INTRODUCTION TO THE NEUROPEPTIDE Y FAMILY

The neuropeptide Y (NPY) system is a multireceptor/multiligand system consisting of four receptors in humans and three polypeptides that bind and activate them with different affinity and potency. The NPY receptors belong to the class A or rhodopsin-like G-protein coupled receptors (GPCR). Five receptors have been cloned from mammals so far, Y1, Y2, Y4, Y5, and y6 but only four of the members are functional in humans (hY1, hY2, hY4, hY5; Table 1). The y6 receptor however is active in rabbit and mouse (Starback et al., 2000). The existence of an additional receptor subtype (Y3) was suggested by pharmacological studies of several human, rat, and rabbit tissues including the human adrenal medulla. This receptor subtype is characterized by a much lower affinity for PPY, compared to NPY (Gehlert, 1998; Lee and Miller, 1998). However, since all attempts to clone this receptor subtype were unsuccessful so far, the existence of Y3 is not very likely.

Neuropeptide Y receptors (NPYR) generally couple to Gs or Gi proteins, which leads to the inhibition of adenylate cyclase and finally to the inhibition of cAMP accumulation (Cabrele and Beck-Sickinger, 2000) and modulation of Ca2+ and K+ channels (Holliday et al., 2004). Besides this, it has been described that Y2 and Y4 receptors also couple to the Gq protein increasing inositol 1,4,5-trisphosphate (IP3) production via the activation of the phospholipase C-β (PLC) in rabbit smooth muscle cells (Misra et al., 2004).

Neuropeptide Y, peptide YY (PYY), and pancreatic polypeptide (PP) are the native ligands of the NPY family. NPY is the most abundant peptide in the mammalian brain and has been suggested to adopt a largely open structure. In surface association processes makes this system suitable for the design of subtype selectivity studies. In this review we focus on the latest findings within the NPY system, we summarize recent mutagenesis studies, structure activity relationship studies, receptor chimera, and selective ligands focusing also on the binding mode of the native agonists.

Keywords: GPCR, NPY, YR, subtype selectivity, ligand side, receptor side

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Table 1 | Amino acid sequence of the NPY ligands.

| Peptides | Amino acid sequence |
|----------|---------------------|
| nNPY     | YS KPDNPGEADAPAE DLARYYSALRHYINLITRQRY |
| nPYY     | YPIKEAPEADASPPEELNRYYASLRRHYLNVLTRQRY |
| nPP      | APLEPVYPGDNATPEGMAGYAADLRRYINMLTRPRY |

Table 2 | NPYR: sequence length and ligand preference.

| Receptor | hY1 | hY2 | hY4 | hY5 |
|----------|-----|-----|-----|-----|
| Native ligand | NPY | NPY | PP | NPY |
| PY | PY | PY |

formation (Baldock et al., 2009; Sousa et al., 2012), regulation of mood and anxiety disorders, the modulation of stress responses (Heilig, 2004), and ethanol intake (Thiele et al., 1998).

Neuropeptide Y family peptides mediate their activity in humans via four receptors. Structurally, these receptors contain two Cys residues in the extracellular regions that form a disulfide bond between extracellular loop I and II. This disulfide bond is a common feature of class A GPCRs and has been confirmed by X-ray crystallography for several members including bovine rhodopsin and the human β2 adrenergic receptor (Pålczewski et al., 2000; Chernev et al., 2007).

The evolution of this system shows that vertebrate ancestors probably had three receptor genes. These genes, possibly located in close proximity in the same chromosomal segment, would be the precursors of the receptor subfamilies. The Y2 subfamily includes the Y2, Y4, and Y6 receptors, the Y2 subfamily comprises Y2 and Y5; (in zebrafish and frogs), and the Y5 subfamily consists of only the Y5 due to lack of close relatives of this receptor (Larhammar, 1996; Blomqvist and Herzog, 1997). The Y5 receptor binds NPY and PYY with comparable affinities. The affinity for PP is slightly lower, but still in the nanomolar range (Gerald et al., 1996). Y5 receptors are mainly expressed in the central nervous system. Tissues with high receptor density include the hippocampus, neocortex, and thalamus (Caberlotto et al., 1997), but is also present in adipose tissue (Castan et al., 1995; Hausman et al., 2008), blood vessels (Cabrele and Beck-Sickinger, 2000), colon, kidney, adrenal gland, heart, and placenta (Wharton et al., 1993). It plays a role in the regulation of food intake (Kanatani et al., 2000b), vasoconstriction of blood vessels (Cabrele and Beck-Sickinger, 2000), heart rate, anxiety (Balasubramaniam et al., 2002), and bone homeostasis (Sousa et al., 2012).

