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Peptide hormone isoforms: *N*-terminally branched PYY3–36 isoforms give improved lipid and fat-cell metabolism in diet-induced obese mice

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The prevalence of obesity is increasing with an alarming rate worldwide and there is a need for efficacious satiety drugs. PYY3–36 has been shown to play a role in hypothalamic appetite regulation and novel analogs targeting the Y2 receptor have potential as drugs for the treatment of obesity. We have designed a series of novel PYY3–36 isoforms, by first adding the dipeptide Ile–Lys *N*-terminal to the N^{α} of Ser-13 in PYY13–36 and then anchoring the *N*-terminal segment, e.g. PYY3–12, to the new Lys N^{ε}-amine. We hypothesized that such modifications would alter the folding of PYY, due to changes in the turn motif, which could change the binding mode to the Y receptor sub-types and possibly also alter metabolic stability. In structure-affinity/activity relationship experiments, one series of PYY isoforms displayed equipotency towards the Y receptors. However, an increased Y2 receptor potency for the second series of PYY isoforms resulted in enhanced Y receptor selectivity compared to PYY3–36. Additionally, acute as well as chronic mice studies showed body-weight-lowering effects for one of the PYY isoforms, which was also reflected in a reduction of circulating leptin levels. Interestingly, while the stability and pharmacokinetic profile of PYY3–36 and the *N*-terminally modified PYY3–36 analogue were identical, only mice treated with the branched analogue showed marked increases in adiponectin levels as well as reductions in non-esterified free fatty acids and triglycerides.

Keywords: PYY3–36; Y2 receptor agonists; obesity; adiponectin; food intake

Introduction

Globally, obesity is increasing with an alarming rate and the health consequences are many and serious, including insulin resistance and type II diabetes, cardiovascular disease, osteoarthritis and certain types of cancers, to mention just a few of the many diseases strongly associated with obesity. The most dramatic and efficient treatment of obesity is Roux-en-Y gastric bypass whereby the gastric volume is radically reduced and food is conveyed directly into the upper jejunum [1]. Aside from marked weight-loss Roux-en-Y gastric bypass surgery often leads to a rapid resolution of type II diabetes [2] and an increase in plasma levels of a number of appetite-regulating hormones, such as glucagon-like peptide-1 (GLP-1), pancreatic polypeptide (PP) and peptide YY (PYY3–36) [3]. PYY3–36, a native Y2 receptor agonist, is involved in appetite regulation, and thus Y2 receptor agonists are potentially a novel class of compounds for the treatment of obesity [4]. Although the body-weight-lowering effect of PYY3–36 has been debated in literature [5], numerous studies in rodents indeed show that chronic Y2 agonism lowers body weight [6–8]. Moreover, several studies in humans demonstrate that PYY3–36 lowers caloric intake and body weight [4,9].

PYY is a member of the PP-fold family of peptides that also consists of PP and neuropeptide Y (NPY) (the sequences are listed in Table 1) [10]. The PP-fold peptides bind and activate a family of G-protein coupled receptors, the so-called Y receptors (Y1, Y2, Y4 and Y5) [11]. Full length PYY (PYY1–36) is released postprandially from endocrine L-cells lining the gut [12] and subsequent to secretion *N*-terminally truncated by dipeptidyl peptidase IV (DPP-IV) to produce PYY3–36 [13,14]. PYY1–36 is a powerful but non-selective agonist stimulating both the Y1 and Y2 receptors [11,15], leading to numerous actions especially on the gastrointestinal function [16]. The *N*-terminally truncated form of PYY (PYY3–36) has a high affinity towards the Y2 receptor, and, to a lesser extent, the Y1 receptor [15]. Moreover, the anorectic and body-weight-lowering effects of peripherally administered PYY3–36 is believed to be

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Table 1. Sequences of human NPY, PP, PYY1–36, PYY3–36, as well as main chain of the PYY isoforms (See also Scheme 1)

Peptide	Sequence
PP	H-APLEPVYPGDNATPEQMAQYAADLRRYIN MLTRPRY-NH_2
NPY	H-YPSKPDNPGEDAPAEDMARYYSALRHYI NLITRQRY-NH_2
PYY1–36	H-YPIKPEAPGEDASPEELNRYYASLRH YLNLVTRQRY-NH_2
PYY3–36	H-IKPEAPGEDASPEELNRYYASLRHYL NLVTRQRY-NH_2
Series 1 isoform example (**3**)[a]	H-IKPEAPGEDA- H-IKSPEELNRYYASLRHYLNLVTRQRY-NH_2
Series 2 isoform example (**5**)[b]	H-IKPEAPGPRRP- H-IKSPEELNRYYASLRHYLNLVTRQRY-NH_2

[a] Series 1; GEDA turn, peptide **3**.
[b] Series 2, GPRRP turn, peptide **5**.

mediated by central Y2 receptors, possibly located in the arcuate nucleus of the hypothalamus [4]. Native PYY3–36 display a rather short half-life; in addition, its ability to activate the Y1 receptor can potentially counteract the anorectic effect. Thus, we aimed at developing more selective (Y2 over Y1) PYY3–36 analogs.

Herein, we present data from a novel class of PYY isoforms, which display changes in the selectivity towards the Y receptors, body-weight-lowering effects in different mice models, and, finally, additional beneficial effects on circulating lipids as well as on fat-cell metabolism. While the body-weight-lowering effects of PYY3–36 and a PYY3–36 isoform (peptide **3**, Table 1) were similar, interestingly, the infusion of peptide **3** to DIO-mice for 2 weeks led to significantly higher adiponectin levels compared to PYY3–36, which is an additional positive effect on fat-cell metabolism.

Peptide design

The sequences of the PP-fold peptides consist of a *C*-terminal receptor-recognizing segment, an amphipathic α-helix and a more flexible *N*-terminus [17–20]. It has been hypothesized that all members of the PP-fold family of peptides bind to the membrane prior to interaction with the receptors [19,21]. The *N*-terminal segments (residue 1–12) have proven very important for the Y receptor selectivity and essential for Y1 receptor activity, which has been shown by *N*-terminally truncating both PYY1–36 and NPY [22,23]. Pro-14 of the turn motif of PYY1–36 is highly important for controlling back-folding in solution; moving Pro-14 to position 13, as seen in the NPY sequence, abolishes all hairpin structures [24]. Consequently, the [^{13}Pro,^{14}Ala]-PYY1–36 mutant shows a destabilized *C*-terminal α-helix. Moreover, Ser-13 is important for PYY1–36 back-folding in solution, due to the hydrogen bonding of the hydroxyl group to the amide proton on Glu-15 [24]. As seen by the latter, small modification to the turn motif of PYY can lead to significant changes of the structure of the peptide. Moreover, sequential changes to the *N*-terminus can lead to major alterations in the potency to the Y receptors [22,23,25].

