Semisynthesis and Characterization of the First Analogues of Pro-Neuropeptide Y

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Dedicated to Prof. Manfred Mutter on the occasion of his 60th birthday

Enzymatic cleavage of prohormone neuropeptide Y (proNPY) leads to mature neuropeptide Y (NPY), a widely distributed neuropeptide with multiple functions both peripherally and centrally. A single dibasic pair of amino acids, Lys^{38}-Arg^{39}, represents the recognition motif for a class of hormone-processing enzymes known as prohormone convertases (PCs). Two members of this PC family, PC1/3 and PC2, are involved in proNPY cleavage. The aim of this work was to establish an effective method for the generation of full-length 69-amino acid proNPY analogues for further studies of prohormone convertase interaction. We have chosen two ligation sites in order to perform the semisynthesis of proNPY analogues by expressed protein ligation (EPL). By using the intein-mediated purification system (IMPACT) with improved conditions for intein splicing, we were able to isolate proNPY 1 – 40 and proNPY 1 – 54 fragments as C-terminal thioesters. Peptides bearing N-terminal cysteine instead of the naturally occurring Ser^{41} and Thr^{55} residues, respectively, were generated by solid-phase peptide synthesis. Moreover, labels (carboxyfluorescein and biotin) were inserted into the peptide sequences. The synthesis of the [C^{41}]proNPY 41 – 69 fragment, which proved to be a difficult peptide sequence, could be achieved by the incorporation of two pseudo-proline derivatives. Western blot analysis revealed that all five proNPY analogues are recognized by monoclonal antibodies directed against NPY as well as against the C-flanking peptide of NPY (CPON).

KEYWORDS:

expressed protein ligation · fluorescence probes · prohormone neuropeptide Y · protein modifications · solid-phase synthesis

Introduction

Like many other neurotransmitters, neuropeptide Y (NPY) is derived from a precursor protein, the 69-amino acid pro-neuropeptide Y (proNPY). A family of mammalian proteases responsible for the specific endoproteolytic processing of prohormones and proproteins has been identified.[1, 2] Basic residues, single or in pairs, display the recognition motif for these subtilisin/kexin-like prohormone convertases (PCs). The seven members so far known include PC1/3, PC2, furin/PACE, PACE4, PC4, PC5/6 and PC7/SPC7/LPC/PC8. Differences in their tissue distributions and subcellular localization lead to the specific production of their target proteins.[3] ProNPY undergoes cleavage at a single dibasic site, Lys^{38}-Arg^{39}, as has been shown to be carried out in vivo and in vitro by PC1/3 and/or PC2.[4–6] Further processing by a carboxypeptidase-B-like enzyme and an amidating enzyme, peptidylglycine-amidating-monooxygenase (PAM), yields C-terminally amidated, mature, biologically active NPY and the C-terminal flanking peptide of NPY (CPON), of so far unknown function (Scheme 1).[7] The 36-amino acid amide NPY is widely distributed both peripherally and centrally, and is one of the most abundant neuropeptides in the brain. Together with peptide YY (PYY) and pancreatic polypeptide (PP) it composes the so-called NPY family. A variety of physiological effects has been attributed to NPY. Stimulation of food intake, secretion of luteinizing hormone, and release of growth hormone and insulin suggest an important role in the pathophysiology of obesity and diabetes.[8, 9] Furthermore, NPY is involved in the regulation of memory retention,[10] circadian rhythm,[11] neuronal excitability, anxiety and depression,[12] and can also act as a potent vasoconstrictor in skeletal muscle, heart, kidney and brain.[13] All these activities of NPY in mammals are mediated by at least three NPY receptors (designated as Y_1-, Y_2- and Y_5-receptors), which belong

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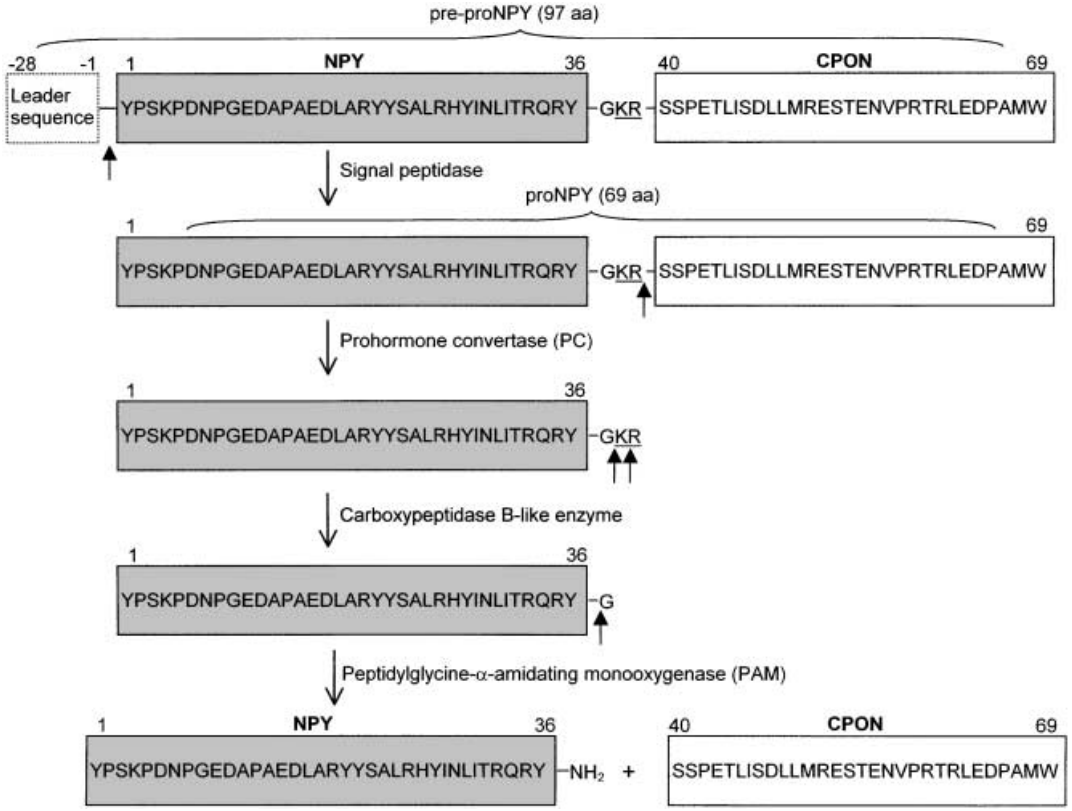


Scheme 1. *Biosynthesis of prohormone neuropeptide Y (proNPY). After removal of a leader sequence from the 97-amino acid (aa) precursor of proNPY (pre-proNPY), further processing of the 69-mer proNPY results in the formation of the amidated 36-amino acid NPY and CPON peptide. The dibasic cleavage site Lys^{38}-Arg^{39}, recognized by the prohormone convertases, is underlined.*

to the superfamily of heptahelical G-protein coupled receptors.[14] Specific inhibition of the conversion of biologically inactive proNPY into active NPY could provide a novel approach to the development of new therapeutic drug targets. Intervention in the biosynthesis of NPY, however, requires knowledge of the processing mechanisms involved. We were previously able to show that proNPY is processed by PC1/3 and PC2,[6] which are primarily expressed in endocrine and neural cells, and accordingly in neuropeptide-rich regions. In contrast, widely distributed prohormone convertases such as furin and PACE4 were not able to cleave proNPY. So far, it still remains unclear which parts of the proNPY sequence—in addition to the dibasic cleavage site—are required for the complete processing by PCs, and which might therefore represent potential targets for the development of processing inhibitors.