The Y2 receptor is predominantly expressed in hippocampal neurons, in the thalamus, hypothalamus, and parts of the peripheral nervous system (Waldowson, 1993; Cabrele and Beck-Sickinger, 2000). It is mainly found in pre-synaptic neurons and exerts its action through the regulation of neurotransmitter release (Wahlstedt et al., 1986; Potter et al., 1989). Typical effects correlated with activation of this receptor include enhanced memory retention, the regulation of the circadian rhythm, angiogenesis (Flood and Morley, 1989; Golombek et al., 1996; Grifkoff et al., 1996; Zukowska-Grojec et al., 1998) and bone formation (Baldock et al., 2009). This receptor consists of 381 amino acids and its preferred agonists are NPY and PYY (Table 2).

The Y4 receptor subtype is the only member of the family with the endogenous agonist PP; while PYY and NPY can still activate this receptor with minor potency (Table 2). It consists of 375 amino acids and is mainly expressed in the gastrointestinal tract (Lundell et al., 1995; Ferrier et al., 2002) but also in the brain (Bard et al., 1995), as well as pancreas and prostate (Lundell et al., 1995). It plays a role in the regulation of feeding (Asakawa et al., 1999; Sainsbury et al., 2010), circadian ingestion and energy homeostasis (Eidebrunner et al., 2009), colonic transit (Morita et al., 2010), and stimulation of the late-maturing hormone release (Stein et al., 1999).

The Y6 receptor subtype is expressed in different splice variants, composed of 445 and 455 amino acids, respectively (Table 1). The N-terminus of the longer isoform is extended by 10 amino acids. However, these differences in the sequence of the receptor isoforms do not result in differences in their pharmacological profile (Rodriguez et al., 2003). Both receptor isoforms bind NPY and PYY with comparable affinities. The affinity for PP is slightly lower, but still in the nanomolar range (Gerald et al., 1996). Y6 receptors are mainly expressed in the central nervous system. Tissues with high receptor density include the hippocampus and hypothalamus. The Y6 receptor subtype has been shown to be strongly involved in food intake (Gerald et al., 1996). Other possible roles of the Y6 receptor are the regulation of the circadian rhythm (Matsumoto et al., 1996b; Grifkoff et al., 1996) and reproduction through inhibition of LH release (Raposinho et al., 2001).
The Y6 receptor encodes a 371 amino acid protein that has been cloned from rabbit, mouse, and chicken among others (Bromée et al., 2004). However, the sequence in humans and monkeys contains a frame shift mutation in the third intracellular loop, resulting in a non-functional truncated receptor protein (Matsumoto et al., 1998a; Michel et al., 1998).

As it has been previously described, the binding affinity of each subtype selective ligands and receptors to modulate these characteristics. Developing selective ligands for NPY receptors is a perfect candidate in which to investigate important positions on the peptides are N- or C- terminal truncations.

Another approach to investigate subtype selectivity is the alanine-scan or Ala-scan: this means that each residue in the sequence is one by one individually substituted with Ala. When an Ala occurs naturally in a certain position, this residue is then changed to Gly. In this scan, only the functional groups are substituted permitting the investigation of ionic interactions as well as dipole-dipole and hydrophobic interactions. Once all the analogs are synthesized they must be tested at all the receptor subtypes to determine the distance between two segments. Finally, the use of cyclization can significantly constrain the conformation of a ligand. Several cyclization techniques can be applied, the most commonly used are: cyclization by disulfide formation between two Cys residues, cyclization by lactamization of N- and/or C-terminus or by the N- and C-group-containing side chains Lys, Orn, Dab, Asp, and Glu and backbone to side-chain cyclization. Recent studies also use click reactions to cyclize peptides using triazoles to mimic disulfide bridges (Holland-Nell and Meldal, 2011) and peptide stapling to increase the propensity to form α-helices, therefore improving pharmacological properties (Verdino and Walensky, 2007).