Given the importance of the turn motif of PYY for back-folding and α-helix stability, we describe two series of branched PYY isoforms. The PYY13–36 sequence was extended *N*-terminally with the dipeptide Ile–Lys and then the *N*-terminal PYY segment, e.g. PYY1–12, PYY2–12 or PYY3–12, was anchored to the new Lys N^{ε}-amine (Table 1). The dipeptide Ile–Lys was chosen, as it represents the native PYY3–4 segment. The branched structure provided an additional positive charge at *N*-terminal amine (Ile N^{α}) near the native PYY α-helix. This created the first series of peptides. In the second series, the GEDA tetrapeptide motif was exchanged for the pentapeptide turn motif GPRRP, which was inspired by the GPRRG sequence reported by DeDrado [26] for *de novo* design of proteins. We hypothesized that this would guide the branched peptide into a favourable Y2 receptor binding conformation possibly via an altered back-folding. In addition, this motif carries a double positive charge, which could interact with the Glu–Glu motif.

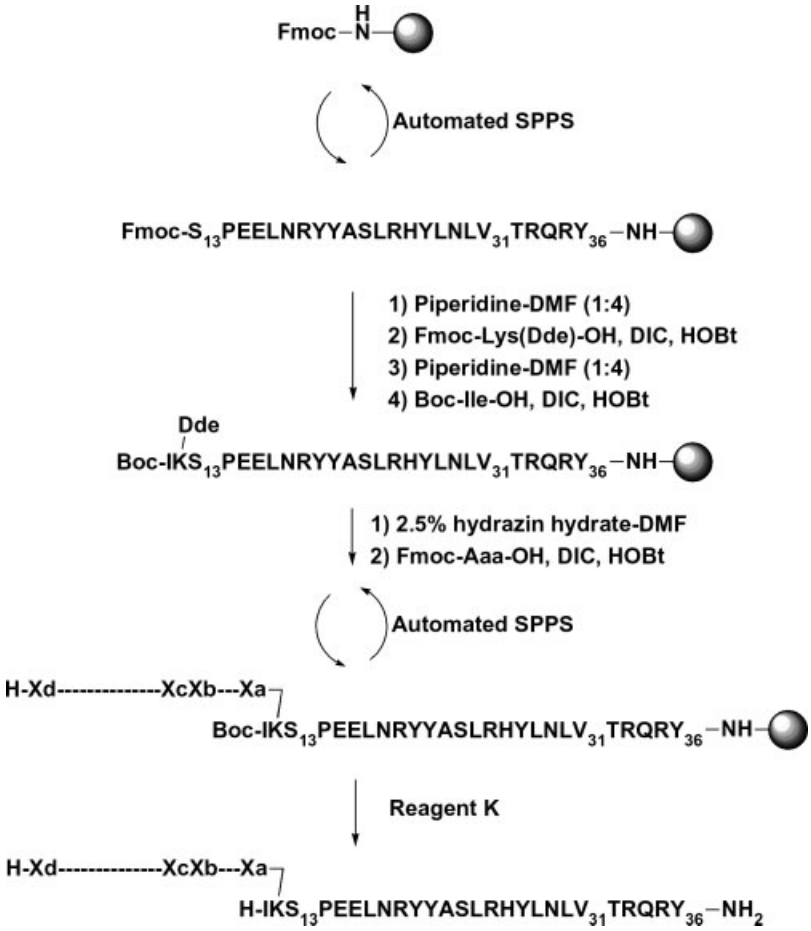
Experimental Section

Materials

All solvents and reagents used for peptide synthesis were of analytical reagent grade and were obtained from Fluka, Advanced Chemtech (USA), Sigma-Aldrich (Denmark or India), Chemimpex (Wood Dale, USA), Chemlabs (Bangalore, India) or Spectrochem Pvt. Ltd. (Mumbai, India). TentaGel S Rink amide resin was obtained from Fluka. Human embryonic kidney 293 cells (HEK293 Flp-In T-Rex), and pcDNA3.1 vector were purchased from Invitrogen (Denmark). Dulbecco's modified Eagle's medium (D-MEM), fetal calf serum, penicillin–streptomycin solution, phosphate-buffered saline (PBS), sucrose and 99% glycerol were all obtained from Sigma-Aldrich (Denmark). FuGENE 6 transfection reagent, complete protease inhibitor cocktail tablets and bovine serum albumin (BSA) were purchased from Roche (Denmark). 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS), $MgCl_2 \cdot 6H_2O$, $CaCl_2$, and NaCl were obtained from AppliChem GmbH (Darmstadt, Germany). (2S,3S)-1,4-bis-sulfanylbutane-2,3-diol (DTT) was obtained from GE Healthcare (Denmark) and radioactive labeled agonists were purchased from Phoenix Pharmaceuticals (Burlingame, USA). 96-well filtration MultiScreen HTS, DV plates were obtained from Millipore (Denmark). [^{3}H]-myo-inositol (PT6-271) was purchased from Amersham (UK).

Peptide synthesis

The peptides were prepared by automated peptide synthesis on a Symphony parallel synthesizer (PTI) by standard solid-phase peptide synthesis (SPPS) on TentaGel S Rink amide resin (0.24 mmol g^{-1} loading) with 9-fluorenylmethyl-oxycarbonyl (Fmoc) for protection of N^{α}-amino groups except for the *N*-terminal Ile of the main chain, where Boc (*tert*-butoxycarbonyl) was used. Side-chain protecting groups were *tert*-butyl (Ser, Thr, Tyr, Asp, Glu), *tert*-butoxycarbonyl (Lys), 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde, for branching at Lys), 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf, for Arg), and trityl (Trt, for Asn, Gln, His). N^{α}-Fmoc amino acids (6.0 equiv) were coupled using diisopropylcarbodiimide (DIC) (6.0 equiv) as coupling agent and 1-hydroxybenzotriazole (HOBt) (6.0 equiv) as additive in DMF for 120 min. N^{α}-Fmoc deprotection was performed using piperidine-DMF (1:4) for 3 min, followed by piperidine-DMF (1:4) for 17 min. The Lys(Dde) residue was side-chain deprotected with 2.5% (v/v) hydrazine hydrate in DMF at room temperature for 15 min. The peptide amides were released from the solid support by treatment with reagent K (trifluoroacetic acid (TFA)-phenol-thioanisol-1,2-ethanedithiol (EDT)-H_2O (82.5:5:5:2.5)) for 2.5 h. The TFA solutions were concentrated by nitrogen flow and the



Scheme 1. Reaction scheme describing the solid-phase synthesis of the PYY isoforms. Underlined sequences represent the inherent α-helical segment. Residues Xa–Xb shows the position of the turn motifs and residues Xc–Xd illustrates the position of the branched *N*-terminal segments derived from the native PYY sequence.

compounds were precipitated with diethylether to yield the crude materials as white powders.

