown in Table 3, these mutants exhibited a nearly complete loss of binding (5−11% compared to that

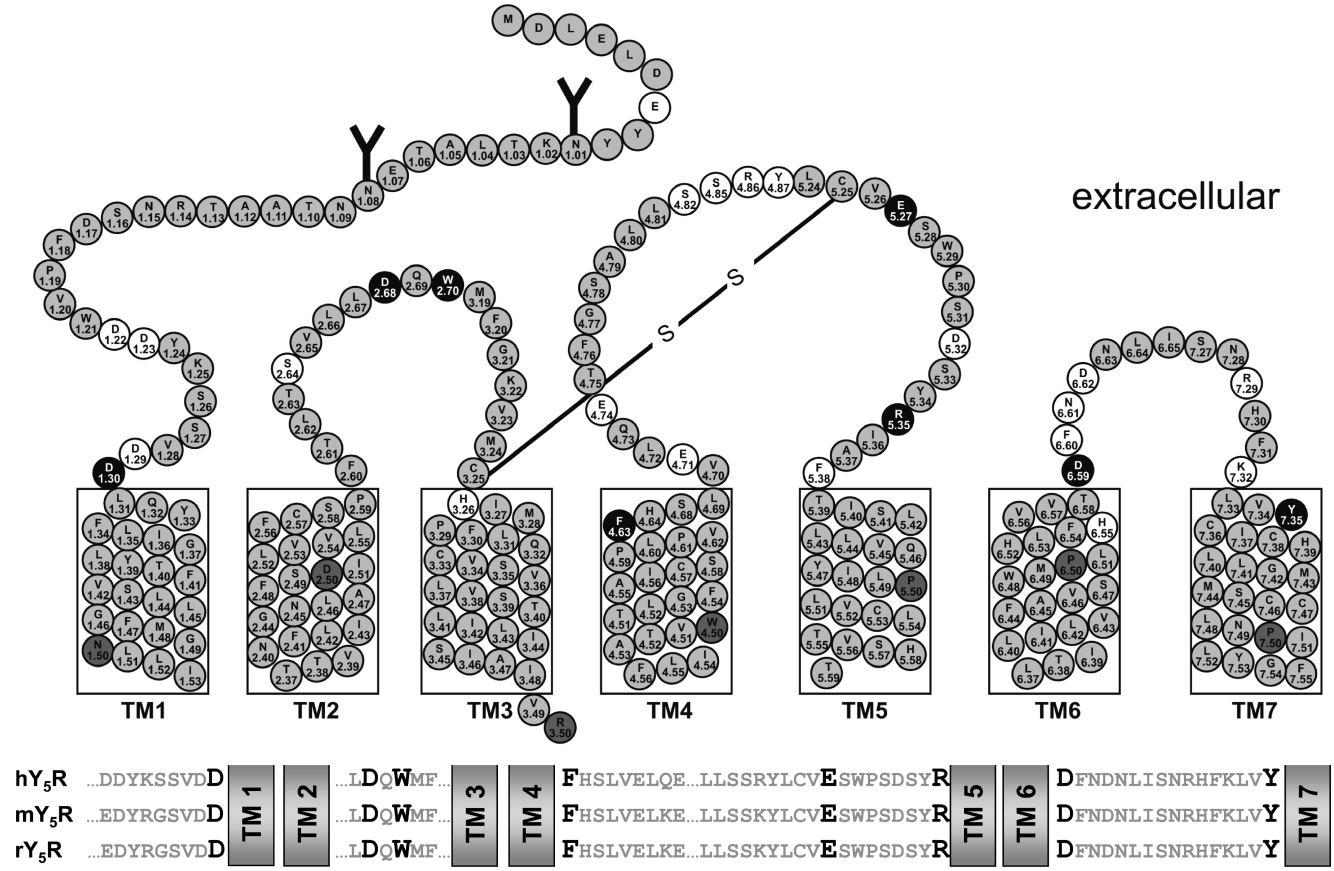

FIGURE 3: Identification of conserved residues important for binding within the sequence of the human Y_5 receptor. A decreased potency and loss of binding were observed for the receptor mutants, in which the residues highlighted in black were replaced by Ala. The Ala substitution of residues shown in white remains unchanged in binding affinity compared to the wild-type receptor (data not shown). For the numbering of the residues, the most conserved residue of each transmembrane helix shown in dark gray was used according to ref *23*.