The aim of this work was to synthesize modified proNPY analogues as suitable tools for an enzymatic assay. The analogues should include chemical modifications such as a carboxyfluorescein (CF) or a biotin label, by which processing could be followed easily, by spectroscopic methods for CF or by affinity chromatography for biotin. Solid-phase peptide synthesis (SPPS),[15] the method of choice for the incorporation of chemical modifications into peptides, is associated with difficulties for peptides longer than $\approx$50 amino acids. The recombinant expression of full-length 69-amino acid proNPY has been described previously,[4, 6] but no site-specific introduction of these chemical modifications is yet possible. The method of expressed protein ligation (EPL)[16] circumvents these disadvantages by combining recombinant methods and chemical synthesis. In a chemoselective reaction, a recombinant peptide/protein segment bearing a C-terminal thioester is joined to a second synthetic peptide containing an N-terminal cysteine residue to yield a native peptide bond at the site of ligation. Two different ligation sites were chosen, because a cysteine had to be incorporated into the sequence in place of the naturally occurring amino acids Ser^{41} or Thr^{55}. While cysteine in position 41 is located next to the dibasic cleavage site of the PCs, thus possibly providing an inhibitory analogue, Cys^{55} is far away from the cleavage site.

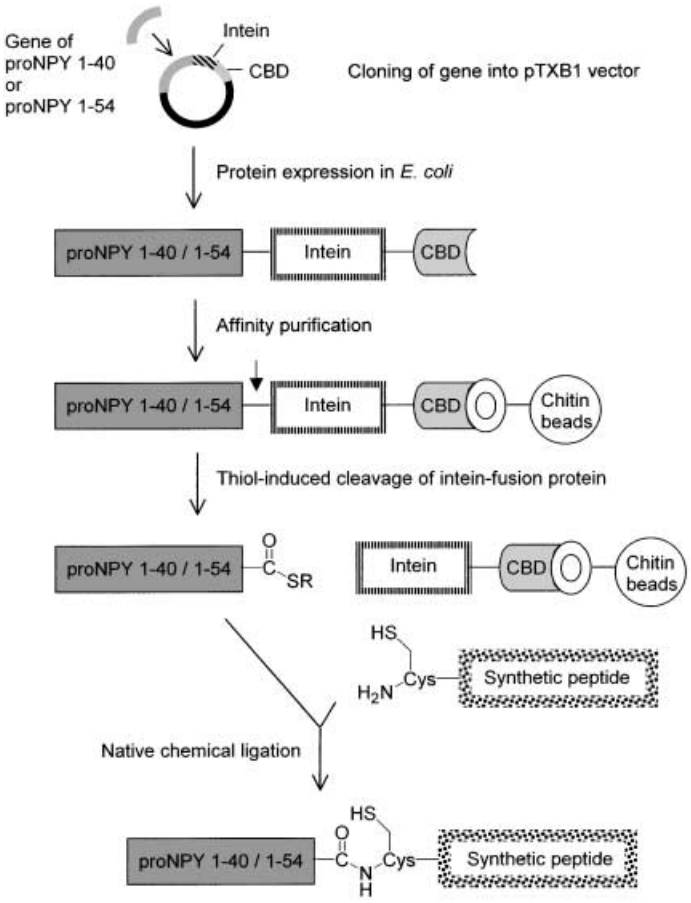
In this paper we report the synthesis and characterization of the first chemically modified proNPY analogues. Five proNPY analogues—two of them containing a CF label, one a biotin label and two without any label—were assembled by use of EPL. With improved conditions for the intein-mediated purification system (IMPACT, Intein-Mediated Purification with an Affinity Chitin-binding Tag)[17] we were able to generate the thioester intermediate by a recombinant approach and in high yield. Solid-phase peptide synthesis of the cysteine fragment [C^{41}]proNPY 41 – 69, which proved difficult to synthesize, could be achieved by incorporation of two pseudo-proline derivatives. The final native chemical ligation (NCL) step yielded the desired proNPY analogues. Western blot and dotblot analyses revealed

that all five proNPY analogues are recognized by antibodies directed against NPY as well as against the CPON. Binding epitopes of anti-proNPY antibodies[18] could be confirmed and further characterized by use of proNPY segments.

Results

Strategy

In order to synthesize proNPY (Scheme 1) and its site-specifically modified analogues for further characterization of prohormone-convertase interaction, we employed the EPL approach for protein semisynthesis. A schematic representation of this strategy is illustrated in Scheme 2. Accordingly, proNPY 1 – 40 and proNPY 1 – 54 fragments have been isolated as C-terminal thioesters in the initial step by use of 2-mercaptoethanesulfonic acid (MESNA) for induction of the cleavage of the intein-fusion protein. Subsequent ligation with proNPY-derived synthetic peptides possessing an N-terminal cysteine resulted in the full-length proteins. Derivatives of proNPY with chemical labels such as carboxyfluorescein and biotin were obtained similarly. Western blot analyses have confirmed the recognition of proNPY analogues by specific antibodies against NPY and CPON peptides.



Scheme 2. *Representation of the semisynthesis of proNPY analogues by EPL. Target proteins (proNPY 1 – 40/1 – 54) were expressed in* E. coli *with the C-terminal intein-CBD tag. A soluble fusion precursor was loaded on a chitin column and thiol-mediated cleavage of the intein-fusion protein was induced. The eluted MESNA-thioester (R = $CH_2CH_2SO_3H$) underwent chemical ligation with synthetic peptides bearing N-terminal cysteine residues.*

Purification of fusion proteins

The proNPY 1 – 40 and proNPY 1 – 54 genes, amplified by PCR, were cloned in-frame into the C-terminal vector pTXB1 upstream of the mini-intein from the GyrA gene of *Mycobacterium xenopi* (*Mxe* intein)[19] and the chitin-binding domain (CBD) from *Bacillus circulans*.[20] Both fragments were introduced without any additional vector-derived amino acids. We observed no significant difference between the expression in the *E. coli* bacterial strains BL21(DE3) and ER2566. In order to maximize the yield of the fusion proteins, inductor concentrations, temperature and expression time were varied. Whereas no fusion protein was detected before the induction (Figure 1 A, lane 1), expression under optimized conditions led to the isolation of 200 – 300 mg

