Research Article

Journal of PeptideScience

Received: 27 January 2009 Revised: 5 July 2009 Accepted: 6 July 2009 Published online in Wiley Interscience: 8 September 2009

(www.interscience.com) DOI 10.1002/psc.1170

Modifying the conserved *C*-terminal tyrosine of the peptide hormone PYY3-36 to improve Y2 receptor selectivity

Søren L. Pedersen,[a,b] Birgitte Holst,[c] Niels Vrang[b,d]* and Knud J. Jensen[a]*

The Y2 selective PYY derived peptide PYY3-36 was recently shown to play a role in appetite regulation. Novel PYY3-36 analogs with high selectivity for the Y2 receptor could be potential drug candidates for the treatment of obesity. The *C*-terminal pentapeptide segment of PYY3-36 is believed to bind to the Y receptors. Tyr-36 is highly conserved across species and only few successful modifications of Tyr-36 have been documented. PYY3-36 analogs were prepared using solid-phase peptide chemistry and tested for binding to the Y1, Y2 and Y4 receptor subtypes by radioligand displacement assay. The Y2 receptor agonists with the best affinity and selectivity were further investigated for activity towards the Y1 and Y2 receptor subtypes. Unexpectedly, modifications of Tyr-36 were well-tolerated, and the analogs of PYY3-36 in which the Tyr-36 hydroxyl group was substituted with a halogen or an amino group were particularly well tolerated and yielded an improved selectivity and approximately equipotent affinity to the Y2 receptor. These modifications could be used to design new potential drug candidates for the treatment of obesity.

Keywords: PYY; PYY3-36; Y2 receptor agonist; gut hormone; tyrosine analogs; obesity

Introduction

More than 35 peptides with either stimulatory or inhibitory effects on appetite have been identified to date. [1] A number of these peptides are produced by endocrine cells lining the gut and are released during ingestion and digestion of a meal. One well-known gut hormone recently implicated in appetite regulation is the 36 amino acid linear 'peptide tyrosine tyrosine' (PYY) first isolated from porcine intestine. [2] PYY is secreted by intestinal L-cells following ingestion of a meal. [3] After secretion, PYY is *N*-terminally truncated by dipeptidyl peptidase IV (DPP-IV) to produce PYY3-36. PYY and PYY3-36 exert a number of actions on gastrointestinal functions including inhibition of pancreatic exocrine secretion, [4–7] inhibition of gastrointestinal motility, [8–11] inhibition of gastric acid secretion [12,13] and stimulation of gallbladder contraction [14]. Recently, PYY3-36 was shown to inhibit food intake in rats and humans. [15,16] Subsequently, several studies in rats and mice using intraperitoneal [17–19] or intravenous [20,21] administration of PYY3-36 have shown acute reductions of food intake. Chronic administration of PYY3-36, in different animal models, has been shown to reduce body weight, [19,22] further supporting a role of PYY3-36 in appetite and weight control.

PYY belongs to the PP-fold family of peptides together with NPY and pancreatic polypeptide (PP). PP is a circulating peptide hormone produced and released from the endocrine pancreas in response to ingestion of food. [23] The peptide hormone alters biliary function and inhibits pancreatic secretion, as well as gastric and intestinal motility [24–26] and was recently shown to inhibit food intake in humans. [15,16] The second family member, NPY, acts as a neurotransmitter and is abundant in the central and peripheral nervous system. [27] The physiological functions of NPY are numerous, e.g. related to feeding, memory, blood pressure, cardiac contractility and intestinal secretion. [28] All three peptides bind to the family of Y receptors; the Y1, Y2, Y4 and Y5 subtypes. While PP is a potent Y4 receptor agonist, it displays very low affinity to the Y1, Y2 and Y5 receptors. NPY and PYY1-36 have very similar binding profiles, and their orexigenic effects are believed to be predominantly mediated via a nanomolar affinity to hypothalamic Y1 receptor. PYY3-36 is believed to be the endogenous ligand for the Y2 receptor to which it binds with sub-nanomolar affinity, however, it also binds with sub-micromolar affinity to both Y1 and Y4 receptors. [29] The appetite suppressing properties of PYY3-36 is believed to be mediated by central Y2 receptors. [16] Interestingly, recent data from rodents and humans suggests that PYY3-36 administration could interact with Y1 and/or Y5 receptors, and thereby counteract the anorectic effects of Y2 stimulation. [30,31] If this is indeed the case, novel highly Y2 selective compounds should show more anorectic and body-weight lowering potential.

* *Correspondence to: Knud J. Jensen, University of Copenhagen, Faculty of Life Sciences, IGM-Bioorganic Chemistry, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark. E-mail: kjj@life.ku.dk*

* *Niels Vrang, Gubra ApS, Ridebanevej 12, 1870 Frederiksberg C, Denmark. E-mail: niels@gubra.dk*

a *University of Copenhagen, Faculty of Life Sciences, IGM-Bioorganic Chemistry, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark*

b *Rheoscience A/S, Glerupvej 2, 2610 Rødovre, Denmark*

c *University of Copenhagen, Faculty of Health Sciences, Department of Neuroscience and Pharmacology, Blegdamsvej 3B, 2200 København N, Denmark*

d *Gubra ApS, Ridebanevej 12, 1870 Frederiksberg C, Denmark*

Table 1. Sequences of human NPY, human PP, human PYY1-36 and [^{36}X]-PYY3-36

Peptide	Sequence	*C*-terminal[a]
PP	H-APLEPVYPGDNATPEQMAQYAADLRRYINML	TRPRY-NH_2
NPY	H-YPSKPDNPGEDAPAEDMARYYSALRHYINLI	TRQRY-NH_2
PYY1-36	H-YPIKPEAPGEDASPEELNRYYASLRHYLNLV	TRQRY-NH_2
[^{36}X]-PYY3-36[b]	H-IKPEAPGEDASPEELNRYYASLRHYLNLV	TRQR**X**-NH_2

[a] Conserved pentapeptide.
[b] Human PYY3-36: X = Y.