**Y1 RECEPTOR**

N- and C-terminal truncations confirm the importance of these two segments for Y1 receptor binding. N-terminally truncated analogs are not well accepted by the Y1 receptor as can be seen in studies using the shortened sequences NPY(3–36), (13–36), and (18–36). These show only micromolar affinities for this receptor and even the truncation of the first amino acid NPY(2–36), results in a loss of affinity (Beck-Sickinger and Jung, 1995). C-terminal truncations show the importance of the amide group in the binding with the receptor (Hoffmann et al., 1996). Centrally truncated analogs containing the spacer Aib and structurally constrained analogs showed that the N- and C-terminal fragments must have a certain length to bind with a good affinity to the receptor (Kirby et al., 1995b). Furthermore, using an Ala-scan it was found that, Pro2, Pro3, Arg19, and Tyr20 are important for ligand affinity. Also the amino acids from positions 27 to 36 were found to be crucial for the peptide, especially position 27. Moreover, positions 33 and 35 showed to be extremely important, as Ala analogs at these positions produced a dramatic loss in binding of >5000-fold over wt peptide (Figure 3; Beck-Sickinger et al., 1994; Cabrele and Beck-Sickinger, 2000; Lindner et al., 2008b). The importance of Arg19 was further confirmed as this residue was found to form a subype-specific ionic interaction with Asp39 of the receptor (Merten et al., 2007). The Tyr on position 36 was also found to be relevant for the ligand binding mode and receptor preference of small antagonists in structure-activity relationships, the knowledge of these binding subtypes is of great interest. Furthermore, to investigate the binding mode and receptor preference of small antagonists or non-peptidic drugs, knowledge of the bioactive conformation is of major importance. Constraining the ligand conformation and testing the peptide on several receptor subtypes, can provide information about its bioactive conformation and receptor selectivity. Several strategies can be used to investigate structure activity relationships constraining the conformation of small peptides (Beck-Sickinger, 1997). First of all, non-proteinogenic amino acids can be incorporated, reducing the number of angle combinations that a natural amino acid could adopt, and thereby decreasing the flexibility of the peptide. One example of a non-proteinogenic amino acid is Aib (aminoisobutyric acid). This residue is the most commonly used in this kind of study. Secondly, the use of several templates and amino acid linkers to induce a desired conformation might be also a good strategy, although this does not always lead to the desired effect because of other amino acids within the sequence. The use of more flexible linkers such as Ahx (6-aminohexanoic acid) or ß-amino alkanoic acids might be a better method to determine the distance between two segments. Finally, the use of cyclization can significantly constrain the conformation of a ligand. Several cyclization techniques can be applied, the most commonly used are: cyclization by disulfide formation between two Cys residues, cyclization by lactamization of N- and/or C-terminus or by the N- and C-group-containing side chains Lys, Orn, Dab, Asp, and Glu and backbone to side-chain cyclization. Recent studies also use click reactions to cyclize peptides using triazoles to mimic disulfide bridges (Holland-Nell and Meldal, 2011) and peptide stapling to increase the propensity to form α-helices, therefore improving pharmacological properties (Verdino and Walensky, 2007).

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binding; this position does however tolerate the exchange to Phe, but not Ala, Bpa, or His. Similar results were obtained using a D-amino acid scan (Kirby et al., 1993).