Purification was accomplished by RP-HPLC (Agilent 1200 series) on a preparative column (Zorbax-Eclipse XDB-C18, 7-μm particles, 21.2 × 250 mm^2) using the following solvent system: solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA. B gradient elution (0–2 min: 5%, 2–5 min: 5–25%, 5–50 min: 25–60%) was applied at a flow rate of 20 ml min^{-1} and column effluent was monitored by UV absorbance at 220 nm. Identification and quantification were carried out by LC-MS (Agilent Technologies LC/MSD VL) using the eluent system A–B (solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA). The eluent system A–B was applied on a C18 analytical column (Zorbax-Eclipse XDB, 80 Å 5 μm 4.6 × 150 mm^2) where the B gradient elution (0–25 min:5–50%) was applied at a flow rate of 1.0 ml min^{-1}. Quantification and characterization data are given in Table 5.

Circular dichroism spectroscopy

UV CD spectra were recorded on a JASCO J-815 circular dichroism instrument using rectangular Hellma quartz cells with a light path of 1 mm. The peptide solutions were approximately 20 μM in 10 mM phosphate buffer, pH 7.4. The absolute concentration was determined spectroscopically (tyrosine absorption at 274 nm, using ε = 1420 cm^{-1} M^{-1}). The mean residue ellipticity (MRE) was calculated according to Yang *et al*. in which the number *n* refers to the number of residues [27].

Radioligand displacement assay

Cell culture and receptor expression

The SK-N-MC cells were cultured in a 1:1 mix of HAM F12 and D-MEM 1885, containing 15% (v/v) fetal calf serum (FCS), 1% non-essential amino acids and 1% (v/v) penicillin–streptomycin in humidified atmosphere of 5% carbon dioxide and 95% air, at 37 °C. The human embryonic kidney (HEK293 Flp-In T-Rex) derived cell line was cultured in D-MEM, containing 10% (v/v) fetal calf serum (FCS) and 1% (v/v) penicillin–streptomycin. Cells were grown as monolayers in humidified atmosphere of 5% carbon dioxide and 95% air, for 48 h at 37 °C. Using serum-free D-MEM and FuGENE 6 transfection reagent, HEK293 Flp-In T-Rex cells were transiently transfected by pcDNA3.1 vectors which encode either the human Y2 or Y4 receptor (FuGENE 6/pcDNA3.1, 3:1). The transfected HEK293 Flp-In T-Rex cells were set to express the receptors in humidified atmosphere of 5% carbon dioxide and 95% air, for another 48 h at 37 °C.

Preparation of membrane fractions

The SK-N-MC and the transiently transfected HEK293 Flp-In T-Rex cells were washed with PBS and homogenized in cold

homogenization buffer (50 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.0 mM DTT, complete protease inhibitor cocktail (one tablet per 50-ml buffer)). An equal amount of 0.6 M sucrose was added to the cell mixture. The homogenate was centrifuged (10 000 *g*, 10 min at 4 °C). Cell pellets were washed in washing buffer (SK-N-MC cells: 50 mM TRIS (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.2% (w/w) BSA fraction V. HEK293-Flp-In-T-Rex: 50 mM TRIS (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50-ml buffer), 1.0% (w/w) BSA fraction V), and subsequently the suspensions were centrifuged (10 000 *g*, 10 min at 4 °C). The pellets were re-suspended in glycerol containing binding buffer (50 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50-ml buffer), 1.0% (w/w) BSA fraction V, 20% (v/v) glycerol) and the concentration was adjusted to an OD600 of 1.6.

Binding affinity

All binding experiments were performed in 96-well filtration MultiScreen HTS, DV plates and each concentration point was performed as triplicates. To the unlabeled peptide (25 µl) at concentrations between 10 pM and 10 µM, cell-membrane suspension (3.5 µl), binding buffer (61.5 µl, 50 mM TRIS, pH 7.5, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50-ml buffer), 1.0% (w/w) BSA fraction V)) and radioligand solution (10 µl; Y1R: ^{125}I-[31Leu,34Pro]-pNPY; Y2R: ^{125}I-PYY3–36; or Y4R: ^{125}I-PP; specific activity of 800–1000 Ci/mmol) were added. After 1–2 h of incubation at room temperature the assay was terminated by filtration. Finally, the membrane-receptor-ligand complexes were washed twice in cold TRIS buffer (50 mM TRIS, pH 7.5, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$) containing 5.0% BSA fraction V. Bound radioactivity was determined as counts per minute (Wallac 1470 Wizard™ Automated Gamma Counter). Each data set was merged and normalized such that 0% was the smallest value (cpm) and 100% the largest value. The binding data was analysed with GraphPad Prism 5.0 (GraphPad Software, Inc. San Diego, CA, USA). The displacement experiments were replicated until the standard error of mean (SEM) of log IC_{50} was below 0.1 ($n = 2–5$).

Functional assay

Transfections and tissue culture

COS-7 cells were grown in Dulbecco's modified Eagle's medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine and 0.01 mg ml^{-1} gentamicin. Cells were transfected with 10 µg cDNA of wild type Y1 or Y2 receptors and 10 µg cDNA of a Gα Δ6qi4myr [28] using the calcium phosphate precipitation method with chloroquine addition. The chimeric G-protein allowed the Gαi-coupled receptors to signal through the signal transduction pathways known for the Gαq-coupled receptors. Receptors from the PP-fold family of peptides – the Y1 and Y2 receptors – were cloned from a human cDNA library and expressed in a pcDNA3.1 vector.

Phosphatidylinositol turnover

The assay was performed as previously described [29] and only described in brief in the following. One day after transfection, COS-7 cells were incubated for 24 h with 5 µCi of [^{3}H]-*myo*-inositol in 1 ml medium, washed twice in buffer, 20 mM HEPES, pH 7.4, and were subsequently incubated in 0.5 ml buffer supplemented with 10 mM LiCl at 37 °C for 30 min. After stimulation with various concentrations of PYY3–36 or the synthetic analogues for 45 min at 37 °C, cells were extracted with 10% ice-cold formic acid followed by incubation on ice for 30 min. The generated [^{3}H]-inositol phosphate was purified on Bio-Rad AG 1-X8 anion-exchange resins. Determinations of each measuring point were made in duplicates. The functional assays were replicated three times.

