Review

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Chemical modification of neuropeptide Y for human Y₁ receptor targeting in health and disease

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Abstract: As a very abundant neuropeptide in the brain and widely distributed peptide hormone in the periphery, neuropeptide Y (NPY) appears to be a multisignaling key peptide. Together with peptide YY, pancreatic polypeptide and the four human G protein-coupled receptor subtypes hY₁R, hY₂R, hY₄R and hY₅R it forms the NPY/hYR multiligand/multireceptor system, which is involved in essential physiological processes as well as in human diseases. In particular, NPY-induced hY₁R signaling plays a central role in the regulation of food intake and stress response as well as in obesity, mood disorders and cancer. Thus, several hY₁R-preferring NPY analogs have been developed as versatile tools to unravel the complex NPY/hY₁R signaling in health and disease. Further, these peptides provide basic lead structures for the development of innovative drugs. Here, the current research is summarized focusing on the development of differently sized hY₁R-preferring NPY analogs as well as their advances with respect to hY₁R profiling, potential therapeutic applications and targeted cancer imaging and therapy. Finally, major limitations and innovative strategies for next generation hY₁R-preferring NPY analogs are addressed.

Keywords: carborane; G-protein coupled receptor signaling; peptide glycosylation; peptide lipidation; peptide modification; targeted cancer therapy.

Introduction

Neuropeptide Y (NPY) is a very abundant neuropeptide in the brain (Holzer et al., 2012) and was first isolated from porcine brain in 1982 (Tatemoto, 1982). It belongs to the so-called gastrointestinal-brain peptides (Murphy and Bloom, 2006) and is evolutionary highly conserved (O'Hare et al., 1988; Blomqvist et al., 1992). Owing to its strong appetite stimulating effect, NPY is considered as the most potent orexigenic neuropeptide (Mercer et al., 2011). Moreover, it is also involved in several regulatory cycles, e.g. concerning the cardiovascular (Tan et al., 2018) and immune system (Farzi et al., 2015), bone homeostasis (Horsnell and Baldock, 2016), sleep (Dyzma et al., 2010), stress response and anxiety (Heilig, 2004). These physiological functions explain its implication in human diseases like obesity (Loh et al., 2015), hypertension and atherosclerosis (Zhu et al., 2016), stress and mood disorders (Rasmusson, 2017) as well as cancer (Tilan and Kitlinska, 2016). NPY mediates its pleiotropic functions by interacting with four different G protein-coupled receptor (GPCR) subtypes, the human Y receptors (hYRs), with the hY₁R subtype being most frequently implicated in all of the mentioned physiological and pathophysiological processes. To understand the diverse (patho-)physiology of the NPY/hY₁R signaling, hY₁R-preferring NPY analogs constitute important pharmacological tools. They can be used, on the one hand, for hY₁R profiling at the corresponding sites of interest (Larhammar et al., 2017) and on the other hand, for the development of hY₁R-selective drugs and drug shuttles to target NPY-related diseases (Pedrazzini et al., 2003; Li et al., 2015). Chemical modification of NPY is a fundamental strategy to obtain hY₁R-preferring ligands and to introduce functional cargoes while maintaining their biological activity.

Here, the development and applicability of NPY analogs for hY₁R profiling and therapeutic targeting are summarized. The first part provides an introduction to the NPY/hYR system and the (patho-)physiological relevance of NPY/hY₁R signaling. The second part focuses on differently sized hY₁R-preferring NPY analogs with agonistic and antagonistic properties, whereas the last

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part addresses current limitations and introduces suitable modification strategies to overcome these problems.