Positions 7, 25, 26, 31, and 34 were revealed to be important for subtype selectivity (Figure 1A). Modifications in positions 25 and 26 showed that [D-Arg25]NPY and [D-His26]NPY bind selectively to the Y1 receptor (Mullins et al., 2001). Also the introduction of Pro in position 34, present in pancreatic polypeptide, redirected the affinity of the peptide to Y2/Y5 receptors. Apart from Gln34, an additional exchange in Asn7 introducing Phe at this position, a similar residue like the Tyr present on the hPP, yielded [Phe7, Pro34]pNPY. This is a selective Y1 receptor binder and illustrated the importance of an aromatic residue in this position (Soll et al., 2001). Also the combination of Pro34 with an exchange in position 31 by Leu contributes to a Y1/Y4/Y5 receptor selective profile (Fuhlendorff et al., 1990; Cabrele et al., 2000). All this strongly indicates the importance of N- and C-terminal fragments for the Y2 receptor subtype.

The synthesis of small selective ligands has also been a topic of interest in the past years and many peptides have been synthesized and characterized. Although the first experiments with short- or medium-sized pNPY truncations showed low binding affinity at the Y2 receptor, in recent years several short antagonists, mimicking the NPY C-terminus have been synthesized such as, GR231118 (1229U91 or GW1229), T-241, and T-190. Unfortunately, these ligands also have Y2 agonistic properties (Figure 2; Parker et al., 1998). Taking the short NPY analog NPY (28–36) and the antagonist GR231118, Zwanziger et al. (2009) designed a set of 19 short peptide analogs. Only [Pro30, Nle31, Bpa32, Leu34]NPY(28–36) displayed Y2 receptor selectivity and was able to activate the receptor (Figure 1B). Follow-up investigations were made by Hofmann and colleagues (Neuropeptides, accepted) on position 32. The authors could further stabilize the peptide by replacing Bpa by Bip (biphenylalanine) and could switch the activity from hY2 receptor to hY2/hY4 receptors by introducing an ortho-carboranoyl moiety. Other small peptide antagonists are BW1911U90 and [5β-3MACE]NPY(25–36); Figure 2; Koglin et al., 2003), and examples of known non-peptidic antagonists are BIBP3226, BIBO3304, LX357897, J-194870 (Figure 4A; Rudolf et al., 1994; Hipshkind et al., 1997; Winland et al., 1998; Sjodin et al., 2006; Antal-Zimanyi et al., 2008).

Y2 RECEPTOR

As with the human Y1 receptor, the Y2 receptor binds NPY and PYY with comparable affinities. Besides these two native high-affinity ligands, a number of Y2-selective NPY-derived peptide
agonists have been synthesized in the past. Interestingly, in contrast to all other Y receptors, Y2 receptors allow large truncations of the peptidic ligands without loss of affinity (Beck-Sickinger and Jung, 1995) and also cyclizations between N- and C-terminally located residues are tolerated (Kirby et al., 1993). Most commonly used Y2 receptor selective NPY-analogs are the N-terminally truncated NPY(3–36) and NPY(13–36). Even larger N-terminal truncations and centrally truncated analogs can bind to the Y2 receptor with nanomolar affinity (e.g., NPY(18–36), NPY(22–36), NPY(26–36), [Ahx5–24]NPY; Figure 1B; Beck et al., 1989; Fournier et al., 1994; Beck-Sickinger and Jung, 1995; Keire et al., 2000b). An Ala-scan of the complete NPY peptide revealed only few positions to be highly important (Figure 3). The substitution of Pro5 to Ala led to a 600-fold loss of affinity. Accordingly, all other important residues except Pro5 are located in the C-terminal part of NPY. The individual substitution of Arg19, Tyr20, Tyr27, and Asn29 in the NPY peptide showed a 30- to 40-fold lower affinity. A more dramatic effect could be observed for the residues Leu31 (1000-fold lower affinity), Arg35 (1350-fold), Gln34 (150-fold), and Tyr36 (17500-fold; Figure 3; Cabrele and Beck-Sickinger, 2000; Eckard et al., 2001). Interestingly, the introduction of a Pro residue at position 34 is not tolerated at the Y2 receptor, which is in contrast to the effect observed on the other Y receptor subtypes (Beck-Sickinger et al., 1994; Keire et al., 2000b; Eckard et al., 2001). Although Tyr36 may not be substituted by Ala, the introduction of Hty (homotyrosine) or p-substituted Phe in PY(3–36) is well tolerated at the Y2 receptor, but almost completely abolishes binding of the modified NPY analogs at Y1 or Y4 receptors (Pedersen et al., 2009).