Table 3: Functional Characterization of Wild-Type and hY_5 Receptor Mutants

	binding assay[a]	IP_3 assay[b]		cell-surface expression[c]
residue of the receptor	specific binding compared to wild type (%)	EC_{50} (nM)	*x*-fold over wild type	compared to wild type (%)
hY_5 wild type	100	5.8 ± 3.1	1	100
N-Terminal Region				
hY_5 $Asp^{1.30}Ala$	20 ± 7	87 ± 29	15	135 ± 61
First Extracellular Loop				
hY_5 $Asp^{2.68}Ala$	8 ± 7	1192 ± 335	206	120 ± 32
hY_5 $Asp^{2.68}Glu$	11 ± 3	125 ± 13	22	100 ± 17
hY_5 $Asp^{2.68}Asn$	5 ± 2	142 ± 15	24	119 ± 36
hY_5 $Asp^{2.68}Arg$	8 ± 5	727 ± 266	126	178 ± 25
hY_5 $Trp^{2.70}Ala$	16 ± 3	93 ± 46	16	157 ± 14
Second Extracellular Loop				
hY_5 $Phe^{4.63}Ala$	6 ± 5	99 ± 23	17	157 ± 40
hY_5 $Glu^{5.27}Ala$	9 ± 2	1899 ± 1339	329	172 ± 93
hY_5 $Arg^{5.35}Ala$	13 ± 7	33 ± 17	6	83 ± 9
Third Extracellular Loop				
hY_5 $Asp^{6.59}Ala$	10 ± 4	1303 ± 381	226	113 ± 9
hY_5 $Tyr^{7.35}Ala$	3 ± 6	227 ± 68	39	157 ± 74

[a] The specific binding of the mutant was compared to the specific binding of the wild-type receptor. [b] The potency was determined by the signal transduction assay. [c] The cell-surface expression compared to the wild-type receptor was observed via ELISA.

of the wild type) and showed a significant decrease of potency. When Asp is replaced by Glu, the EC_{50} value is 25-fold increased (EC_{50} = 125 ± 13 nM). This indicates a steric limitation at that contact point, because insertion of a methylene group into the side chain resulted in a loss of affinity. A similar effect was also observed for the mutant $Asp^{2.68}Asn$ (EC_{50} = 142 ± 15 nM), confirming the importance of the negative charge. In comparison to the Ala substitution, a lower EC_{50} value was found for the Asn substitution of $Asp^{2.68}$. Considering this difference, the conclusion that polar attractions are involved in the interaction between Arg^{25} of NPY and $Asp^{2.68}$ of the hY_5 receptor is further supported. The potency of the $Asp^{2.68}Arg$ mutant is similar to that of the $Asp^{2.68}Ala$ mutant, because polar or ionic attractions are impossible with these variants.

Receptor and Not the Ligand Decides the Binding Mode. At this stage, two direct contact points between NPY and the hY_5 receptor have been identified. Next, we wanted to study whether these two contact points between NPY and the hY_5 receptor are identical in different ligands. Accordingly, the complementary mutagenesis approach was also performed for PYY and PP (*22*). In analogy to the NPY studies, Arg^{25}, Arg^{33}, and Arg^{35} were substituted individually by Ala in PYY and PP. As shown in Figure 2B, the dose–response curve for [Ala^{25}]-PYY and [Ala^{25}]-PP at the hY_5 receptor mutant $Asp^{2.68}Ala$ is not further shifted to the right compared to unmodified PYY or PP. The same is found for the EC_{50} ratios of [Ala^{25}]-PYY and [Ala^{25}]-PP at the hY_5 receptor mutant $Asp^{2.68}Ala$. The ratios are about 1 or lower and confirm the contact point between Arg^{25} and

Table 4: Comparison of the Potencies of NPY, PYY, and PP with the Potencies of the Ala-Substituted Peptides at the hY_5 Receptor Wild Type and Mutants $Asp^{2.68}Ala$ and $Asp^{6.59}Ala$