The molecular (patho-)physiology of the NPY system

NPY is a tyrosine-rich C-terminally amidated neuropeptide consisting of 36 amino acids, a flexible N-terminal tail and a pronounced C-terminal amphipathic α -helix (Figure 1A) (Monks et al., 1996; Bader et al., 2001). It shares a high sequence homology and structural similarities with the closely related peptide YY (PYY) and pancreatic polypeptide (PP), thus forming the so-called NPY family (Figure 1B) (Larhammar, 1996). In humans, the NPY family peptides mediate cellular information by four human Y receptor subtypes (hY_1R , hY_2R , hY_4R and hY_5R) (Michel et al., 1998). They are members of the β group of rhodopsin-like GPCRs

(Schiöth and Fredriksson, 2005) and consist of an extracellular N-terminus, seven transmembrane-spanning helices connected by three intra- and extracellular loops (ICLs and ECLs, respectively) and an intracellular C-terminus. A putative eighth helix has been described which is located in the C-terminal part (Babilon et al., 2013). hYR exhibit overlapping binding profiles for the different NPY family peptides, thus forming a complex multiligand/multireceptor system with NPY and PYY preferentially binding to hY_1R , hY_2R and hY_5R and PP as the endogenous high-affinity ligand of hY_4R (Pedragosa-Badia et al., 2013). As a commonly proposed mechanism for many peptide-GPCR systems (Schwyzer, 1995), the NPY family peptides have been shown to pre-associate with the cell membrane by their C-terminal helix (Lerch et al., 2004; Thomas et al., 2005) to efficiently reach the receptor by lateral diffusion on the cell surface (Figure 2A) (Schwyzer, 1995). The subsequent binding of the native ligands results in structural changes of the receptor and a stabilization of active receptor conformations

Figure 1: Molecular information on Y-peptides.

(A) Three-dimensional solution structure of human NPY determined by nuclear magnetic resonance spectroscopy (PDB: 1RON). (B) Amino acid sequences of the human NPY family peptides hNPY, hPYY and hPP. Identical positions are marked in grey.

Figure 2: Schematic diagram of NPY binding and hYR-induced signaling.

NPY is depicted as its three-dimensional solution structure (Figure 1). The hYR is embedded into a lipid bilayer representing the cell surface. (A) NPY membrane association and lateral diffusion towards receptor. (B) Receptor activation stimulates inhibitory $G_{\alpha_{i/o}}$ protein (blue) resulting in an inhibition of the adenylyl cyclase (AC, green) and decrease of intracellular cyclic adenosine monophosphate (cAMP) level. (C) Complexation with arrestin adapter protein (red) initiating receptor internalization and further arrestin-dependent signaling cascades (e.g.: mitogen-activated protein kinases (MAPK), nonreceptor tyrosine kinases (TK), and E3 ubiquitin ligases) (Luttrell and Gesty-Palmer, 2010). The complex cross-talk between G protein-dependent and arrestin-dependent signaling is simplified by a left right arrow.

which in turn determine intracellular signal transduction (Niesen et al., 2011). Recently, we could solve the structure of hY₁R with bound antagonists, and by a combination of mutagenesis, cell free receptor expression, crosslinking of full-length NPY and molecular modeling the binding mode of the peptide (Yang et al., 2018). hYRs predominantly signal through pertussis toxin-sensitive inhibitory G $\alpha_{i/o}$ proteins (Herzog et al., 1992), which inhibit the adenylate cyclase resulting in a decrease of the intracellular concentration of cyclic adenosine monophosphate (cAMP) (Figure 2B). G protein signaling is mainly terminated by C-terminal receptor phosphorylation by GPCR receptor kinases (GRKs) and subsequent recruitment of the ubiquitously distributed adapter protein arrestin (Moore et al., 2007). Complexation with arrestin initiates hYR internalization and mediates further signaling cascades as, for example, mitogen-activated protein kinase (MAPK) pathways (Figure 2C) (Lefkowitz and Shenoy, 2005; Gurevich and Gurevich, 2006). G protein signaling and arrestin recruitment, the two main downstream events after hYR activation, act in an interdependent manner in order to provide a fast, direct cellular response and a sustained long-lasting effect during hYR internalization and intracellular trafficking (Luttrell and Gesty-Palmer, 2010). We could recently identify the formation of a triple complex of arrestin, G-protein and ligand activated receptor for Y₁R and the binding mode of arrestin at the receptor in the so-called ‘tail-conformation’ (Wanka et al., 2018).