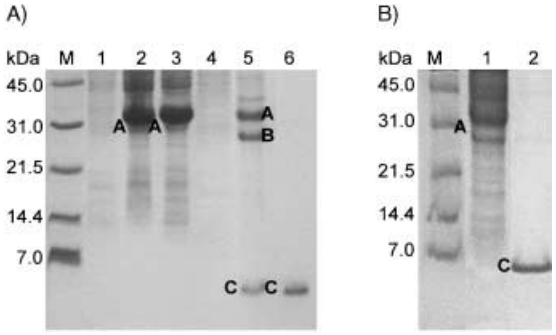


Figure 1. *Isolation of proNPY 1 – 40 and 1 – 54 thioester, analysed by SDS-PAGE. A) Purification of proNPY 1 – 40-intein-CBD fusion protein (lanes 1 – 3) and isolation of proNPY 1 – 40 thioester (lanes 4 – 6). Lane M represents the protein marker, lane 1 cells before induction, lane 2 crude extract after cell lysis, lane 3 supernatant after lysis, lane 4 second column flow-through, lane 5 the cleavage of intein-fusion protein (after 1 day with MESNA), and lane 6 eluted MESNA-thioester. B) Isolation of proNPY 1 – 54 thioester. Lane M represents the protein marker, lane 1 supernatant after lysis, and lane 2 eluted MESNA-thioester. Soluble fusion proteins (bands A) revealed high binding efficiency on chitin. Thiol-mediated cleavage of the intein-fusion protein was monitored directly on the column. Whereas intein-CBD tag (Part A, band B) remained bound to chitin beads, generated thioesters were eluted (bands C).*

of fusion precursors from one litre of bacterial culture. Soluble fusion proteins (Figure 1 A, lane 3 and Figure 1 B, lane 1, bands A) represented up to 40% of the total amount of protein in the bacterial extract.

Generation of protein thioester

The fusion proteins showed better than 95% binding efficiency on the chitin column (Figure 1 A, lane 4). Since stringent column washing reduced non-specific binding of other bacterial proteins, the purity of the loaded protein remained very high. The intein splicing proceeded after thiol addition and was monitored directly on the column (Figure 1 A, lane 5). The conditions for

thioester formation were optimized with respect to the thiol type, pH and temperature applied for the cleavage of the intein-fusion protein. The efficiency of the cleavage showed a strong dependency on the thiol used for processing of the fusion proteins (Figure 2). Whereas some thiols (such as dithiothreitol, DTT) displayed up to 85% splicing activity, thiophenol (TF) and carboxypropyl mercaptan (SCP) did not appear to be very efficient even after prolonged reaction times. Interestingly, we did not achieve any differences in intein splitting either in the pH

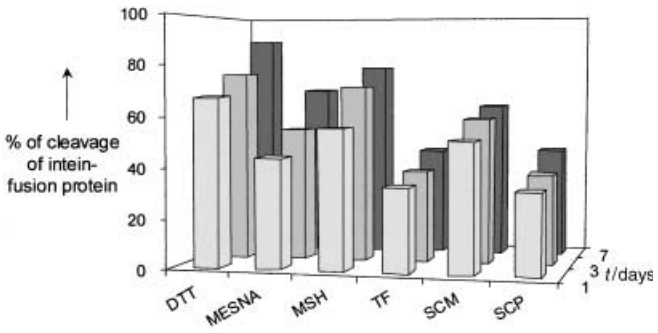


Figure 2. Cleavage of the intein-fusion protein on the chitin column mediated by different thiols and their different time dependencies. The cleavage of intein-fusion protein was carried out at 0.1 M thiol concentration at pH 8 and at room temperature. Thiol abbreviations are as follows: dithiothreitol (DTT), 2-mercaptoethanesulfonic acid (MESNA), 2-mercaptoethanol (MSH), thiophenol (TF), carboxymethyl mercaptan (SCM), carboxypropyl mercaptan (SCP).

range between 6 and 8.5 or at room temperature and at 4 °C. Enhancement of the thiol concentration provoked an improvement of the splicing efficiency by approximately 10% when the concentration was doubled. We thus achieved up to 75% cleavage of the intein-fusion protein by use of MESNA at a final concentration of 0.25 M. After elution of the generated thioester from the chitin column, further MESNA-mediated splicing resulted in the processing of more than 85% of the initially loaded fusion protein. Both the eluted proNPY 1–40 and the eluted proNPY 1–54 thioester showed up to 95% purity (bands C in Figure 1A, lane 6 and Figure 1B, lane 2). MALDI mass spectrometry revealed N-terminal methionylation of both fragments (up to 95%).

Protein thioester stability

In order to prevent thioester hydrolysis and to identify optimal storage conditions, stability tests were performed. As in previous reports,[21] the pH value significantly influenced the rate of hydrolysis. Moreover, the thioester moiety also proved to be critical. After cleavage of the intein-fusion protein for one day at pH 7.5 at room temperature, 33% of the generated MESNA-thioester, or 78% of the SCP-thioester, respectively, was already hydrolysed. However, the hydrolysis rate dropped to half the value when the intein splicing was performed at pH 6. MESNA-thioesters turned out to be very stable (less than 5% hydrolysis) when a low pH and a low temperature was applied for the intein splicing and for further thioester storage. Finally, the thioesters were purified by reversed-phase HPLC and their identities were confirmed by MALDI mass spectrometry.