***In vitro* metabolism of PYY3–36 and PYY isoforms**

Mouse serum stability

PYY3–36 and peptide **1**–**5** were dissolved in 195 µl protease free water to a final peptide concentration of 107 µM. To the peptide solution, 5 µl of freshly prepared mouse serum (male NMRI mice) was added. After incubation at 37 °C for 0, 60, 120 and 300 min, 20 µl of the peptide–serum mixture was deproteinated with 60 µl of ethanol. Subsequent to centrifugation (14 000 rpm, 4 °C, 15 min), the supernatant was analysed by LC-MS (Dionex Ultimate 3000 system equipped with a PDA UV detector, MSQ Plus Mass Spectrometer, Thermo), using eluent system A–B (solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA) which was applied on a C18 analytical column (Phenomenex, Gemini, 110 Å 3 µm C18 particles, 4.60 × 50 mm^2) where an A gradient elution (0–12 min : 5–100%) was applied at a flow rate of 1.0 ml min^{-1}. A control (deproteinated mouse serum without novel peptide or PYY3–36 added) showed no trace of mouse PYY3–36 or any other protein or peptide (data not shown).

Saline stability

PYY3–36 and peptide **3** (10 µM) were incubated in 0.9% saline at 37 °C for 14 days. The samples were analysed by analytical RP-HPLC at day 2, 7 and 14.

***In vivo* experiments**

All experiments were conducted in accordance with internationally accepted principles for the care and use of laboratory animals and the protocols were approved by the Danish Committee for Animal Research (intravenous PK study and acute mice study) or the Institutional Animals Ethics Committee of Aurigene Discovery Technologies Ltd, Bangalore, India (chronic mice study).

Intravenous pharmacokinetic study

Animals

Fifty-two male NMRI (Naval Medical Research Institute) mice (Charles River), 8–9 weeks old at the time of arrival at the In Vivo Pharmacology Department of Rheoscience were used. Upon arrival (day 7), the animals were kept on a 12 : 12-h light–dark cycle (lights on at 03 : 00 AM) in a temperature-controlled environment (22–24 °C). The mice had *ad libitum* access to standard chow (Altromin 1324, Brogaarden ApS, Denmark).

General procedure

At day 5, the mice were weighted and randomized according to body weight and divided into dosage groups ($n = 3$) which were terminated at $t = 0$, 2, 5, 15, 30, 60, 120 and 240 min. Only PYY3–36 and peptide **3** were investigated. The compounds (or vehicle) were administered by intravenous route (*i.v.*) at $t = 0$ min. Dosage for both peptides was 0.25 µmol kg^{-1}

(2 ml kg^{-1}). Exact concentration of the peptide solutions were determined spectroscopically (tyrosine absorption at 274 nm, using $\varepsilon = 1420\ cm^{-1}\ M^{-1}$). At the time of termination, eye-blood was collected in cold EDTA tubes (containing 0.6 TIU (trypsin inhibitor unit) of aprotinin per milliliter of blood) and the mice were immediately euthanized by decapitation. Immediately after collection, the blood in the EDTA tubes was gently mixed several times to inhibit the activity of proteases.

Bioanalysis

The blood samples were centrifuged at 1600 × *g* for 15 min at 40 °C, plasma was collected and stored at −70 °C until analysis. Plasma concentrations of PYY3–36 and peptide **3** were determined by PYY ELISA kit (for human PYY, Phoenix Pharmaceuticals, lot #600448; LLoQ 10 pg ml^{-1}). The interference from endogenous PYY was corrected by subtracting the mean binding values of PYY in the control group from the mean binding values of PYY3–36 and peptide **3** at different time points in the *in vivo* study. The human PYY ELISA kit (Phoenix Pharmaceuticals) was tested using dilutions of PYY3–36 and peptide **3** respectively, and showed that PYY ELISA kit was very useful for measuring both PYY3–36 as well as peptide **3**. The standard curve showed a linear range between 0.1 and 1 ng ml^{-1} for PYY3–36 and 0.1–3 ng ml^{-1} for peptide **3** (not shown).

Acute mice study

Animals

The studies were carried out in lean male NMRI mice (Charles River), 8–9 weeks old. Animals were kept on a 12 : 12-h light–dark cycle (lights on at 03 : 00 AM) in a temperature-controlled environment (22–24 °C). Food for the entire experiment was standard chow (Altromin 1324).

Sample preparation

Vehicle for all experiments was 0.9% saline. Compounds were administered via Alzet osmotic mini-pumps (model 1003; 100 µl; 1.0 µl h^{-1}, 3 days of delivery). The animals were given either vehicle (0.9% saline), PYY3–36, peptide **2** or **3** in concentrations of 0.25 µmol kg^{-1} day^{-1}. Pumps were filled in the morning of day 0 and 'primed' for 4 h (pump filled and kept in 0.9% saline at 37 °C until operation, approximately 4–6 h).

General procedure

The animals ($n = 7$) were given water and food for the entire experiment (Altromin 1324; Petersen, Ringsted, Denmark) on the day of arrival. Upon arrival, the animals were transferred to single cages in which they were housed until the start of the experiment (∼10 days). Body weight was recorded on the morning of day 0 (07 : 00–08 : 00 AM) and the mice were randomized. On day 0, animals were anaesthetized using gas anaesthesia (halothane) and Alzet osmotic pumps (model 1003) implanted subcutaneously in the lower back and surgical between 12 : 00 and 14 : 00 PM, wound closed with surgical staples. Povidone iodine solution was applied topically on surgical site. Following the operation, the mice were allowed to recover, and subsequently transferred back to their cages. Pentazocin (5 mg kg^{-1} SQ) was administered once as analgesic. Body weight was recorded in the morning between 08 : 00 and 10 : 00 AM from days 0 to 6.

Chronic DIO-mice study

Animals

Studies were carried out in male C57BL/6J mice. Animals were kept on a 12 : 12-h light–dark cycle (lights on at 07 : 00 AM) in a temperature-controlled environment (22–24 °C) with free access to food and water.

Sample and pump preparation

Vehicle for all experiments was 0.9% saline. Compounds were administered via Alzet osmotic mini-pumps (model 2002; 200 µl; 0.5 µl h^{-1}, 14 days of delivery). The animals were given either vehicle, PYY3–36 (batch no: IN1009-041) or peptide **3** (batch no: IN1094-071) in concentrations of 0.25 µmol kg^{-1} day^{-1} or 0.1 µmol kg^{-1} day^{-1}. The pumps were filled the day before the start of the experiment and 'primed' overnight (pump filled and kept in 0.9% saline at 37 °C).