The NPY system is regulated on different levels to enable its pleiotropic mode of action. On a molecular level individual hYR subtypes display distinct binding modes for the NPY family peptides (Lindner et al., 2008). It has been shown that, for example, the hY₁R and hY₄R primarily use position arginine 35 of NPY family peptides as key anchor point, whereas hY₂R and hY₅R use position arginine 33 (Merten et al., 2007). Based on mutagenesis and structural as well as computational data, binding modes for the endogenous ligands of hY₁R (Yang et al., 2018), hY₂R (Kaiser et al., 2015) and hY₄R (Pedragosa-Badia et al., 2014) have been proposed and reveal very different binding poses, depending on the receptor subtype. While for hY₁R the ligand NPY binds rather flatly and aspartate in position 6.59 of the receptor mainly interacts with arginine in position 35 (Yang et al., 2018), for hY₂R the binding pose is rather steep, and it is arginine in position 33, that docks to aspartate^{6.59} (Kaiser et al., 2015). Distinct local expression patterns of the NPY family peptides and receptors contribute to a spatial regulation of hYR subtype specific signaling and mediation of biological effects. PYY and PP are predominantly expressed in the digestive system and activate hY₂R and hY₄R which decrease gastrointestinal

motility and food intake (Holzer et al., 2012), the latter referred to as anorexigenic effect. In contrast, NPY is widely expressed in the central, peripheral and enteric nervous system, from where it is released into circulation and distributed into the gut and the spleen (Minor et al., 2009). Furthermore, it is expressed in endothelial cells, platelets and macrophages (Ericsson et al., 1987; Singer et al., 2013) as well as in various cell types of the adipose tissue (Kos et al., 2007). This results in a high abundance of NPY in the brain and a basal peripheral circulation level (Hirsch and Zukowska, 2012). In the brain, NPY is able to induce anorexigenic effects by hY₂R activation (Batterham et al., 2002), whereas it predominantly induces strong orexigenic effects by hY₁R and hY₅R (Nguyen et al., 2012). While NPY/hY₅R signaling is limited to the central nervous system owing to the exclusive central expression of hY₅R (Durkin et al., 2000), NPY/hY₁R and NPY/hY₂R signaling is widely distributed and involved in further physiological and pathophysiological actions. Referring to specific hY₁R-mediated effects, NPY reduces depression-like behavior (Stogner and Holmes, 2000; Farzi et al., 2015) and anxiety (Wahlestedt et al., 1993; Tasan et al., 2016), inhibits pain (Naveilhan et al., 2001; Malet et al., 2017) decreases bone formation (Baldock et al., 2007; Sousa et al., 2016), stimulates neurogenesis (Decressac et al., 2009) and vasoconstriction (Lundberg and Modin, 1995; Tan et al., 2018). These diverse effects emphasize NPY as crucial multi-level signaling peptide and the high (patho-)physiological relevance of NPY/hY₁R signaling.

NPY analogs: structure, physiological and therapeutic application

Owing to its multitude of diverse physiological and pathophysiological implications, the hY₁R subtype is an interesting target in the field of biomedical research and drug development. To unravel its biological complexity, a full understanding of its molecular basis and the discrimination between single signaling pathways involved in different biological effects are necessary. For that purpose, hY₁R subtype selective NPY analogs with different functional properties appear to be versatile tools.

As for most peptide analogs derived from natural peptides, NPY analogs can be categorized according to their functional properties compared to native NPY. With respect to the efficacy, which describes the magnitude of cellular response after receptor activation, they are either

Figure 3: Signaling modes of NPY and its analogs.