	hY_5 receptor wild type		hY_5 receptor $Asp^{2.68}Ala$		hY_5 receptor $Asp^{6.59}Ala$	
peptide	EC_{50} (nM)	EC_{50} (Ala^x-peptide)/ EC_{50} (peptide)	EC_{50} (nM)	EC_{50} (Ala^x-peptide)/ EC_{50} (peptide)	EC_{50} (nM)	EC_{50} (Ala^x-peptide)/ EC_{50} (peptide)
pNPY	4.9 ± 1.6	1	1192 ± 335	1	1303 ± 381	1
[Ala^{25}]-pNPY	50 ± 21	10	736 ± 145	0.6	>6000	>4.6
[Ala^{33}]-pNPY	185 ± 49	38	>3000	>2.5	276 ± 8	0.2
[Ala^{35}]-pNPY	1592 ± 209	324	nd[a]		nd[a]	
hPYY	5.2 ± 2.5	1	1435 ± 378	1	1247 ± 765	1
[Ala^{25}]-hPYY	167 ± 46	32	397 ± 282	0.3	6677 ± 1153	5.4
[Ala^{33}]-hPYY	63 ± 41	12	3424 ± 693	2.4	34 ± 5	0.03
[Ala^{35}]-hPYY	4527 ± 71	874	>7000	4.9	nd[a]	
hPP	26 ± 10	1	1387 ± 234	1	4709 ± 279	1
[Ala^{25}]-hPP	211 ± 45	8	1778 ± 782	1.3	>12000	>2.5
[Ala^{33}]-hPP	671 ± 245	26	>6000	>4.3	839 ± 237	0.2
[Ala^{35}]-hPP	3628 ± 621	141	nd[a]		nd[a]	

[a] nd = not detectable with sufficient accuracy.

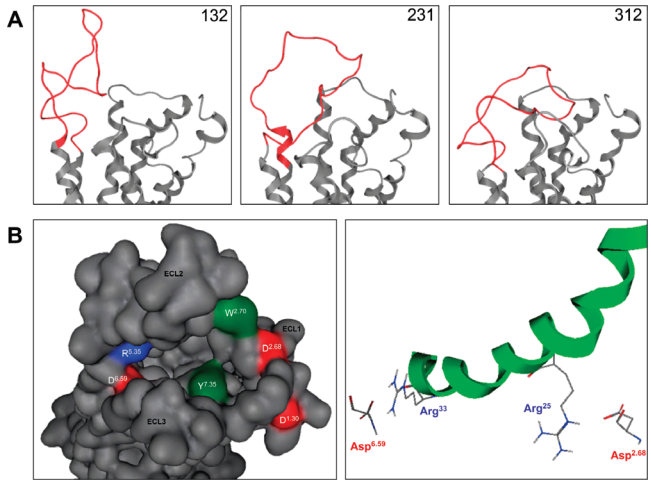

FIGURE 4: Computational model of the hY_5 receptor. (A) Various models result for the extracellular loops dependent upon the order of loop modeling demonstrated for ECL2 (cf. the text for details). (B) For the docking studies, the hY_5 receptor model (132) was used. Because of the open structure, the residues identified to be important for ligand binding are accessible from the extracellular space. The two contact points between NPY and the hY_5 receptor are shown.

$Asp^{2.68}$ also for PYY and PP. Thus, the interaction between Arg^{25} of PYY/PP and $Asp^{2.68}$ of the hY_5 receptor is the same as in NPY.

Similar studies were performed for the previously identified interaction, in which Arg^{33} of NPY binds to $Asp^{6.59}$ of the hY_5 receptor (*15*). Overlaying dose−response curves have been observed for [Ala^{33}]-PYY and PYY as well as for [Ala^{33}]-PP and PP at the hY_5 receptor mutant $Asp^{6.59}Ala$. Thus, the second contact point between NPY and the hY_5 receptor can be confirmed for all members of the neuropeptide Y family (cf. Figure 2C).

Modeling and Docking Studies. The modeling of the extracellular loops resulted in different receptor structures. Dependent upon the order of loop modeling, various conformations of the ECLs were obtained. In particular, the longest and most flexible loop ECL2 might, therefore, exist in different unclosed or closed structures (cf. Figure 4A). Thus, it will have an open structure if ECL1 is modeled first and ECL3 next (132). If ECL2 is modeled first followed by ECL3 and ECL1 (231), the position of ECL2 will be intermediate, and if the third loop is modeled first (312), ECL2 will cross the molecule along the membrane surface (cf. Figure 4A). Recently, the three-dimensional structure of the human β_2-adrenergic receptor has been resolved (*28*), in which ECL2 shows an open structure. This is in contrast to the crystal structure of rhodopsin, in which ECL2 crosses the molecule along the membrane surface (*29, 37*). However, the relative positions of the TM helices in the rhodopsin structure and the β_2-adrenergic receptor are similar. Considering the length of the structurally variable ECLs, the presented strategy to model only the TM helices with bovine rhodopsin as the template and build the loops de novo seems to be justified.