Taken together, these data underline the importance of the C-terminal part of the peptide ligand for high-affinity binding to the Y2 receptor, despite the fact that the binding pocket for NPY at the Y2 receptor seems to be less narrow than the ones of Y1 or Y4 receptors.
A number of selective high-affinity antagonists at the Y2 receptor have been published so far. The most widely used compound in pharmacological studies is BIIE0246 (Figure 5A; Doods et al., 1999). In order to identify compounds with improved bioavailability, and brain permeability, further studies have been conducted. A number of molecules and scaffolds have been reported as highly selective and affine small molecule Y2 receptor antagonists (Figure 5A) including JNJ-327787 (Bona-aventure et al., 2004; Jablonowski et al., 2004), SF-11, SF-21, SF-22, SF-31, SF-41 (Brothers et al., 2010), MLO72 to MLO75 (Saldanha et al., 2009), JNJ-M1020028 (Shoblock et al., 2010; Swanson et al., 2011), a series of substituted 3-chloranilides (Lunniss et al., 2009, 2010), CYM 9484, and CYM 9552 (Mittapalli et al., 2012).

Y4 RECEPTOR

The Ala-scan of the NPY (Eckard et al., 2001) revealed that again Arg30 and Arg31 are crucial for receptor affinity. Ala substitutions in these positions led to a dramatic loss in binding. Positions

![Chemical structures of non-peptidic antagonists for Y1 and Y4 receptors. (A) Antagonists for the Y1 receptor (Rudolf et al., 1994; Hipskind et al., 1997; Wieland et al., 1998; Balasubramaniam et al., 2001; Sjodin et al., 2006; Antal-Zimanyi et al., 2008); (B) Antagonists for the Y4 receptor (Ziemek et al., 2007).](image)

![Chemical structures of non-peptidic antagonists of Y2 and Y5 receptors. (A) Y2 receptor antagonists (Doods et al., 1999; Saldanha et al., 2009; Shoblock et al., 2010; Swanson et al., 2011); (B) Y5 receptor antagonists (Criscione et al., 1998; Kanatani et al., 2000a; Rueger et al., 2000; Kakui et al., 2006).](image)
Tyr^{22}, Tyr^{27}, Arg^{29}, Thr^{32}, and Tyr^{38} are also important residues in the ligand and showed a loss in binding affinity (30–to-60-fold), whereas Pro^{2}, Pro^{7}, and Tyr^{21} proved to be less relevant, causing only a slight loss in affinity (5–to-10-fold) when changed to Ala (Figure 3).

In a follow-up study using hNPY (Merten et al., 2007), Arg residues 33 and 35 were confirmed to be essential for receptor activation, showing a dramatic effect when changed to Ala in position 33. Position Arg^{21} had been found to interact with Asp^{30} of the receptor.

Because this receptor subtype has its own selective ligand, peptide research is more focused on improving proteolytic stability and increasing bioavailability of the peptide. However, a number of specific ligands have been published in the past years for this receptor. As previously described, position 34 of NPY peptides is a key residue to introduce Y4 receptor selectivity to NPY and PYY, whereas in PP when exchanging Pro^{24} for Gln the peptide acquires Y2 agonistic properties without losing Y4 receptor activity. Some of the analogs published like [Gln^{34}]-hPP, the so called Clanepitide (Schwartz, 2006), which is selective for Y2 and Y4 receptors, contains this exchange (Figure 1C). Other small peptide agonists described also as Y1 receptor antagonists are: GR23-1118 ([D-[Trp^{32}]NPY] and [Ala^{31}, Abn^{34}]NPY with a ∼15-fold decreased affinity compared to pNPY (Cabrele and Beck-Sickinger, 2000).