Functional properties of (A) native NPY and (B) hY_1 R-prefering NPY analogs with respect to hY_1 R-mediated G protein signaling (blue) as well as arrestin recruitment (red), respectively. The diagrams represent the peptide concentration-dependent cellular response after hY_1 R activation.

full agonists, if they are able to induce maximal receptor activation like NPY (Figure 3A), or partial agonists, if the efficacy is lower compared to NPY (Figure 3B upper left). In case they possess a high binding affinity but lack in receptor activation potency they are denoted as antagonists (Figure 3B upper right). Furthermore, analogs which are able to discriminate between different signaling pathways, as, for example, prefer G protein signaling over arrestin recruitment or vice versa (Figure 3B lower left and right), are termed biased ligands (Violin et al., 2014).

All of these different types of ligands possess high potential in both research as well as therapeutic applications. While selective agonists are important to specifically induce a desired effect, antagonists are necessary for corresponding blocking experiments to understand the mechanism of action and to elucidate structure-function relationships (Tatemoto, 1990). At the same time, they can be used in order to inhibit pathways that are overregulated in disease. In the case of NPY, this would include conditions such as epilepsy (Iughetti et al., 2018) or chronic pain (Lin et al., 2004; Diaz-delCastillo et al., 2018). Biased agonists can be used in order to assign a certain biological effect to a distinct signaling pathway. For example, in the case of NPY/ hY_1 R signaling they can help to clarify whether the strong orexigenic effect of NPY is induced by G protein signaling or is mediated by arrestin downstream signaling or both. This knowledge would help in finding therapeutics that target a certain pathway in disease without affecting others leading to less side effects.

Full-length NPY analogs

Because of a limited access to detailed structural information about the hY R subtype binding pocket and missing crystal structures of the ligand-receptor complex, the development of NPY analogs with hY_1 R-preference has mainly been accomplished by trial-and-error-based structure-activity relationship (SAR) studies. In-depth SAR studies of NPY revealed the very C-terminal segment NPY (28–36) as minimal core sequence with arginine 33, arginine 35 and tyrosine 36 to be crucial for hY_1 R binding and isoleucine 31, threonine 32 and glutamine 34 to be essential for hY_1 R activation and subtype preference (Figure 4A) (Cabrele and Beck-Sickinger, 2000). Accordingly, $[Leu^{31},Pro^{34}]NPY$ (Fuhlendorff et al., 1990) and $[Phe^7,Pro^{34}]NPY$ (Soll et al., 2001) display full agonistic properties at hY_1 R and a pronounced hY_1 R preference over hY_2 R (Figure 4B).

So far, $[Leu^{31},Pro^{34}]NPY$ has been used successfully to investigate hY_1 R expression profiles in cell lines (Hofliger et al., 2003) and tissues (Reubi et al., 2001). Furthermore, it served as hY_1 R agonist in several *in vitro* and *in vivo* studies to elucidate the hY_1 R-mediated orexigenic (Fekete et al., 2002; Lecklin et al., 2003), vasoconstrictive (Abounader et al., 1995) as well as antidepressive and anxiolytic effect (Morales-Medina et al., 2012). Based on these studies, hY_1 R agonists have been suggested as suitable drug leads for mood disorders such as the post-traumatic stress disorder (Hendriksen et al., 2012). In contrast to $[Leu^{31},Pro^{34}]NPY$, $[Phe^7,Pro^{34}]NPY$ has rather

Figure 4: Chemical modification of used peptides.

(A) Relevant amino acid positions in pNPY for high hY₁R binding obtained from Ala-Scan (Beck-Sickinger et al., 1994) (gray) and chemical structures of Asn⁷, Ile³¹ and Glu³⁴, which are additionally important for introducing hY₁R selectivity. (B) Selected full-length hY₁R-preferring agonists. The substituted amino acid positions are in bold and emphasized by their chemical structure.

been used for hY₁R targeting in cancer, as discussed further down.