Because of its open structure, the hY_5 receptor model (132) was selected for further analysis. In this receptor model, most of the critical residues are surface-exposed and the two Asp residues, which are the counterparts of Arg^{25} and Arg^{33} of NPY, should be accessible for the ligand, as shown in Figure 4B. Whereas RosettaDock performs the docking calculations with flexible side chains, the backbones of the binding partners are kept fixed to make the docking experiments computationally feasible. Thus, larger movements of the ECLs or a conformational change of the C-terminal α helix of NPY cannot be captured within this approach. A detailed analysis of the solution structure of NPY [RCSB entry 1RON (*35*)] revealed that the N terminus is rather flexible, while the C-terminal α helix is well-structured. Thus, we assume that this helix is kept upon binding to the hY_5R. This is supported by the observation that the docked peptide ligand NPY fits well into the binding groove and fulfills the experimentally determined interactions (Arg^{25}−$Asp^{2.68}$ and Arg^{33}−$Asp^{6.59}$).

DISCUSSION

GPCRs form a large superfamily with many important physiological functions. As such, they have become important targets in pharmaceutical research (*38*). Analysis of the ligands as well as the receptors by site-directed mutagenesis and molecular modeling are essential to elucidate the binding site (*39*).

The interest in the Y_5 receptor exploded when pharmacology identified this receptor as one that may mediate the feeding response of NPY (*40, 41*). Thus, it is now up to intense interest to design a selective Y_5 receptor antagonist that might safely reduce food intake as a potential antiobesity

drug (*42–44*). Up to now, however, no major breakthrough was obtained in clinical studies and some promising compounds seem to inhibit food intake by non-NPY Y_5-related mechanisms (*45*). Understanding of the binding of the native ligands to this receptor subtype accordingly might help for a more successful and specific antagonist design. Therefore, we provide here an extensive mutational study on the hY_5 receptor. The first hY_5 receptor mutants have been described recently, in which three acidic residues of the extracellular loops were replaced by Ala and one of these residues was identified as the direct counterpart for Arg^{33} of NPY (*15*). In contrast to the Y_1 receptor that is extensively investigated by mutational studies, no further amino acid replacements in the Y_5 receptor sequence have been reported up to now (*46–49*).

The Y_5 receptor subtype was first cloned from rat tissues in 1996 (*40, 50*). The gene of this receptor encodes a 445 amino acid−protein that is very well conserved in mammalian species (88−90% overall amino acid identity and 95−98% identity, when the third intracellular loop is not taken into account) (*51*). In contrast, the Y_5 receptor only has a relatively low sequence similarity of 30% compared to other Y receptor subtypes. Three native ligands are known for the Y receptor family, named NPY, PYY, and PP (*16, 19, 52*). The most potent agonists at the hY_5 receptor are NPY and PYY; however, PP shows still a rather high affinity (*41, 53, 54*).

Recent studies described that in particular three Arg residues Arg^{25}, Arg^{33}, and Arg^{35} as well as two Tyr residues Tyr^{27} and Tyr^{36} in NPY are involved in binding to the hY_5 receptor (*15–17, 52*). Here, we provide the evidence that these Arg residues and Tyr^{36} as well as the amidated C terminus are participating in the binding for all three ligands not only in NPY.

Consequently, we assume a similar binding mode for these three peptides at the hY_5 receptor. Furthermore, the rationale for the lower affinity of PP in comparison to the higher affinity NPY and PYY was found. In contrast to PYY and NPY, the replacement of Tyr^{27} to Ala in PP does not cause a loss of binding affinity, which indicates that this contact point between PP and the hY_5 receptor is missing. This might explain the reduced affinity of PP to the hY_5 receptor.

To characterize the attraction between NPY and the hY_5 receptor, the binding affinity was measured in the presence of mono- or bivalent ions. In comparison to monovalent ions, bivalent ions are able to reduce the Debye length of charged residues to a greater extent. Thus, the effective reach of electrostatic attraction is dramatically decreased in the presence of bivalent ions compared to monovalent ions. In conclusion, we demonstrated here that electrostatic attractions are responsible for binding of NPY to the hY_5 receptor and can now assume that the Arg residues of NPY form ionic, polar, or π-cationic interactions with residues in the hY_5 receptor. Acidic and aromatic residues are considered as potential counterparts. Four acidic residues ($Asp^{1.30}$, $Glu^{5.27}$, $Asp^{2.68}$, and $Asp^{6.59}$) and two aromatic residues ($Trp^{2.70}$ and $Tyr^{7.35}$) were identified as possible counterparts for these Arg residues. The Tyr residues of NPY possibly form π-cationic interactions with positively charged residues or hydrophobic interactions with aromatic amino acids in the hY_5 receptor. Only one positively charged amino acid residue, $Arg^{5.35}$, was identified to be critical for binding of NPY. However, the identified residues could also play a role to form the active receptor conformation or in proper folding of the receptor instead of being a direct binding contact.