General procedure

The animals ($n = 9$) were aged 6–8 weeks at the time of arrival to the *in vivo* facility. The mice were housed 5 per cage for 20 weeks before the initiation of the experiment. During this period, mice were given free access to water and a high energy diet containing 60% energy from fat (Diet # D12492; Research Diets, New Jersey, USA). Two weeks prior to pump implantation, the animals were transferred to individual cages. Seven days before the experiment started and until the end of the experiment, body weight and food intake were recorded daily at 09.00 AM. The animals were randomized according to body weight on the day before implantation of the Alzet osmotic mini-pumps. On day 0, animals were anaesthetized using anaesthesia gas (halothane) and Alzet osmotic pumps (model 2002) implanted subcutaneously in the lower back and surgical wound closed with surgical staples. Povidone iodine solution was applied topically on the surgical site. Following the operation, mice were allowed to recover, and then transferred back to their cages. Pentazocin (5 mg kg^{-1} SQ) was administered once as analgesic. On the morning (09 : 00 AM) of day 14 of the experiment, blood samples from the *ad libitum* fed mice were obtained for analysis of plasma triglycerides, total cholesterol, free fatty acids, leptin and adiponectin.

Plasma analysis

For measuring plasma triglycerides, total cholesterol and non-esterified free fatty acids (NEFA), blood were sampled from the retro-orbital plexus into pre-cooled EDTA coated tubes (K_3-EDTA, 1.6 mg ml^{-1}). Samples were immediately centrifuged at 4800 × *g* for 15 min and stored at −80 °C until analysis. NEFA were measured in duplicates using a colorimetric enzyme assay kit purchased from FA115, Randox Laboratories, Antrim, United Kingdom). Triglycerides (kit #30364) and total cholesterol (kit #30183) were measured using Labkit from Chemelex S.A. (Barcelona, Spain). For the analysis of plasma leptin and adiponectin blood was sampled into pre-cooled EDTA tubes (K_3-EDTA, 1.6 mg ml^{-1}) and stored at −80 °C until analysis. Leptin and adiponectin levels were measured using mouse leptin (EZML-82K) and mouse adiponectin (EZMADP-60K) ELISA kit, respectively (Millipore, Denmark). Fluorescence readout was measured using SpectraMax Gemini Spectrofluorometer (Molecular Devices Corporation, USA).

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Results and Discussion

Peptide synthesis

Human PYY3–36 and the PYY isoforms were all assembled using the Fmoc/*t*-Bu strategy by automated solid-phase peptide synthesis on a Rink amide handle on TentaGel resin. The N^{α}-Fmoc-protected amino acids were coupled using DIC and HOBt as coupling reagent and additive, in DMF, with coupling times of 2 h. The Fmoc group was removed using 1 : 4 piperidine-DMF, for 3 + 17 min. The sequences of PYY3–36 and resin-bound PYY13–36 were assembled using standard Fmoc/*t*-Bu chemistry as described above. The sequence of the resin-bound PYY13–36 was additionally modified by coupling of a Dde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl) side-chain protected Lys followed by Fmoc removal and coupling of a N^{α}-Boc-protected Ile (Scheme 1). The Dde group was removed using 2.5% (v/v) hydrazine hydrate in DMF making the ε-amino group available for elongation of the peptide branch (Scheme 1, a–d), which additionally was performed by standard Fmoc/*t*-Bu chemistry.

Series 1 maintained the turn motif from native PYY (PYY9–12, GEDA); however, in series 2 the turn motif (Xa–Xb, Scheme 1) was exchanged with the less-flexible GPRRP sequence, which was derived from the GPRRG sequence that was reported to induce a β-turn in helix–loop–helix structures by Degrado *et al.* [26]. The GPRRP sequence includes a Gly ($i + 1$) and two Pro ($i + 2$ and $i + 5$) residues in a full pentapeptide turn as well as two Arg ($i + 3$ and $i + 4$) residues providing a double positive charge. The additional Pro in GPRRP compared with GPRRG should provide a more rigid turn. The branched *N*-terminal segments (Xc–Xd, Scheme 1), were either PYY1–8, PYY2–8 or PYY3–8 and the branched *N*-terminus was synthesized as a free amine (Scheme 1). After completion, the peptides were side-chain deprotected and simultaneously cleaved from the solid support using reagent K. Finally, the peptides were purified by preparative RP-HPLC, quantified by analytical RP-HPLC and characterized by mass spectrometry. The final products were obtained with >95% purity (Table 5).

The secondary structures of PYY3–36, and peptides **1**–**5** in solution were assessed by circular dichroism (CD) spectroscopy in 10 mM phosphate buffer. The spectra obtained were typical of an α-helical structure, showing characteristic minima at 208 and 222 nm (Figure 1). The degree of α-helicity generally showed that the branch modifications were well tolerated and only showed small changes in the overall secondary structure of the native α-helix. The mean molar ellipticity (ME) – i.e., per molar peptide – of peptide **3** was reduced compared to PYY3–36, which indicates a slight destabilisation of the α-helix, in contrast to peptide **5** which exhibited a moderately increased ME.

In vitro pharmacology

To characterize the influence of the alteration of secondary structure of the PYY isoforms, both the novel and the native peptides were assayed by a radioligand displacement binding assay (competition assay) based on membranes from SK-N-MC and HEK293 Flp-In T-Rex cell lines expressing the human Y1, Y2 and Y4 receptor sub-types, respectively. ^{125}I-[^{31}Leu,^{34}Pro]-pNPY for Y1R, ^{125}I-hPYY3–36 for Y2R and ^{125}I-hPP for Y4R were used as radioligands. The analogues were additionally evaluated for activity in a signal transduction assay (functional assay). Peptide **1**, branched isoform of PYY1–36, showed equal Y receptor affinities compared to PYY3–36. The signal transduction assay confirmed the equipotency towards the Y2 receptor (Table 2); however, the high potency towards the Y1 receptor was also established. Peptide **2** (the branched isoform of PYY2–36) had a Y receptor binding profile very similar to PYY3–36, as did peptide **3** (branched isoform of PYY3–36). Though the competition binding of peptide **3** showed Y receptor affinities comparable to native PYY3–36, the functional assay demonstrated a marginal increase in Y2 over Y1 selectivity (Table 2).