Short NPY analogs

In the past decades, NPY research increasingly focused on size reduction as a powerful tool to develop short NPY analogs with improved hYR selectivity profiles and different functional properties. Short NPY analogs are highly attractive because of their facile synthesis, lower production costs and improved labeling efficiencies (Zwanziger et al., 2009). Yet, the development of short NPY analogs with hY₁R preference emerged as major hurdle because, among all hYR subtypes, hY₁R is most sensitive towards N-terminal and central truncations of NPY (Cabrele and Beck-Sickinger, 2000). In addition to the C-terminal core sequence NPY (28–36), the systematic alanine scan of NPY revealed the N-terminal residues proline 2 and proline 5 as well as the central residues arginine 19 and tyrosine 20 as highly sensitive (Figure 4A) (Beck-Sickinger et al., 1994). Both the N-terminal and C-terminal part have been suggested to stabilize the bioactive conformation by intramolecular interactions (Cabrele and Beck-Sickinger, 2000). Interestingly, short C-terminally derived NPY analogs with single amino acid substitutions or conformational restrictions were shown to maintain a high hY₁R binding affinity. This led to the development of first and second generation hY₁R antagonists (Figure 5B and C) which have been used successfully in *in vitro* and *in vivo* competition studies to verify hY₁R expression in the brain and to block hY₁R-mediated food intake (Kanatani et al., 1996) which nicely confirms the [Leu³¹,Pro³⁴]NPY studies (Fekete et al., 2002). Further, they have been suggested as useful tool to elucidate the pathophysiological role of NPY in feeding behavior (Kanatani et al., 1996).

A thorough SAR study with the C-terminal nonamer NPY (28–36) revealed the first and so far, shortest

hY₁R-preferring NPY analog with partial agonist properties (Zwanziger et al., 2009). In addition to proline 30 and leucine 34, which have been shown to be crucial for high hY₁R binding affinity of C-terminally derived NPY analogs (Figure 5B) (Leban et al., 1995), the substitution of isoleucine 31 by norleucine and threonine 32 by a bulky aromatic benzoylphenylalanine (Bpa) (Figure 5D) led to agonistic properties while maintaining a nanomolar binding affinity (Zwanziger et al., 2009), however, signaling efficiency reached only about 20% of the NPY response at hY₁R. Very recently it was shown, that N-terminal elongation by a lysine carrying a bulky, hydrophobic moiety such as adamantyl or a carboranyl cluster (see next chapter) at the N^ε-position led to full G-protein signaling and arrestin-3 recruitment/internalization of hY₁R, yielding in the first full truncated hY₁R agonist based on NPY (Hofmann et al., 2018). In addition, substitution with a phenyl ring instead of carboranyl or adamantyl gave a G-protein biased ligand, that no longer was able to induce internalization of hY₁R (Hofmann et al., 2018).

The presented repertoire of hY₁R-preferring NPY agonists and antagonists substantially contributed to the characterization of the hY₁R physiology and pathophysiology. They further provide potential lead structures for next generation hY₁R-preferring NPY analogs, especially for the development of hY₁R-preferring biased NPY analogs.

Targeted breast cancer imaging and therapy

Apart from therapeutic NPY analogs, which mediate their beneficial effects by either stimulating (agonists) or blocking (antagonist) hY₁R activation, hY₁R-preferring NPY analogs have been suggested as shuttles for peptide receptor targeting (Walther et al., 2011). This concept evolved

Figure 5: Selected short hY_1R antagonists and agonists derived from NPY.

Sequences are aligned with respect to Ile^{28} and residues differing from the native NPY sequence are in bold. (A) C-terminal core sequence NPY (28–36). (B) First generation of monomeric high-affinity hY_1R antagonist (BVD15) (Daniels et al., 1995; Leban et al., 1995) and the conformationally restricted short linear hY_1R antagonist $[\text{}^{32,34}\beta\text{ACC}]\text{NPY}$ (Koglin et al., 2003). (C) Second-generation of short hY_1R antagonists containing dimeric structures (Balasubramaniam et al., 1996, 2001). (D) First short NPY analogs with partial (top) and full (bottom) hY_1R agonist properties (Zwanziger et al., 2009; Hofmann et al., 2018). βACC : β -aminocyclopropane carboxylic acid; Bpa: 4-phenylphenylalanine; Bip: biphenylalanine; Dpr: diaminopropionic acid; Nva: norvaline.