Solid-phase synthesis of C-terminal proNPY segment analogues

The assembly of the N-terminal cysteine fragment [C^{41},K^{68}]pro-NPY 41–69 by automated SPPS proved more difficult than expected. With use of the Peptide Companion software package (CSPS Pharmaceuticals, San Diego, USA), synthesis was predicted to involve only easy or intermediate coupling steps. However, a high rate of mis-couplings could be demonstrated (Figure 3A). In order to identify the critical coupling steps, the synthesis was interrupted at different stages and small-scale cleavage of these peptide fragments was performed. Analysis of the fragments showed that the first coupling reactions (proNPY 50–69) were yielding a peptide with >95% homogeneity, while amino-acetylation by all the subsequent amino acids (Leu^{49}–Cys^{41}) was incomplete. These coupling steps were therefore performed by manual attachment of the corresponding amino acids under conditions recommended for overcoming difficult peptide sequences.[22, 23] Various solvents (*N*,*N*-dimethylformamide(DMF)/dichloromethane (DCM), DCM and dimethyl sulfoxide (DMSO)), different coupling reagents (*N*-[1*H*-benzotriazol-1-yl-*N*-oxy-tris-(dimethylamino)methylene]-*N*-methyl-methanaminium hexafluoroborate *N*-oxide (TBTU) and *N*-[1*H*-azabenzotriazol-1-yl-*N*-oxy-tris-(dimethylamino)methylene]-*N*-methyl-methanaminium hexafluorophosphate *N*-oxide (HATU)), several resins (polyethyleneglycol-Co-Wang (PEG-Co-Wang), pyridine-Co-Wang), coupling times and increasing numbers of coupling replications were applied, but none of these changes were capable of improving the synthesis significantly. In order to change the peptide backbone properties and thus reduce interchain association during the SPPS assembly, secondary amino acid surrogates were introduced.[24] The insertion of Fmoc-Leu(Fmoc-Hmb)-OH (Fmoc = fluorenylmethoxycarbonyl)-in positions 45 and 49, with its backbone amide-protecting group 2-hydroxy-4-methoxybenzyl (Hmb),[22, 25] resulted in an increased yield of the complete sequence of [C^{41},K^{68}]proNPY 41–69, but large amounts of side products were still obtained, particularly because of the problematic coupling of the amino acid immediately following Leu(Hmb) (Asp^{48}, for example). Further attempts to improve the synthesis yield focused on the incorporation of oxazolidine pseudo-proline dipeptides (ΨPro) introduced recently by Mutter et al.[26] Three different applications were compared: a) incorporation of Fmoc-Ile-Ser-($\Psi^{Me,Me}$)pro-OH in place of Ile^{46} and Ser^{47}, b) incorporation of Fmoc-Ser(*t*Bu)-Thr-($\Psi^{Me,Me}$)pro-OH in place of Ser^{54} and Thr^{55}, and c) incorporation of both pseudo-proline dipeptides. Whereas the first two strategies suffered from incomplete coupling steps (Figure 3B and Figure 3C), and the absence of the N-terminal cysteine residue (Figure 3C), approach c provided the desired peptide in sufficient purity (60–70%) and quantity (Figure 3D). After purification of the peptide by preparative RP-HPLC, homogeneity of >95% was achieved.

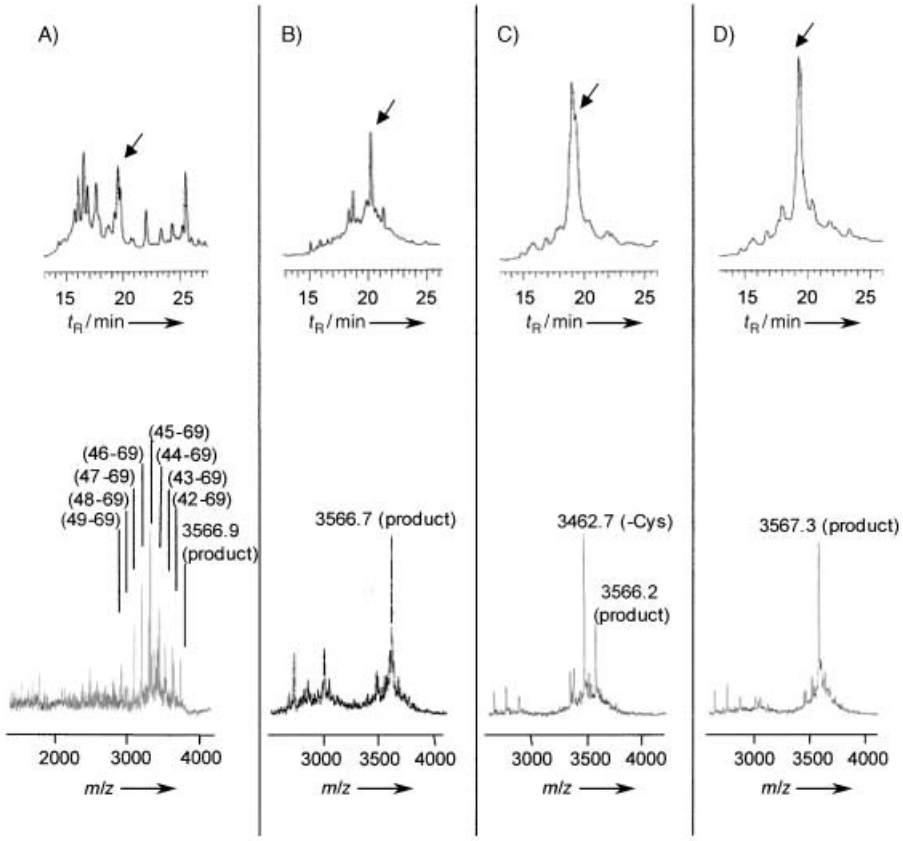


Figure 3. *Solid-phase synthesis of the N-terminal cysteine fragment [C^{41},K^{68}(Dde)]proNPY 41 – 69. The target peptide was analysed by RP-HPLC (top), with the eluting system 10 to 70% A (0.08% TFA in acetonitrile) over 30 min at a flow rate of 0.6 mL min^{-1}, and by MALDI-MS (bottom) after SPPS; A) in one single automatic run under standard Fmoc-based peptide synthesis conditions, B) including Ile-Ser($\psi^{Me,Me}$)pro-OH in positions 46 and 47, C) including Ser-Thr-($\psi^{Me,Me}$)pro-OH in positions 54 and 55, and D) with incorporation of both pseudo-proline derivatives. Arrows indicate product according to retention time and MS.*

Automated SPPS of the shorter N-terminal cysteine fragments [C^{41}]proNPY 41 – 49 and [C^{55},K^{68}]proNPY 55 – 69 did not raise any synthesis problems, crude peptides of $>80\%$ homogeneity being obtained. Furthermore, introduction of chemical labels (CF, biotin) at Lys^{68} was accomplished in one coupling step.[27]

Generation of proNPY analogues by chemical ligation

Ligations were carried out with both reactants in the millimolar concentration range. A molar excess of peptide of up to threefold relative to the thioester did not affect the coupling reaction. We performed the ligation in the presence of MESNA, which—besides the high cleavage activity of intein-fusion protein—also possesses the attributes of a suitable cofactor for EPL.[28] However, the ligation efficiency decreased by 5 – 10% at MESNA concentrations of less than 0.1 M. In order to maintain the reducing environment necessary for the reduction of cysteine residues, tris(2-carboxyethyl)phosphine (TCEP) was added to the ligation mixture. It has been reported that thiol additives such as TF can accelerate the rate of fragment condensation.[29] In our experiments, however, none of these thiols displayed any significant effect on the ligation progress, whereas basic conditions and higher temperature favoured the coupling reactions.[29] A high yield of ligation ($\approx 80\%$) was achieved after 30 h at a pH 8, while a comparable yield of ligation at pH 7 was identified only after 48 h. Furthermore, we observed no substantial differences in the ligation efficiency with respect to the size and to the chemical modification of the ligated synthetic peptides. Ligation products were already detectable after 30 min, approximately 20 – 25% ligation having been accomplished after the first 4 h. Purification of all ligation products was performed by RP-HPLC techniques.