The second series of PYY analogues (containing the GPRRP modified turn motif) were able to displace the Y2 receptor selective radioligand ^{125}I-hPYY3–36 with high affinities, showing 3- (peptide **4**) and 2.5-fold (peptide **5**) improvements in IC_{50} values, respectively, over the native peptide. The potencies from the functional assay confirmed the improved agonistic effects of peptide **4** and **5**, displaying a 5- and 8-fold, respectively, increase in potencies compared to PYY3–36. The di-arginine containing motif of peptide **4** and **5** increases the net charge from +1 (PYY3–36) to +6 and the pI from approximately 9.3 to approximately 10.8. In addition to the possible changes in secondary and tertiary structure of the series 2 analogues, we speculate that the increase in net charge affects either the binding to the membrane or the Y receptors in a manner favourable for Y2 receptor potency. Interestingly, these two peptides displaced the Y1 receptor selective radioligand ^{125}I-[^{31}Leu,^{34}Pro]-pNPY with very low apparent affinities – the IC_{50} was shifted 28- and 12-fold, respectively, for peptides **4** and **5**, compared to PYY3–36. However, the potencies obtained from the functional study showed that these peptides actually activated the Y1 receptor

Table 2. Affinities and potencies of PYY3–36 and the novel PYY isoforms towards the Y receptors. The main chain sequence of the PYY isoforms: H-IKSPEELNRYYASLRHYLNLVTRQRY-NH_2

		Binding affinity, IC_{50} (nM)			Potency, EC_{50} (nM)	
Peptide	Branched sequences	Y1R[a]	Y2R[b]	Y4R[b]	Y1R[c]	Y2R[c]
PYY3–36		19 ± 5	0.47 ± 0.08	261 ± 33	90 ± 11	5.6 ± 2.1
1	H-YPIKPEAPGEDA-	10 ± 1.5	0.88 ± 0.05	239 ± 15	12 ± 7	5.6 ± 3.2
2	H-PIKPEAPGEDA-	25 ± 16	0.90 ± 0.01	277 ± 29	80 ± 22	4.3 ± 2.9
3	H-IKPEAPGEDA-	19 ± 5	0.95 ± 0.07	617 ± 24	118 ± 19	4.6 ± 2.4
4	H-YPIKPEAPGPRRP-	229 ± 22	0.17 ± 0.02	275 ± 80	4.5 ± 3.3	1.1 ± 0.3
5	H-IKPEAPGPRRP-	535 ± 182	0.20 ± 0.05	524 ± 155	46 ± 23	0.7 ± 0.3

[a] SK-N-MC cells expressing the Y1 receptor.
[b] Transfected Y2 or Y4 receptors in HEK293 Flp-In T-Rex cells.
[c] Transfected Y1 or Y2 receptors as well as Gα Δ6qi4myr in COS-7 cells.

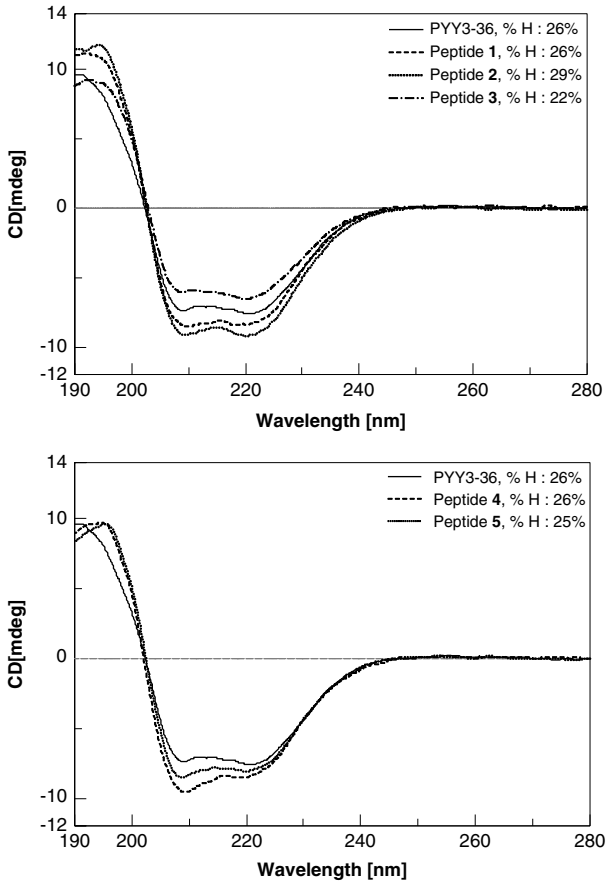

Figure 1. Circular dichroism (CD) of PYY3–36 and peptides **1**–**5** in phosphate buffer as well as degree of α-helicity (% H) for each compound.

with a high potency. An explanation for the discrepancy between apparent affinity observed in competition binding studies and the potency observed in functional characterization is most likely related to the fact that a heterologous competition binding study has been used [30]. In heterologous competition studies, the radioligand differ chemically from the investigated ligand, which allow the radioligand to trap the receptor in one conformation and high concentration of the ligand is required to induce the conformational changes before binding [30]. Another possible explanation for the discrepancy between affinity and potency could be that the functional assay allow for intracellular amplification of the response, which may increase the potency.

It is well known that *N*-terminally truncated versions of both NPY, such as NPY3–36 and NPY13–36, and *N*-terminally truncated versions of PYY, such as PYY3–36 and PYY13–36, improves the Y receptor specificity (Y2 over Y1) [23]. Recently, even shorter versions of PYY, the *N*-terminal PEGylated PYY22–36 analogues, were shown to retain some Y2 activity while greatly reducing Y1 activity [31–33]. Interestingly, however, while PEGylation and *N*-terminal truncation also reduces Y2 affinity (10- to 50-fold), our *N*-terminal branched peptides retain or increase a high affinity towards the Y2 receptor.

Mouse serum stability

The serum stability of native PYY3–36 and the PYY isoforms was evaluated after 60, 120 and 300 min by analytical RP-HPLC.

Table 3. Metabolic digestion in diluted mouse serum[a] of native PYY3–36 and peptide **1**–**5**

Peptide	Undigested peptide[b] (%)		
	After 60 min	After 120 min	After 300 min
PYY3–36	>95	93	69
1	88	89	n.d.
2	>95	83	53
3	94	88	69
4	~50	<5	<5
5	>95	<5	<5

[a] Water-mouse serum (40 : 1) at 37 °C.
[b] Analysed by analytical RP-HPLC.

The first series of PYY analogues showed no significant variation in serum stability compared to PYY3–36 during a 300-min incubation period at 37 °C (Table 3, entry 1–4). PYY3–36 and peptides **1**–**3** were primarily digested by carboxy-peptidases cleaving at the amino side of Arg-33 and Arg-35. Likewise, peptides **4** and **5** were digested in a similar mode, however, unexpectedly rapidly (>10 times as fast). Due to the unexpected low half-life in serum peptides **4** and **5** were not further investigated.