and progressed particularly in the field of cancer research, bearing an enormous capacity for conventionally undruggable primary and metastatic cancers (Bidwell, 2012). Major drawbacks of conventional cancer diagnosis and therapy include limited accessibility to the cancerous tissue, high drug doses, excessive cytotoxicity, the occurrence of multiple drug resistance and off-target side effects (Mohanty et al., 2011).

hY_1R has been shown to be overexpressed in numerous epithelial, endocrine and embryonal tumors (Korner and Reubi, 2007) and has been found in 85% of primary breast cancer tumors and in 100% of lymph node metastases derived from hY_1R -positive tumors (Reubi et al., 2001, 2002). Interestingly, in contrast to the breast tumor, the hY_2R subtype is primarily expressed in normal breast tissue, with no expression of hY_1R (Reubi et al., 2001). This differential expression pattern of both subtypes allows the

hY_1R -over- hY_2R -preferring NPY analog $[\text{Phe}^7, \text{Pro}^{34}]\text{NPY}$ to be used as shuttle to specifically guide covalently linked diagnostic or therapeutic agents into malignant cells of solid breast cancer tumors and metastases by hY_1R -mediated internalization (Figure 6) (Reubi, 2003).

As proof of concept, radioactively $^{99\text{m}}\text{Tc}$ -labeled $[\text{Phe}^7, \text{Pro}^{34}]\text{NPY}$ has been demonstrated to selectively reach the breast cancer tumor after injection into the foot vein in a first-in-man study (Khan et al., 2010). In-depth studies on $[\text{Phe}^7, \text{Pro}^{34}]\text{NPY}$ revealed lysine 4 as highly tolerant position for the incorporation of chelator-based radiolabels for radio imaging and therapy (Zwanziger et al., 2008), boron-rich carborane clusters for boron neutron capture therapy (BNCT) (Ahrens et al., 2011), cytostatic agents (Bohme and Beck-Sickinger, 2015) and peptide toxins (Ahrens et al., 2015b) for targeted therapy (Figure 6). To expand the scope of $[\text{Phe}^7, \text{Pro}^{34}]\text{NPY}$ -based

Figure 6: Schematic illustration of peptide receptor targeted diagnosis (left) and therapy (right).

After injection, labeled peptide analogs are expected to reach the tumor or metastatic lesions through blood circulation and extravasation (Reubi, 2003). While agonists are able to selectively accumulate inside the tumor cells by receptor-mediated internalization, antagonists are able to accumulate on the cell surface.

targeted therapy, further suitable positions were screened resulting in a highly active triply-modified carborane variant for BNCT (Ahrens et al., 2015a).

With respect to the size of peptide shuttles, shorter analogs have been described to possess improved *in vivo* kinetics in terms of enhanced tumor penetration (Wester and Kessler, 2005). In first attempts to develop short hY_1R radiotracers the first generation hY_1R antagonist BVD15 (Figure 5B) was used, which resulted in a chelator-based radiolabeled variant with a low-nanomolar hY_1R binding affinity and selectivity (Guérin et al., 2010). Antagonists have been described to be suitable for peptide receptor imaging as they are able to efficiently accumulate on the cell surface (Figure 6, antagonist) (Wild et al., 2014). However, peptide agonists are highly preferred, especially for peptide receptor targeted therapy, owing to their ability to induce receptor-mediated endocytosis and thereby ensure selective intracellular accumulation (Figure 6, agonist). Accordingly, the short hY_1R agonists mentioned above (Figure 5D) have been suggested as promising lead structures for next generation hY_1R -preferring NPY targeting peptides (Zwanziger et al., 2009; Hofmann et al., 2018).