Analysis of ligating fragments and ligation products

The ligation products were analyzed by SDS-PAGE (Figure 4A, lane 5 – 9) and their identities were confirmed by MALDI-MS. Western blot analysis was performed by use either of streptavidin or of antibodies raised against NPY (NPY02) or the CPON (CPON01). Streptavidin selectively identified the ligation product containing the biotin label (Figure 4C, lane 9). Whereas NPY02 recognized both thioester fragments (Figure 4D, lanes 1 and 2) and all ligation products (Figure 4D, lanes 5 – 9), CPON-fragments were not stained as expected (Figure 4D, lanes 3 and 4). The cross-reactivity of CPON01 was unexpected, because the CPON-cysteine fragments including the 55 – 69 sequence supposed to be labelled by CPON01[18] were not detected by Western blot analyses. (Figure 4E, lanes 3 and 4). We therefore additionally performed dotblot analyses, revealing that [C^{41}]proNPY 41 – 69 and [C^{55}]proNPY 55 – 69, as well as their chemically modified analogues, are recognized by CPON01. As expected, CPON01 recognized all full-length proNPY analogues and their chemically modified counterparts (Figure 4E, lanes 6 – 9), but neither the N-terminal thioester fragments (Figure 4E, lanes 1 – 2) nor the short [C^{41}]proNPY 1 – 49 protein (Figure 4E, lane 5).

Discussion

The biosynthesis of NPY, as deduced from its precursor protein proNPY, has become of major interest in recent years.[4–6, 30–33] Knowledge of the mechanisms and of possible sites for intervention or even inhibition could provide new insights into the physiological activities of NPY and might finally result in the

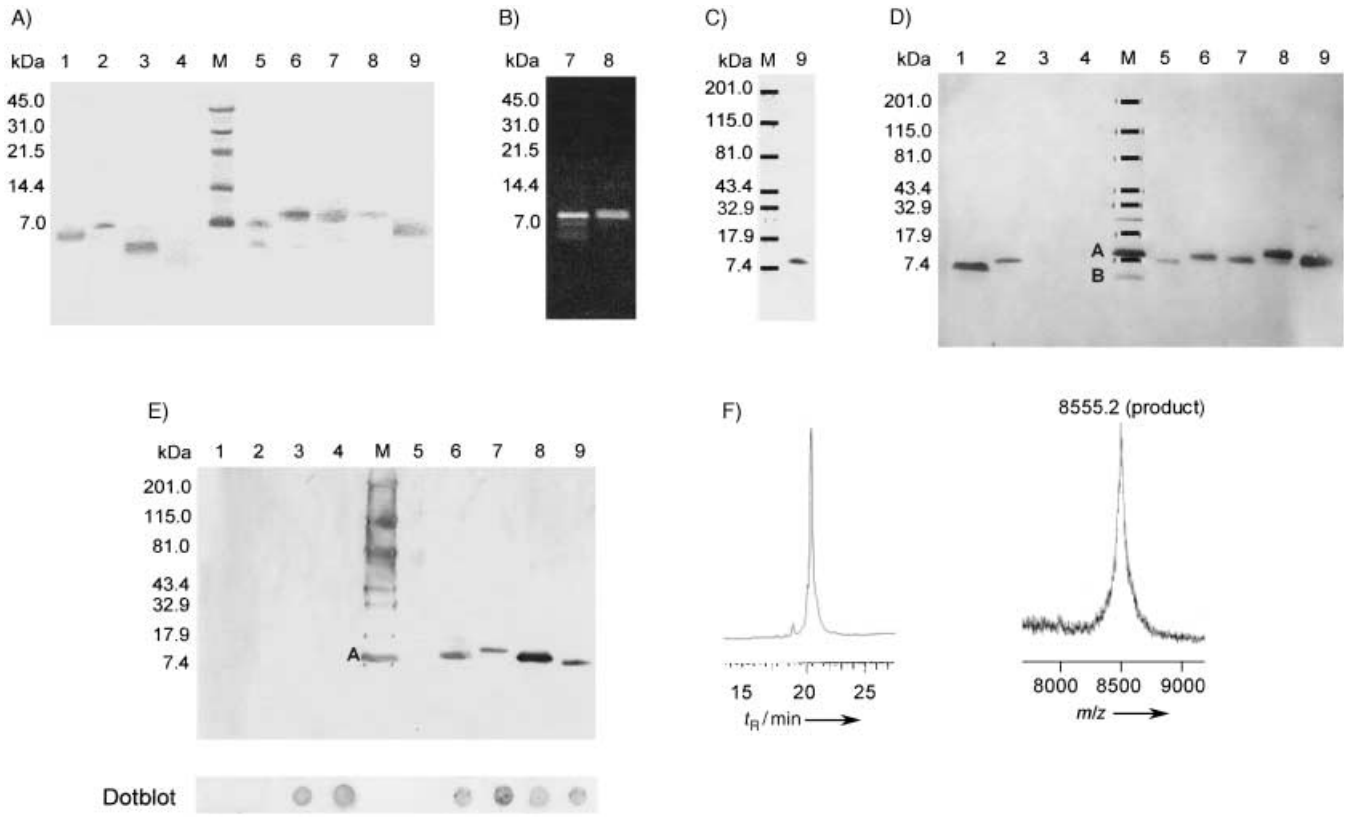


Figure 4. *Analysis of ligation fragments and products by SDS-PAGE and Western blotting. A) SDS-PAGE analysis: lane 1 proNPY 1 – 40 thioester, lane 2 proNPY 1 – 54 thioester, lane 3 synthetic [C^{41},K^{68}]proNPY 41 – 69, lane 4 synthetic [C^{55},K^{68}]proNPY 55 – 69, lane 5 ligated [C^{41}]proNPY 1 – 49, lane 6 [C^{41},K^{68}]proNPY 1 – 69, lane 7 [C^{41}, K^{68}(CF)]proNPY 1 – 69, lane 8 [C^{55},K^{68}(CF)]proNPY 1 – 69, and lane 9 [C^{55},K^{68}(biotin)]proNPY 1 – 69. Lane M represents a protein marker. The bands were stained with GelCode blue reagent. B) Visualization of [C^{41}, K^{68}(CF)]proNPY 1 – 69 (lane 7) and [C^{55},K^{68}(CF)]proNPY 1 – 69 (lane 8) on the gel by UV irradiation at 312 nm prior to gel staining. C) Western blot analysis of ligation product [C^{55},K^{68}(biotin)]proNPY 1 – 69 by use of streptavidin labelling. D) and E) Recognition of ligation fragments (lane 1 – 4) and ligation products (lane 5 – 9) by NPY02 (D) and CPON01 (E). The sample order is identical to that in (A). NPY (band B) and proNPY (band A) were supplemented to the protein marker. In (E), the dotblot data are also shown. F) RP-HPLC (left) and MALDI-MS (right) profile of the ligation product [C^{41}, K^{68}(CF)]proNPY 1 – 69.*