Body-weight-lowering effects in acute mouse study

The effect of 3 days of subcutaneous mini-pump administration of PYY3–36, peptides **2** and **3** on body weight in male NMRI mice were investigated (Figure 2). The two novel PYY analogues were selected for *in vivo* investigation based on their potency and serum stability. Pre-treatment analysis showed PYY3–36 and peptide **3** to be stable in 0.9% saline over a period of 14 days (data not shown) similar to what has previously been reported for PYY3–36 [7]. On the first day of the study, the animals were implanted with an Alzet osmotic mini-pump providing a continuous administration of either vehicle (0.9% saline), PYY3–36, peptides **2** or **3** in peptide concentrations of 0.25 μmol kg^{-1} day^{-1}. A reduction in body-weight gain was observed at day 3 for animals treated with PYY3–36 (~6%), peptide **2** (~4%) and **3** (~7%), as well as at day 6 for PYY3–36 (~2%), peptide **2** (~2%) and **3** (~3%) treated animals.

Pharmacokinetic analysis of PYY3–36 and peptide 3

On the basis of affinity, potency, serum stability and the acute *in vivo* data peptide **3** was further investigated. The pharmacokinetic profile of PYY3–36 and peptide **3** were examined following an intravenous bolus injection. The calculated half-life of both compounds was approximately 35 min.

Chronic body-weight reduction in DIO-mice and adipose tissue biomarkers

We next examined the effect of 14 days of administration of PYY3–36 and peptide **3** by osmotic mini-pumps in male diet-induced obese C57BL/6J mice. Mice were randomized according to body weight and implanted subcutaneously in the back with an Alzet osmotic mini-pump delivering either vehicle (0.9% saline), PYY3–36 (0.25 or 1.0 μmol kg^{-1} day^{-1}) or peptide **3** (0.25 or 1.0 μmol kg^{-1} day^{-1}) (Figure 3). Both doses of PYY3–36 and peptide **3** reduced food intake and body weight depending on the dose. A one-way analysis of variance (ANOVA) test [34]

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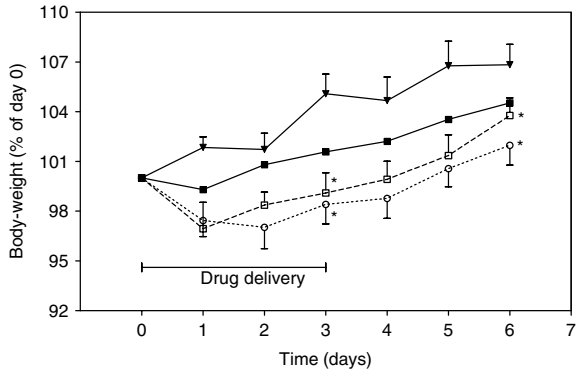

Figure 2. Acute mini-pump experiment in lean male NMRI mice (3 days). Vehicle (▼), 0.25 μmol kg^{-1} day^{-1} PYY3–36 (□), 0.25 μmol kg^{-1} day^{-1} peptide **2** (■) and 0.25 μmol kg^{-1} day^{-1} peptide **3** (○). Values are means ± SEM ($n = 7$). One-way ANOVA followed by a 'Dunnett's multiple comparison test': * $P < 0.05$ compared to vehicle.

followed by a 'Dunnett's multiple comparison test' at day 7 and 14 showed PYY3–36 and peptide **3** reduced body weight significantly compared to vehicle (Figure 3A). The reductions in body weight were correlated to a reduction in food intake during the first week of the study (Figure 3B and C). The degree of body-weight loss (~10%) during the first 7 days was compatible to previous reported PYY3–36 analogues [4,6,7,13,35].

After the final dosing at day 14, blood was collected to measure leptin, non-esterified free fatty acids, triglycerides, cholesterol and adiponectin (Table 4). In accordance with the body-weight loss, both PYY3–36 and peptide **3** treated mice had lower levels of circulating leptin at termination (Figure 4A). Interestingly, adiponectin levels were increased to a larger extent in peptide **3** treated mice when compared to PYY3–36 (0.25 μmol kg^{-1} day^{-1}; $P < 0.005$ and 1.0 μmol kg^{-1} day^{-1}; $P < 0.01$) (Figure 4B). Increases in plasma adiponectin concentrations are particularly interesting from a metabolic point of view as adiponectin has been shown to possess anti-diabetic, anti-atherogenic and anti-inflammatory properties [36,37]. Also, peptide **3** led to higher reductions in triglycerides and free fatty acids than PYY3–36, suggesting that peptide **3** exert additionally beneficial effects on fat-cell metabolism. Although it can be speculated that the differential effects of peptide **3** on adiponectin levels and plasma lipids could be due to an alteration in Y2/Y1 selectivity, the change in this selectivity ratio is rather modest and therefore other factors should be considered. First, the PYY3–36 isoform exemplified by peptide **3** may have an altered back-folding; the branch and the additional *N*-terminal amino group could lead to a changed secondary structure in the solution, thus leading to an altered distribution. It is also possible that peptide **3** activated other receptors than the Y receptors. The orphan GPCR, glucocorticoid-induced receptor (GIR, GPR83), has previously been speculated to be a target for PYY3–36.