We have recently reported on the identification of direct counterparts between NPY and the hY_5 receptor (*15*). It was described that Arg^{33} of NPY interacts with the $Asp^{6.59}$ of the hY_5 receptor. Using the described complementary mutagenesis approach, we identified a second contact point between NPY and the hY_5 receptor. The Arg^{25} of NPY interacts via ionic and polar attractions with the $Asp^{2.68}$ of the hY_5 receptor. For the other identified residues in the hY_5 receptor, binding partners are currently being investigated.

In the model of the hY_5 receptor, in which the second loop shows an open structure, the two interaction points are accessible for NPY. Because these residues are located near the ends of the TM helices, their relative positions are mainly determined by the orientation of the TM helices. Because of the fact that the hY_5 receptor is the first Y receptor subtype, for which a second contact point with NPY could be identified, docking studies have been performed to identify the orientation of the binding pocket. Our model suggests that the C-terminal α helix of NPY remains intact upon binding to the hY_5 receptor. Furthermore, most of the residues identified by the mutagenesis studies to be critical for NPY binding are located within the postulated binding pocket of the $hY_5R(132)$ model. However, further direct interaction partners between the hY_5 receptor and NPY could not yet be identified. Because of the high flexibility of the second loop, we speculate that this loop closes after binding of the ligand and keeps the ligand in the binding pocket. The high conservation of $Trp^{2.70}$ in most peptide GPCRs implies its structural relevance, possibly by structuring the ECL1 via formation of a hydrophobic cluster. The residue $Tyr^{7.35}$ forms the bottom of the proposed binding grove and is accessible too. Although this aromatic residue 7.35 is found only in a few peptide GPCR families, such as the QRFP and NPFF receptors, $Phe^{7.35}$ has already been reported to be critical for activation of the melanocortin-4 receptor (*55*).

For the other two members of the neuropeptide Y family, PP and PYY, the same two interaction points could be confirmed. It was previously shown that Arg^{35} of PP interacts with $Asp^{6.59}$ of the hY_4 receptor in contrast to the contact point in the hY_5 receptor (Arg^{33}−$Asp^{6.59}$). Besides, the $Asp^{2.68}$ residue is highly conserved in the hY_5 receptor of different species as well as in the hY_4 receptor. However, a similar interaction in the hY_4 receptor is not suggested because Arg^{25} of PP is not involved in binding to this Y receptor subtype (*15*). Finally, in this study, we presented the evidence that the receptor and not the preformed conformation of the ligand decides the binding mode in the neuropeptide Y multireceptor/multiligand system, because we clearly demonstrated that the three members of the neuropeptide Y family have the same contact points within the hY_5 receptor but different interaction partners in the hY_1 and hY_4 receptors. In the past, it was speculated that the preorientation of the ligand on the membrane decides the binding mode, which according to our results can now be excluded (*56*).

The multireceptor/multiligand problem was recently addressed in the orexin system. Several amino acid residues in both ligands were substituted by Ala and functionally tested on both receptors OxR_1 and OxR_2; however, contact points could not be identified (*4, 5*). This is consistent with the Y receptor system in which distinct residues are involved

in binding to the various Y receptor subtypes (*15*). However, by identifying the binding partners, we could show that, even when the ligands have important residues on more than one receptor, they could interact differently and bind to different partners in the receptor subtypes.

In conclusion, our comprehensive mutagenesis data on the Y_5 receptor provides three novel, important contributions. We identified a second direct interaction point between NPY and the hY_5 receptor that now allows for the description of the ligand−receptor interaction on a molecular level and in a 3D model. Next, we could prove that all three ligands bind to the hY_5 receptor in the same manner but different to the binding mode in hY_1 and hY_4. Accordingly, the membrane-bound peptide conformation does not contribute to receptor selectivity. Third, we identified one lost contact in the PP−hY_5 interaction that might explain the reduced affinity of PP to the hY_5 receptor, compared to NPY and PYY.

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