development of novel therapeutics, similar to angiotensin converting enzyme (ACE) inhibitors.[34] The main interest in proNPY processing is focused on its enzymatic cleavage by PC, as this is the only hormone-specific cleavage step. Together with distinct cellular expression or intracellular localization of both substrate and enzyme, the cleavage of prohormones by PC depends mainly on the catalytic selectivity of the enzyme,[35] which is strongly influenced by the surrounding amino acids at the substrate cleavage site. Previous studies, mainly on the pro-oxytocin/neurophysin system, showed that the dibasic cleavage site is flanked by sequences organised in β-turns or, as proposed alternatively, in omega loops,[36] which participate in enzyme recognition.[37–40] In vitro cleavage of shortened proNPY substrates showed several positions within the sequence of the peptide to play a crucial role for interaction with the converting enzyme.[41] Since it has been suggested that the length of the substrate discriminates between PC1/3 and PC2 processing activity,[6] further characterization of the relevant sequence of proNPY for the specific prohormone-enzyme interaction has to be investigated on full-length proNPY substrates.

The IMPACT system has been used for the recombinant synthesis of proNPY fragments (proNPY 1 – 40 and proNPY 1 – 54). For the first time, EPL has been applied as an expression system for proNPY synthesis. Beside single-column affinity purification of recombinant proteins, the IMPACT system also allowed the isolation of target thioesters without any additional vector-derived amino acids. The effect of the residue adjacent to the cleavage site of *Mxe* intein and the effect of other conditions (temperature, pH value) on the cleavage of intein-fusion protein have been studied before.[17, 42] Serine, the C-terminal residue of proNPY 1 – 40 and proNPY 1 – 54, was reported not to be compatible with thiol-inducible *Mxe* intein cleavage of intein-fusion proteins.[42] In contrast, we were able to detect more than 85 % splicing activity mediated by MESNA. However, we have shown a strong dependency of the cleavage of the intein-fusion protein on the thiol applied for thioester formation. Moreover, the thioester hydrolysis also proved to be thiol-dependent. By use of optimized conditions for on-column intein splicing, we were able to achieve high yields of the pure thioester product (30 – 40 mg from one litre of bacterial culture). Thus, we have demonstrated the feasibility of the IMPACT system as an efficient expression system for the recombinant synthesis of proNPY-derived analogues.

Automated SPPS of the N-terminal cysteine fragment [C^{41},K^{68}]proNPY 41 – 69 posed more problems than estimated. Difficult coupling steps occurred for amino acids Cys^{41} – Lys^{50},

suggesting that a specific conformation of this sequence is adjacent to the dibasic cleavage site of proNPY. This conformation will obviously be formed even if the CPON is synthesized separately and not linked to the PC cleavage site of proNPY. The improvement of the synthesis by incorporation of secondary amino acid surrogates is in agreement with the presence of the strong association phenomena, the oxazolidine pseudo-proline dipeptide units (ΨPro) proving to be superior to the Hmb peptide backbone protection. By kinking the conformation of the peptide backbone, ΨPro prevents aggregation or secondary structure formation and increases the solubilization of the growing peptide chain. Similarly to the synthesis of chaperonin 60.1 (195 – 217),[43] multiple use of pseudo-proline units was necessary because their effect is only local. The incorporation of both pseudo-proline derivatives therefore provided the most efficient strategy for overcoming this difficult peptide synthesis.

Chemical ligations between the recombinant C-terminal thioester and the corresponding synthetic N-terminal cysteine fragment of proNPY were achieved in high yields (> 80 %) in millimolar concentration ranges of the two reactants and with the addition of MESNA and TCEP at pH 8. In contrast to the frequently applied denaturating conditions for NCL (6 M guanidine or urea), we performed the ligation reactions under native conditions, which made further refolding steps superfluous. We confirmed that MESNA in combination with the reducing agent TCEP is an attractive alternative to the widely used TF as a cofactor to accelerate chemical ligation reactions. While TF caused precipitation, and particularly coprecipitation of reactants and product, MESNA/TCEP kept the reducing environment without causing precipitation. Furthermore, besides the better handling properties (MESNA is odourless), the reaction rate was equivalent to that of TF.

Western blot and dotblot analysis of fragments and ligation products gave further evidence of previously reported epitopes of the antibodies NPY02 and CPON01. The antibody NPY02 can detect all expected substrates (both thioesters and all ligation products). From earlier studies[18] and from our results, the epitope of the CPON01 antibody can be confined to proNPY 55—69, since all segments and all 69-amino acid ligation products containing this sequence are well bound, independently of the modification. Surprisingly, this could not be seen in the Western blot (Figure 4) but was very clear in the dotblot. Obviously, the small fragments are blotted through or cannot easily be analysed by SDS-PAGE. In contrast, neither [C^{41}]proNPY 41 – 49, nor [C^{41}]proNPY 1 – 49 including the first nine amino acids of CPON, nor proNPY 1-54 thioester are recognized, either in Western blot or in dotblot analysis. Accordingly, it can be assumed that CPON01 binds to an epitope in the immediate C-terminal part of proNPY. However, Met^{68} does not seem to be involved in the recognition site, as this residue can easily be exchanged by large and bulky modifications such as Lys(CF) or Lys(biotin). The introduced changes in the sequence of proNPY disturbed the recognition neither of the antibodies nor of streptavidin. This indicates that neither the presence of Cys^{41} in place of Ser^{41} nor of the chemical modifications CF and biotin at position 68 produce dramatic changes in the conformation of the direct partners of CPON01 recognition.

In conclusion, EPL proved to be an efficient method for the semisynthesis of proNPY analogues. The possibility of varying the sequence within the chemically synthesized fragment and of introducing chemical modifications into the analogues makes them valuable tools for assaying proNPY processing by PC. Other modifications—biophysical probes, for example—should allow us to characterise structure-function relationships further, or introduction of statines may provide PC inhibitors.

Experimental Section

Materials: The N^{α}-Fmoc-protected amino acids were purchased from Alexis (Läufelfingen, Switzerland) and Novabiochem (Läufelfingen, Switzerland). The side chain protecting groups were: *tert*-butyl for Asp, Glu, Ser, Thr and Tyr; *tert*-butyloxycarbonyl (Boc) or 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) for Lys, Boc for Trp; trityl for Asn, Cys, Gln and His, and 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg. Fmoc-Leu(Fmoc-Hmb)-OH, Fmoc-Ile-Ser($\psi^{Me,Me}$)pro-OH, Fmoc-Ser(*t*Bu)-Thr-($\psi^{Me,Me}$)pro-OH, *p*-benzyloxybenzyl alcohol resin (Wang resin) and TBTU were obtained from Novabiochem. PEG-Co-Wang and Pyrimidine-Co-Wang resins were obtained from Advanced Chemtech (Louisville, US). *N,N'*-Diisopropylcarbodiimide (DIC) was purchased from Aldrich (Buchs, Switzerland), and HATU from Fluka (Buchs, Switzerland). *N*-Hydroxybenzotriazole (HOBt), 4(5)-carboxyfluorescein (CF), biotin, trifluoroacetic acid (TFA), *N*-ethyldiisopropylamine (DIPEA), thioanisole, ethanedithiol, piperidine and *tert*-butanol were purchased from Fluka, and DMF, methanol, DCM and diethyl ether from Biosolve (Valkenswaard, Netherlands). Acetonitrile was obtained from Merck Eurolab (Bruchsal, Germany) and DMSO from Fluka.