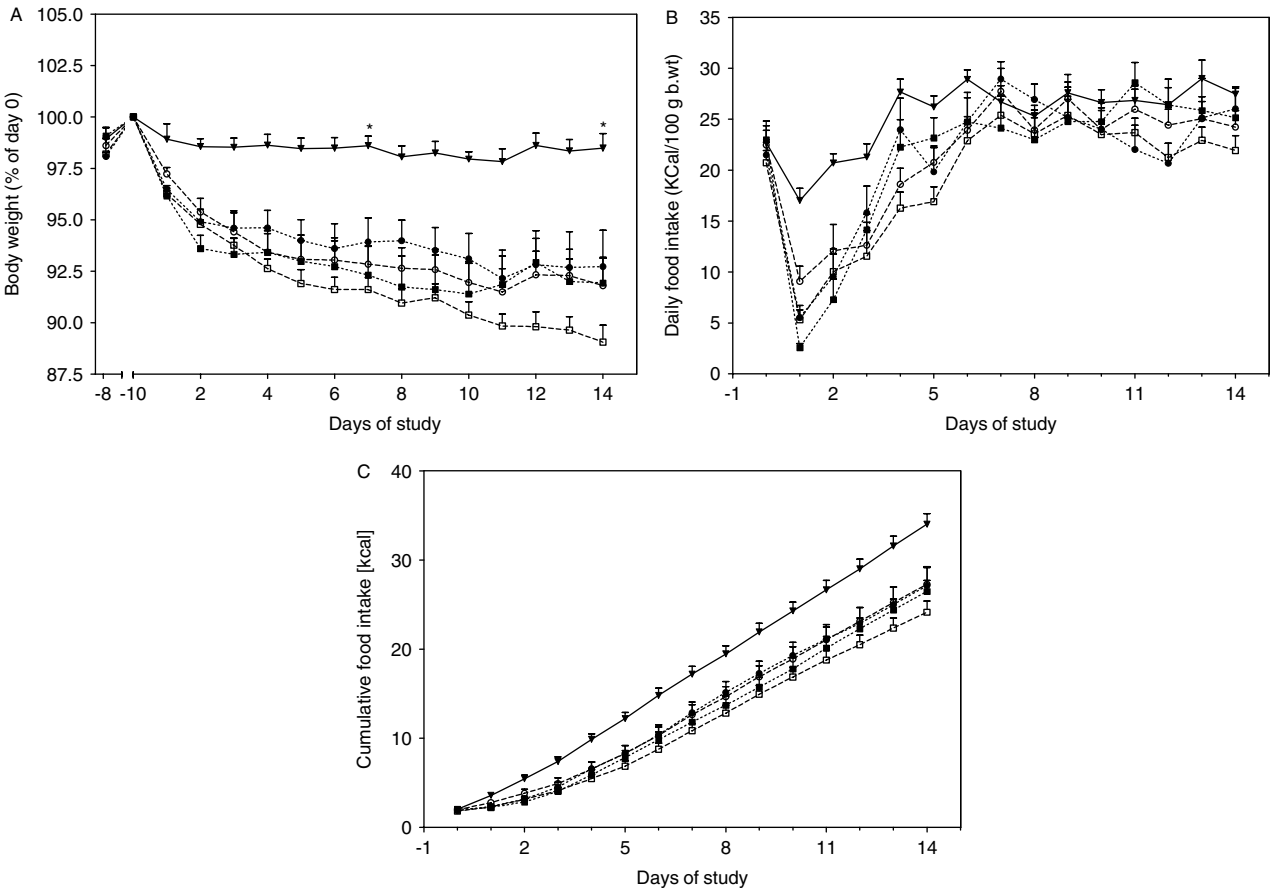

Figure 3. Chronic mini-pump experiment in male DIO C57BL/6J mice (2 weeks). Body weight (A), daily food intake (B) and cumulative feed intake (C). Vehicle (▼), 0.25 μmol kg^{-1} day^{-1} PYY3–36 (□), 1.0 μmol kg^{-1} day^{-1} PYY3–36 (■), 0.25 μmol kg^{-1} day^{-1} peptide **3** (○), 1.0 μmol kg^{-1} day^{-1} peptide **3** (●). Values are means ± SEM ($n = 9$). One-way ANOVA with 'Dunnett's multiple comparison test': * $P < 0.05$ compared to vehicle.

Table 4. Amount of free fatty acids, triglycerides and cholesterol on day 14 Values are means ± SEM ($n = 9$)

	Peptide concentration (μmol kg^{-1} day^{-1})	Free fatty acids (mmol l^{-1})	Triglycerides (mg dl^{-1})	Total cholesterol (mg dl^{-1})
Vehicle		1.26 ± 0.27	217 ± 21	153 ± 56
PYY3–36	0.25	1.07 ± 0.26	191 ± 37	136 ± 53
	1.0	1.00 ± 0.18	201 ± 27	104 ± 27
Peptide **3**	0.25	0.92 ± 0.15*	199 ± 26	111 ± 22
	1.0	0.86 ± 0.22**	187 ± 26*	96 ± 30

One-way ANOVA with 'Dunnett's multiple comparison test': * $p < 0.05$, ** $p < 0.01$ compared to vehicle.

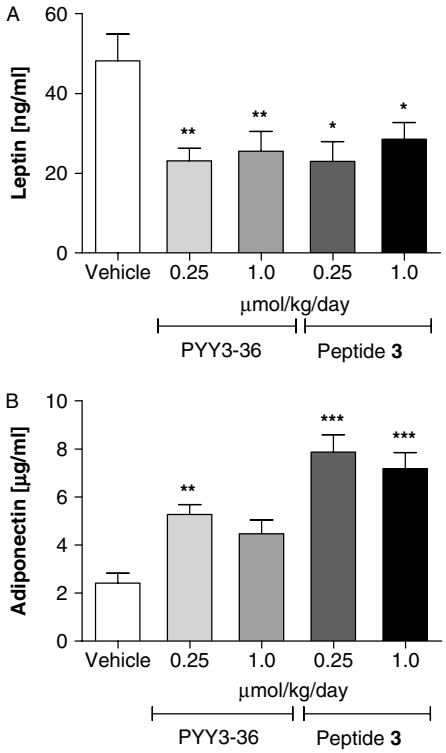

Figure 4. Leptin levels on day 14 (A) and adiponectin levels on day 14 (B). Values are means ± SEM ($n = 9$). One-way ANOVA with 'Dunnett's multiple comparison test' : * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to vehicle.

GIR is found in brain regions such as hypothalamus; however, the pharmaceutical action of the receptor is awaiting [38].

Conclusion

In conclusion, we report the design of PYY analogues with an isoform peptide architecture, their synthesis and pharmacological characterization, which led to the identification of a number of potent Y2 receptor agonists. Particularly, the compounds effectively reduce body weight in both lean and DIO-mice. Interestingly, despite identical stability and PK-profile between peptide **3** and native human PYY3–36, peptide **3** has additional beneficial effects on plasma lipids and presumably fat-cell metabolism reflected in significant increases in adiponectin levels. The discovery that isoforms of PYY hold interesting and unforeseen pharmacological properties should be explored further due to their lipid-lowering effects.

Table 5. Quantification and characterization data of PYY3–36 and the branched PYY isoforms

Peptide	ESI-MS (m/z)[a] Calculated MS	ESI-MS (m/z)[a] Found, $[M+4H]^{4+}$	Purity[b] (%)
PYY3–36	4049.5	1012.8	98
1	4551.2	1138.4	96
2	4388.0	1097.7	95
3	4291.0	1073.4	97
4	4743.5	1186.4	95
5	4482.2	1121.4	95

[a] Identified by ESI-MS on a Agilent Technologies LC/MSD VL.
[b] Quantified by RP-HPLC at 215 nm. Eluent A: 0.1% TFA in H_2O. Eluent B: 0.1% TFA in AcN.

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