Limitations of NPY analogs and strategies for improvement

Although almost all hY_1R -preferring NPY analogs are characterized by a pronounced hY_1R -over- hY_2R preference

compared to NPY, they exhibit a limited overall selectivity pattern due to a persistent nanomolar hY_4R and/or hY_5R activity (Parker et al., 1998; Wyss et al., 1998). The prevalent hY_1R/hY_4R bi-functionality of most of the ligands originates in the close evolutionary relation (Wraith et al., 2000) and is based on a similar binding mode for both receptor subtypes, with arginine 35 as common key contact point (Merten et al., 2007; Pedragosa-Badia et al., 2014). Unfortunately, this fact partially limits their application, especially for *in vivo* studies (Larhammar et al., 2017). In addition to the functional limitation, the intrinsically decreased metabolic stability of peptides, owing to a high susceptibility towards proteolytic cleavage by ubiquitously present proteases, is regarded as a further drawback of hY_1R -preferring NPY peptides (Brothers and Wahlestedt, 2010).

For that purpose, different modification strategies have been developed which are able to simultaneously address functional and metabolic limitations of peptides in order to turn them into versatile tools and drug leads (Ahrens et al., 2012). Among others, such multifunctional modification strategies include the site-specific incorporation of (i) fatty acids, referred to as lipidation (Zhang and Bulaj, 2012), (ii) 1,2-dicarba-*closo*-dodecarboranes (carboranes or carbaboranes), referred to as carboranylation (Scholz and Hey-Hawkins, 2011), and (iii) carbohydrates, referred to as glycosylation (Rodriguez and Cudic, 2013). Although all three modifications are characterized by different physicochemical properties (Table 1), they are considered as highly potent pharmacophores that are able

Table 1: Summary of different chemical modification strategies to improve functional properties of peptides.

Modification	Pharmacophore	Properties	Type of interaction
Lipidation (palmitic acid)		<ul style="list-style-type: none"> – C16 aliphatic chain – Hydrophobic 	Hydrophobic interactions
Carboranylation (1,2-dicarba-closo-dodecarborane ^a)		<ul style="list-style-type: none"> – Icosahedral – Super-hydrophobic – Aromatic 	<ul style="list-style-type: none"> – Hydrophobic interactions – Dihydrogen bonding
Glycosylation (glucopyranose)		<ul style="list-style-type: none"> – Heterocyclic – Hydrophilic 	Hydrogen bonding

For each strategy the chemical structure of a frequently used pharmacophore and its properties are presented.

^aAlso referred to as carborane or carbaborane.

to influence peptide stability and receptor subtype selectivity while maintaining high biological activity through direct or indirect target interactions.

Lipidation of peptides, especially palmitoylation, appears to be an outstanding multi-effective functionalization approach (Table 1). Palmitoyl residues have been shown to properly interact with lipid membranes (Avadisian and Gunning, 2013), thereby enhancing the association of the peptides with the cell surface and increasing their effective concentration in close proximity to the receptor and thus resulting in an enhanced biological activity. Furthermore, for full-length NPY family peptides palmitoylation has been shown to directly influence hYR subtype preferences (Made et al., 2014b) and to improve hYR internalization (Made et al., 2014a). In addition, animal studies with palmitoylated NPY family peptide analogs demonstrated a prolonged effect and an increased half-life (Bellmann-Sickert et al., 2011). Apart from that, in some cases lipidation of peptides bears the potential of traversing the blood-brain barrier (Di, 2015; Maletinska et al., 2015). Also, it can enhance tissue penetration, as shown for lipidized pepducins (Tressel et al., 2011). However, as lipidation also leads to decreased hydrophilicity of the peptide and therefore lower solubility, the beneficial effects have to be carefully balanced against the physicochemical properties of fatty acid modification. Straight forward synthetic strategies have been developed which allow a facile site-specific incorporation of palmitic acid and further fatty acids of different length into peptides by solid-phase peptide synthesis (SPPS) (Made et al., 2014b).