DNA constructs: The DNA that encodes NPY-Gly was received as a gift from Dr. R. Bader.[44] The proNPY 1 – 40 fragment was created by PCR from the pUBK19/pNPY-G vector by use of the forward 5′-GGTGGTCATATGTACCCGTCTAAACCGGAC-3′ and reverse 5′-GGTGGTTGCTCTTCCGCAAGAACGTTTCCCGTAACGCTGACGGGT-3′ primer (Microsynth, Switzerland). Similarly, forward 5′-GGTGGTCATATGTACCCCTCCAAGCCGG-3′ and reverse 5′-GGTGGTTGCTCTTCCGCAGCTTTCTCTCATTAAGAGATCTG-3′ primer (MWG-Biotech AG) were used for the PCR amplification of proNPY 1 – 54 fragment from the cDNA of rat proNPY. Primers were designed to introduce the *Nde*I and *Sap*I sites in the forward and reverse primers, respectively. After digestion and purification, the PCR fragments were inserted into *Nde*I-*Sap*I-treated C-terminal fusion vector pTXB1 (New England Biolabs, NEB). DNA sequencing was used to confirm in-frame cloning of the proNPY 1 – 40 and the proNPY 1 – 54 gene.

Fusion protein expression in *E. coli*: *E. coli* BL21(DE3) or ER2566 cells transformed with pTXB1/proNPY 1 – 40 or pTXB1/proNPY 1 – 54 plasmid, respectively, were grown in LB medium that contained ampicillin (100 μg mL^{-1}) until they reached the mid-log phase and were induced with isopropylthiogalactoside (IPTG, 0.5 mM). Cells were harvested by centrifugation after 6 h expression at 37 °C. Lysis of the cells was performed over one hour at 4 °C in buffer A (tris(hydroxymethyl)aminomethane (Tris-HCl) 20 mM, ethylenediaminetetraacetate (EDTA) 1 mM, NaCl (500 mM), pH 8) in the presence of Triton X-100 (0.5 %), phenylmethylsulfonylfluoride (PMSF; 20 μM), TCEP (0.7 mM) and lysozyme (15 μg mL^{-1}). By addition of 10 μg mL^{-1} DNase I and $MgCl_2$ to a final 5 mM concentration, followed by incubation at room temperature (1 h), the viscosity of the lysate was reduced. Sonication pulses (3 × 50 s) were used to complete the lysis. The soluble protein extract was isolated by centrifugation (12 000 rpm, 30 min, 15 °C). Expression and isolation of the target

protein was monitored by SDS-PAGE (200 V, 120 mA, 45 min), GelCode blue staining reagent (Pierce) was applied for gel staining. Modified sample buffer without DTT (NEB manual), which induces the cleavage of intein-fusion protein, was used for gel analysis. In all cases, N-terminal Met-extension was found as expected when aromatic amino acids were following.[45]

Purification and isolation of protein thioester: A column (Econo Column, Bio-Rad) filled with chitin beads was equilibrated at room temperature with buffer A (10 bed volumes). The extract containing the fusion protein was diluted with buffer A (1:1) prior to column loading. Loading proceeded at 4 °C at a flow rate of 0.5 mL min^{-1} and the first flow-through was subsequently reloaded a second time onto the column. Samples from the flow-through analysed by SDS-PAGE indicated a high binding efficiency of the fusion precursor on the chitin. Washing with solubilization buffer (three bed volumes; Tris-HCl (20 mM), EDTA (1 mM), NaCl (500 mM), urea (2 M), Tween (0.1 %); pH 8) and buffer A (nine bed volumes) reduced nonspecific binding of other *E. coli* proteins. The cleavage of intein-fusion protein was induced by rapid flushing with buffer B (three bed volumes; Tris-HCl (20 mM), EDTA (1 mM), NaCl (500 mM); pH 6) that contained the sodium salt of MESNA (0.25 M). On-column cleavage proceeded at 4 °C for 48 – 70 h. Splicing efficiency was monitored by SDS-PAGE analysis of induced resin slurry (100 μL) mixed with sample buffer (30 μL). The thioester was eluted at room temperature with buffer B in 3 mL fractions and the protein content was determined by Bradford assay. After complete thioester elution, a second MESNA-mediated (0.2 M) cleavage of intein-fusion protein was induced. Analysis of the eluted thioester was carried out by MALDI mass spectrometry (Voyager II, Perseptive) and RP-HPLC on a C18 column (Vydac, 5 μm, 4.6 × 250 mm) with 0.08 % TFA in acetonitrile (A) and 0.1 % TFA in water (B) as eluting systems (gradient 10 to 70 % A over 30 min at a flow rate of 0.6 mL min^{-1}). The purity exceeded 95 %. ProNPY 1 – 40-MESNA thioester, MALDI-MS: *m/z* calcd.: 4938.6; found: 4938.2. ProNPY 1 – 54-MESNA thioester, MALDI-MS: *m/z* calcd.: 6511.4; found: 6512.2.

Analysis of the cleavage of intein-fusion protein: Intein splicing of loaded resin slurry (500 μL) was induced by flushing of three bed volumes of buffer A supplemented with various thiols (0.1 M): DTT, MESNA, 2-mercaptoethanol (MSH), thiophenol (TF), carboxymethyl mercaptan (SCM) or carboxypropyl mercaptan (SCP). Cleavage proceeded at room temperature, with shaking at 650 rpm. Efficiency of intein splicing was monitored by SDS-PAGE analysis of resin slurry after 1, 3 and 7 days. In order to examine the effect of pH, the cleavage of the intein-fusion protein was activated by washing the resin with buffer A (pH 6, 7, 7.5, 8 and 8.5, respectively) containing SCP or MESNA (0.5 M). The rate of cleavage, which was carried out at room temperature, was evaluated after 1, 3 and 7 days.

Thioester stability assay: On-column intein splicing was induced by washing with buffer A (pH 6 or 7.5) containing MESNA and SCP (0.1 M), respectively. The generated thioester was eluted after cleavage for one day at room temperature and further stored at 4 °C, 37 °C or room temperature. Analysis of the spontaneous thioester hydrolysis was performed after 3, 5, and 14 days by RP-HPLC.