An Ala scan of the complete peptide revealed the Pro residues 2, 5, and 8 to be important for the affinity of NPY at the Y5 receptor, further N-terminal truncation of NPY results in a decreased affinity of the peptides. Similarly, larger central truncations of NPY are not tolerated by Y5 receptors. The only centrally truncated analog of NPY with high Y5 receptor affinity is [Ala^{21}−38]pNPY with a ∼15-fold decreased affinity compared to pNPY (Cabrele and Beck-Sickinger, 2000). When investigating the relevance of the N-terminus, successive truncations or substitutions using tags or spacers can be an elegant method. Moreover, C-terminal truncations can provide information about segments relevant for internalization. It is known that this receptor part is involved in arrestin-dependent internalization processes of Y1, Y2, and Y5 receptors (Waller et al., 2011). However, single mutagenesis techniques can be used to investigate important residues for the structure or for ligand receptor interactions. Using this approach, certain residues located in extracellular areas of the protein are mutated to Ala or other amino acids in single substitutions. The residues can be chosen according to its location, charge, aromaticity, hydrophobicity. Moreover, 3-D models are also a good tool to select new targets, although mutagenesis data are needed to refine the models and make them more reliable. Once a relevant residue has been identified, double

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cycle mutagenesis can be used to find the type of interaction that includes both positions. In this technique, peptide analogs containing modifications in positions of interest are investigated with receptor mutants. The aim is to form artificial bonds to prove a native interaction. The introduction of charged residues in the peptide and receptor positions to create a repulsion/attraction, or aromatic residues and hydrophobic residues are feasible ways to prove a ligand-receptor interaction. In order to finally prove a ligand-receptor interaction, a reciprocal mutation approach can be followed, where the residue of interest on the peptide side is exchanged by the residue present on the receptor side and vice versa. In the case of a critical position or segment, the binding affinity of the native ligand should significantly decrease, whereas in signal transduction assays the EC50 or half maximal activation value should increase. Despite all the advantages that these approaches provide, it has to be taken into account that they also present some disadvantages. Thus, when constructing receptor chimeras or receptor mutants, alterations in the receptor structure may arise due to misfolding and therefore may lead to impaired receptor export. Moreover, these modifications might result in a reduced receptor retention time at the cell surface and an enhanced degradation. All together, this might lead to a loss in binding or receptor activity. In order to analyze such phenomena, fluorescence microscopy, cell surface ELISA or radioligand binding studies are a good tool to ensure cell surface expression.

**Y1 RECEPTOR**

In the past years, much effort has been made to characterize this receptor. Using N-terminal truncations and receptor chimera, it could be elucidated that the N-terminal part of NPY receptors does not participate in the binding pocket. N-terminal truncation in the hY1 receptor disrupts the membrane expression; however any eight residues are enough to recover the membrane expression (Lindner et al., 2009).

From all these studies, a number of residues emerged as important for the receptor. First of all, two negatively charged residues are able to establish electrostatic interaction. Asp2.68 and Asp6.59 were found to be important for the peptide as the antagonist was perfectly bound. Taken together, it is very likely that position 6.58 and 7.31 participate in the binding of all tested peptidic ligands and deeper position, Asn6.55 and Asn7.32 showed a loss in PYY binding and also in antagonist binding suggesting that they could play a role in ligand binding (Figure 6A).

Studies with antagonists indicate that the binding of these compounds differs depending on the ligand between transmembrane helices 3 and 7. Taking all the data into consideration, it can be assumed that the binding pattern of the native ligands and the small antagonists overlaps in TM6 because several residues have been found to be relevant in both cases.

**Y2 RECEPTOR**

In this receptor subtype, the N-terminus does not play a role in membrane expression and it does not participate in a subtype specific binding pocket. However, it does play a role in agonist induced internalization processes since the complete truncation slowed down the process, although it could be seen that the exchange of the N-terminal fragment by the hY1 receptor or hY1 receptor fragment did not affect ligand dependent internalization (Lindner et al., 2009).