In contrast to the plain aliphatic carbon chain of fatty acids, carboranes are icosahedral boron-carbon clusters (Table 1). They are very hydrophobic but carry an aromatic character based on their electronic configuration (Scholz and Hey-Hawkins, 2011), thus serving as three-dimensional phenyl ring mimetics. The clusters are covered by a hydrogen shell composed of two C-H group hydrogen atoms and 10 B-H group hydrogen atoms. Because of the comparably low electronegativity of boron, the B-H protons adopt a hydridic behavior (Custelcean and Jackson, 2001), thus enabling intermolecular dihydrogen bonding which has been associated with their unique biological effects (Fanfrlik et al., 2006; Lee et al., 2012). Carboranes are highly stable in various organic solvents and aqueous solutions and insensitive towards oxygen, but they are prone to decomposition under basic conditions. Nonetheless, facile synthesis strategies have been developed to introduce carboranes into peptides by SPPS (Ahrens et al., 2011), mostly as ready-to-use carborane building blocks (Armstrong and Valliant, 2007; Frank et al., 2012; Stadlbauer et al., 2012). Originally, in medicinal chemistry carboranes have been designed as multiple-boron carriers for boron neutron capture therapy (BNCT) (Carlsson et al., 1994). Owing to their unique properties they have been suggested as multi-effective auxiliary units, however, studies on that aspect are scarce. Due to their hydrophobic character, carboranes may be able to cross the blood-brain barrier or induce higher tissue penetration. However, this was only shown until now for L-(4-boronphenyl)alanine, but not for carborane clusters (Roda et al., 2014).

In contrast to lipidation and carboranylation, the introduction of carbohydrates increases hydrophilicity because of their polyhydroxy nature (Table 1). Thus, the overall solubility of peptides can be improved (Sola and Griebenow, 2009). Glycosyl residues have been shown to interact with the peptide backbone by either stabilizing or destabilizing secondary structure elements (Wormald et al., 2002; Otvos and Cudic, 2003). Thereby receptor subtype selectivity can be modulated as already demonstrated for full-length NPY family peptide analogs (Pedersen et al., 2010). Referring to metabolic stability, direct spatial shielding of protease cleavage sites has been described to enhance the half-life of peptides (Powell et al., 1993; Sola and Griebenow, 2009). Modification with D-sugars is especially beneficial, if hepatocytes need to be targeted. These cells overexpress asialoglycoprotein receptors at their surface that lead to internalization of the carbohydrate modified peptide. This implies, that for all other cell types, liver uptake has to be taken into consideration and tested carefully in order to exclude unwanted liver uptake (Ahmed and Narain, 2015). Generally, carbohydrate chemistry is very complex and laborious due to the polyhydroxy nature and regio- and stereochemical issues (Seeberger and Werz, 2007). Nonetheless, convenient SPPS-compatible synthesis strategies have been developed to site-specifically introduce different carbohydrates species into peptides (Kan and Danishefsky, 2009; Yang et al., 2011; Filice and Palomo, 2012; Rodriguez and Cudic, 2013). Apart from functional and metabolic improvements of peptides, glycosyl residues have been suggested as favorable prosthetic groups to incorporate radionuclides such as ^{18}F into peptides to develop peptide-based radiotracer for cancer diagnosis via positron emission tomography (Maschauer et al., 2010).

The multi-functional properties of lipid-, carborane- and glycosyl residues emphasize their potential applicability as promising pharmacophores in order to develop next generation NPY analogs, especially of reduced size, with improved or even new functional properties.

Conclusion

In the last decades, the essential physiological and pathophysiological role of NPY/hY₁R signaling has been demonstrated. Up to now, differently-sized hY₁R-preferring NPY analogs with agonistic and antagonistic properties have been developed which enabled *in vitro* and *in vivo* hY₁R profiling. Furthermore, they served as lead structures for peptide-based diagnostics and therapeutic drugs.

However, a reduced overall selectivity and a decreased metabolic stability limit their *in vivo* application. Thus, innovative modification strategies such as lipidation, carboranylation and glycosylation appear to be highly promising to develop next generation NPY analogs, especially short variants and biased ligands, for an improved physiological and therapeutic hY₁R targeting.

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