Synthesis of proNPY-derived peptides: **(A) General procedure**: The peptides were synthesized by the Fmoc/*t*Bu solid-phase strategy with an automated multiple peptide synthesiser (Syro MultiSynTech) on Wang resin (30 mg, resin loading 0.5 mmol g^{-1}) preloaded with the first C-terminal amino acid. The coupling steps of the N^{α}-Fmoc amino acids were performed by a double coupling procedure (2 × 35 min) with a tenfold excess of amino acid, HOBt and DIC in DMF (2 × 40 min). The Fmoc-protecting group was removed with 40 % piperidine in DMF for 3 min, 20 % piperidine for 7 min and finally 40 % piperidine for 5 min. Cleavage from the resin and deprotection of the amino acid side-chains were accomplished in one step by use of TFA/thioanisole/1,2-ethanedithiol (90:7:3 v/v, 3 h). The peptides were precipitated from ice-cold diethyl ether, collected by centrifugation, washed five times with diethyl ether and lyophilised from water/*tert*-butanol (1:3 w/w). The lyophilised peptides were purified by preparative HPLC on a C18 column (Waters, 5 μm, 25 × 300 mm) and lyophilised again.

(B) SPPS of difficult peptide sequence: The synthesis was interrupted to couple the secondary amino acid surrogates Fmoc-(Fmoc-Hmb)Leu-OH or pseudo-proline dipeptides manually. Fmoc-(Fmoc-Hmb)Leu-OH was introduced at positions 45 and 49 by repeating the coupling of amino acid (two equivalents), HATU and DIPEA (four equivalents) in DMF (0.5 M) for 1 h. The same coupling procedure was applied for the subsequent amino acids, either Thr^{44} or Asp^{48}. The pseudo-proline dipeptide Fmoc-Ile-Ser($\psi^{Me,Me}$)pro-OH was inserted in place of Ile^{46} and Ser^{47}, and Fmoc-Ser(tBu)-Thr-($\psi^{Me,Me}$)pro-OH in place of Ser^{54} and Thr^{55} by double coupling of a threefold excess of dipeptide, DIC, HOBt and DIPEA in DMF (0.5 M) for 2 h. After completion of the synthesis, the final N-terminal Boc-Cys(Trt)-OH was again coupled manually by double coupling of amino acid (5 equivalents), TBTU, HOBt and of DIPEA (10 equivalents) for 2 h.

(C) Introduction of chemical modifications: Chemical modifications were introduced at lysine in position 68. The naturally occurring methionine was replaced by Lys(Dde). Before cleavage of the peptide from the resin, the Dde protecting group was selectively removed by treatment with a 2 % hydrazine solution in DMF and either CF (10 equivalents of CF, HOBt and DIC in DMF (0.5 M) for 30 min) or biotin (5 equivalents of biotin, HOBt, DIC and DIPEA in DMF (0.5 M) for 15 h) was coupled.

(D) Analysis of proNPY-derived peptides: The purified products were characterized by analytical RP-HPLC on a C18-column (Vydac, 5 μm, 4.6 × 250 mm) with 0.08 % TFA in acetonitrile (A) and 0.1 % TFA in water (B) as eluting system (10 to 70 % A over 30 min at a flow rate of 0.6 mL min^{-1}), revealing a purity > 95 %, and by MALDI mass spectroscopy. A yield of 2 – 3 mg of pure peptide/30 mg resin could be obtained. [C^{41}]proNPY 41 – 49, MALDI-MS: *m/z* calcd.: 991.2; found: 992.4. [C^{41},K^{68}]proNPY 41 – 69, MALDI-MS: *m/z* calcd.: 3401.9; found: 3401.0. [C^{41},K^{68}(CF)]proNPY 41 – 69, MALDI-MS: *m/z* calcd.: 3758.2; found: 3758.7. [C^{55},K^{68}(CF)]proNPY 55 – 69, MALDI-MS: *m/z* calcd.: 2174.4; found: 2174.0. [C^{55},K^{68}(biotin)]proNPY 55 – 69, MALDI-MS: *m/z* calcd.: 2041.4; found: 2041.6.

Ligation of proNPY-derived analogues: Ligation reactions were carried out with the reactants (0.3 mM) in Tris-HCl (10 mM) at room temperature in the presence of MESNA (0.1 M) and TCEP (20 mM). The final pH in the ligation mixture was adjusted to 8 and the reaction was allowed to proceed for 24 – 48 h with gentle shaking at 600 rpm. Progress of the ligation was monitored by SDS-PAGE. A broad range marker (Bio-Rad) was used as molecular weight standard. CF-containing samples were directly visualized on the gel by UV irradiation (312 nm) prior to gel staining. Ligation products were purified by semipreparative RP-HPLC or preparative SDS-PAGE (the purity was 80 – 90 %) and analysed by MALDI-MS: [C^{41}]proNPY 1 – 49, *m/z* calcd.: 5786.6, found: 5786.8; [C^{41},K^{68}]proNPY 1 – 69, *m/z* calcd.: 8197.3, found: 8196.5; [C^{41},K^{68}(CF)]proNPY 1 – 69, *m/z* calcd.: 8554.6; found: 8555.2; [C^{55},K^{68}(CF)]proNPY 1 – 69, *m/z* calcd.: 8542.6, found: 8564.9 (Na^+); [C^{55},K^{68}(biotin)]proNPY 1 – 69, *m/z* calcd.: 8409.6; found: 8431.8 (Na^+).

Analysis of ligation fragments and products by Western blotting and dotblotting: For Western blot analyses, samples were separated on a Tricine/SDS gel, prepared as described by Schägger and von

Jagow.[46] Kaleidoscope prestained marker (Bio-Rad) with supplemented NPY (5 μg) and proNPY (5 μg) was used as molecular weight standard. The samples were blotted 60 min (25 V, 180 mA) in a semi-dry electrophoretic transfer cell (Bio-Rad) onto nitrocellulose membrane (Schleicher & Schuell) in a transfer buffer (Tris (25 mM), glycine (150 mM), methanol (20%)). For dotblot analyses, ligation fragments and products were dissolved in water and loaded onto nitrocellulose membrane in a dotblot-cell (Consort N.V., Turnhout, Belgium). All membranes were blocked in 1% BSA in Tris-buffered saline/Tween 20 buffer overnight (TBS/Tween: Tris (25 mM), KCl (3 mM), NaCl (140 mM), Tween (0.5%); pH 7.4). For Western blotting, the membrane was first incubated with a monoclonal antibody directed either against NPY (NPY02) or against CPON (CPON01) at 1:1000 dilution in BSA (0.1%) in TBS/Tween for 90 minutes. Dotblot analyses were performed analogously with CPON01. Anti-mouse antibody conjugated with alkaline phosphatase was used for the second incubation step (1:20 000 dilution in BSA (0.1%) in TBS/Tween) for 1 hour, followed by colorimetric detection with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in Tris-HCl buffer (150 mM; pH 9.6).

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