Mutagenesis studies to identify residues that contribute to ligand binding in the Y2 receptor were initially motivated by the finding that human and chicken Y2 receptors show a significantly different pharmacological profile. The chicken Y2 receptor is able to bind [Leu31,Pro34]-NPY, a peptide agonist selective for mammalian Y1/Y4/Y5 receptors, but was unable to bind BIIE0246, a small molecule antagonist for mammalian Y2 receptors (Salszcz et al., 2000). Sequence comparison and reciprocal mutagenesis revealed three residues in transmembrane helices 3, 5, and 6 that contribute to the binding of BIIE0246. Individual and combined substitution of Glu5.27, Leu5.31, and Leu6.31 in the hY2 receptor decreased the affinity for BIIE0246 to a chY2-like level, whereas substitution of the corresponding residues in the chY2 by the human residues increased the affinity for BIIE0246 (Berghard et al., 2002). Further mutagenesis studies on the human Y2 receptor revealed interaction partners for the native peptidic ligand NPY. Several acidic residues have been tested for their importance for NPY binding. Glu2.27 and Asp5.29 turned out to be highly important for the binding of NPY (Figure 6C). While Asp5.29 is important for all Y receptor subtypes, Glu3.27 only plays a role in the Y2 receptor. Both receptor mutants were tested in a signal transduction assay using pNPY, [Ala25]pNPY, [Ala33]pNPY, and [Ala35]pNPY to identify the interaction partner of the two acidic residues in the peptide. It could be shown that Asp5.29 interacts with Arg6.59 of the peptidic ligand in the Y2 and Y5 receptors, whereas the interaction partner in Y1 and Y3 receptors is Arg3.5. However, no direct interaction partner could be identified for Glu3.27 (Merten et al., 2007). More recent studies investigated additional residues in the Y1 receptor for their impact on the binding of pNPY, PNPY, hPPY (3–36), nPPY(13–36), and the non-peptidic antagonist BIIE0246 (Alberberg et al., 2010; Fallmar et al., 2011). The residues tested, namely Tyr2.24, Glu2.27, Glu5.27, Thr5.31, Leu5.31, Glu6.34, Val6.35, and Tyr7.31, were chosen by similarity to residues in the Y1 receptor subtype, which were proven to be important for ligand binding in this receptor subtype. It could be shown, that of the tested residues, only Tyr2.24 participates in the binding of all tested peptidic ligands and...
the non-peptidic antagonist BIIE0246. The substitution of this residue to Ala resulted in a five- to ninefold reduction in affinity (Akerberg et al., 2010).

The individual substitution of Tyr7.31 by Ala and Gln2.68 by the bigger and more polar residue Asn revealed a lower affinity only for the truncated peptide agonists. The authors hypothesize that Tyr7.31 does not play a role in binding of the full-length peptide, but may contribute to a compensatory interaction for ligands that lack the N-terminal residues. Furthermore, the authors could show that an introduction of a His residue in position 7.31 (the corresponding residue in Y1 receptor) completely abolished the binding of [125I]-pPYY (Akerberg et al., 2010). These findings are somewhat unexpected, since this His residue was shown to be involved in ligand binding in the Y1 receptor (Sjodin et al., 2006). This indicates that position 7.31 is important in both receptor subtypes, but may have different modes of action (Akerberg et al., 2010). For position 2.68, a mode of binding is proposed in which the lack of Asp (a residue common to all other Y receptor subtypes at this position) contributes to the selectivity of truncated peptides [e.g., NPY(3–36)] for the Y2 receptor (Fallmar et al., 2011). The Leu4.60Ala mutant showed a slightly decreased affinity for hPYY(3–36) and a strong loss of affinity for BIIE0246, which may be caused by a weakened or lost hydrophobic interaction. This indicates that this residue is highly important for antagonist binding. The corresponding position in Y1 receptor (Phe4.60) has been shown to be involved in the binding of [125I]NPY, [3H]BIBP3226 (Sautel et al., 1996) as well as [3H]J-104870 (Kanno et al., 2001). This indicates that position 4.60 is involved in the binding of small molecule antagonists at both receptor subtypes, Y1 and Y2.

The Y2 receptor mutants Thr3.40Ile and Gln6.55Ala showed increased affinity for pNPY and hPYY(3–36), but decreased affinity for the non-peptidic antagonist BIIE0246. Taken into account that these positions are located deeper in the transmembrane part...
of the receptor, an indirect effect on the binding of peptidic ligands would be the most likely explanation. However, the decreased binding affinity of 600-fold by a different binding pocket for small molecule antagonists, located more deeply in the transmembrane (Fallmar et al., 2011) and surrounded not only by Thr3.40 and Glu5.27, but also by the nearby residue Glu5.24, which was earlier shown to participate in the binding of BIE2246 earlier (Berglund et al., 2002).

Y5 RECEPTOR
N-terminal truncations and substitutions revealed the importance of this fragment for membrane expression and indicated that the N-terminus is not involved in forming a specific binding pocket. It is likely that this part of the receptor is involved in order to test the influence of the side chain, Glu5.24 was mutated to Ala in order to test the influence of the side chain, Glu5.27 showed a threefold loss in potency. On the other hand, Asp6.59 was mutated to Ala, Glu, Arg, and Asn to test the influence of charge and length of the side chain. The mutation to Ala showed a complete loss of both binding and activity, the exchange to Glu displayed wild-type-like binding and activation. In addition, the mutation to Asn showed indeterminate binding and a 200-fold loss in activation. Finally the exchange to Arg resulted in a dramatic loss in potency (>600-fold) and in no detectable binding (Figure 6B).

Y4 RECEPTOR
The Y4 subtype N-terminus could play a role in ligand binding, since N-terminal truncation of the segment produced a loss in binding activity. Interestingly, the receptor remains on the membrane even when the complete N-terminus is removed (Lindner et al., 2009).

Only few mutagenesis studies have been published so far for the human Y4 receptor. Merten et al. (2007) exchanged three acidic residues in the extracellular domains of Y4 receptor. While the Asp2.68Ala mutant showed wild-type-like pharmacological properties, Asp6.59Ala and Glu5.27Ala displayed a dramatically reduced affinity for NPY. Additional residues were investigated by Lindner et al. (2008b), resulting in identification of a third acidic residue (Asp5.28) which is important for ligand binding at the Y4 receptor. These Ala-mutants have also been tested with NPY analogs in which the Tyr27, Tyr29 and the Arg residues at position 25, 33 and 35 were individually substituted by Ala (Figure 6D). This approach revealed no further loss of affinity for [Ala33]pNPY on the Asp5.28Ala mutant of the receptor, indicating a direct interaction between Ala33 of the peptide and Asp6.59 of the receptor. Similarly, Arg25 of the NPY peptide could be identified as the interaction partner for Asp6.59 of the receptor (Lindner et al., 2008b).

CONCLUSION AND PERSPECTIVES
The NPY system has been extensively characterized in the last years. The modulation of actions mediated by the distinct receptors like, e.g., its involvement in obesity, cancer, and epilepsy are of great importance. Therefore, the development of receptor subtype-selective ligands and structure-activity relationship studies have been a major objective in the past years. Primarily, amino acid scans and truncations have identified the important residues and areas of the ligand with respect to binding at each receptor. The Y receptors have been extensively studied, several important residues have been characterized and some of the binding pockets have been partially characterized. Two subtype-selective interactions have been elucidated so far. A similar binding mode has been identified on NPY receptors, where a common residue Asp89 binds to one of the two C-terminally located Arg of the peptide depending on the receptor subtype. Moreover, a second binding interaction has been found on the Y5 receptor where Asp6086 located at the top of TM2 interacts with Arg23 of the peptide (Merten et al., 2007; Lindner et al., 2008b). This finding would suggest that probably a second interaction could take place in other receptor subtypes. Nevertheless, further investigations have to be performed. It is likely that more interactions between the receptors and the peptides could occur, therefore structure activity relationship studies are still a focus of interest.

The design of short analogs and antagonists have confirmed these findings, indicating that this is a great tool to modulate and study the receptors. Some promising progresses have been achieved in cancer diagnosis using Y1 receptor selective short ligands. However the development of short analogs for treatment of this pathology still remains challenging. Also in anti-obesity drugs, Y2/Y4 receptor selective agonists are in progress and currently in clinical trials of Phase I/I. On the basis of well studied characteristics accounting for receptor subtype selectivity, it is likely that subsequent investigations could be focused on the improvement of pharmacological properties such as stability and half-life. In addition the development of more potent selective ligands might be a focus of interest.

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