Peptide Design

Since the discovery of the PP-fold peptides, a vast number of studies have investigated the PP-fold peptide structure and their Y receptor affinities. The *C*-terminal pentapeptide sequences of native NPY, PYY1-36 and PP are highly conserved and play a pivotal role for the affinity to the binding pocket of the Y receptors (Table 1). [32,33] Diverse interaction between acidic Y receptor residues and the *C*-terminal arginines at position 33 and 35 of the PP-fold of peptides are most likely leading to differences in the docking mode of the ligands to the Y receptors, which could be one reason for the affinity differences. [34] An exception to the conserved *C*-terminal is Pro-34 of PP, which could partly explain its differentiated Y receptor selectivity. The *C*-terminal Tyr is assumed to be essential for NPY binding and changes will lead to dramatic reduction in affinity to both the Y1 and Y2 receptor subtypes. [33] Ala scans of NPY [35] and PYY3-36 [36] both report complete loss of affinity to the Y1 and Y2 receptors when replacing Tyr-36 with Ala. These results strongly indicate that the effect from the aromatic side-chain of Tyr-36 is extremely important for the binding to the Y receptor subtypes. [35,36] NPY has additionally been point-substituted at position 36 by replacing Tyr with Phe, which lowered the affinity (13-fold for Y1R and 11-fold for Y2R) but had no impact on selectivity. [35] However, incorporation of the large, hydrophobic residue 4-benzoyl-phenylalanine, [^{36}Bpa]-NPY, gave a 590-fold decrease in affinity to the Y1 receptor and a moderate eightfold-drop in binding to the Y2 receptor. [37] The steric prerequisite for receptor binding was further explored by introducing a D–Tyr at position 36 of NPY, which resulted in a highly reduced affinity to the Y1 receptor (100-fold), compared to modest 10-fold drop to the Y2 receptor. [38] These results indicate that the Y receptors favour an aromatic side-chain at residue 36, and the size could lead to Y2 receptor selectivity due to steric limitations of the Y1 receptor.

Here we describe a number of [^{36}X]-PYY3-36 analogs; we aimed at increasing Y2 receptor selectivity while maintaining its potency. This series included peptides of unnatural Phe or Tyr analogs, which could aid in understanding the structural requirements in the receptor binding pocket.

Materials and Methods

Materials

The organic solvents and reagents for peptide synthesis were all of analytical reagent grade and were obtained from Iris Biotech GmbH (Germany), except for DMF which was obtained from Sigma-Aldrich (Denmark). TentaGel S Rink Amide resin was obtained from Novabiochem. Milli–Q (Millipore) water was used for RP–HPLC analyses and purifications. Human Embryonic Kidney (HEK) 293 cells (HEK293 Flp-In T-Rex) and pcDNA3.1 vector were purchased from Invitrogen. Dulbecco's Modified Eagle's Medium (D-MEM), FCS, penicillin–streptomycin solution, phosphate buffered saline (PBS), sucrose and 99% glycerol were obtained from Sigma-Aldrich. FuGENE 6 transfection reagent, complete protease inhibitor cocktail tablets and BSA were purchased from Roche. 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS), $MgCl_2 \cdot 6H_2O$, $CaCl_2$ and NaCl were obtained from AppliChem GmbH. (*2S*,*3S*)-1,4-bis-sulfanylbutane-2,3-diol (DTT) was obtained from GE Healthcare, and radioactive labeled agonists were purchased from Phoenix pharmaceuticals. 96-Well filtration multiscreen HTS, DV plates were obtained from Millipore. [^{3}H]-*myo*-inositol (PT6-271) was purchased from Amersham.

Peptide Synthesis

The peptides were prepared by automated peptide synthesis on a Syro II peptide synthesiser (MultiSynTech) by standard SPPS on TentaGel S Rink Amide resin with Fmoc for protection of N^{α}-amino groups. Side-chain protecting groups were *tert*-butyl (Ser, Thr, Tyr), 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf, for Arg), and trityl (Trt, for Asn, Gln, His). N^{α}-Fmoc amino acids (4.0 equiv) were coupled using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide HBTU (3.8 equiv), 1-HOBt (4.0 equiv) and *N*,*N*-DIEA (8.0 equiv) as coupling agents in DMF for 45 min, except unnatural amino acids which reacted for 120 min. N^{α} -Fmoc deprotection was performed using piperidine-DMF (2 : 3) for 3 min, followed by piperidine-DMF (1 : 4) for 12 min. The peptide amides were released from the solid support by treatment with TFA-triethylsilane (TES)-H_2O (95 : 2 : 3) for 2 h. The TFA solutions were concentrated by nitrogen flow and the compounds were precipitated with diethylether to yield the crude materials as white powders. Purification was accomplished by preparative RP-HPLC (Dionex Ultimate 3000 system) on a preparative column (FeF Chemicals, 300 Å 5 μm C4 particles, 2.1 × 200 mm) using the following solvent system: solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA. B gradient elution (0–50 min: 10–60%) was applied at a flow rate of 10 ml min^{-1} and column effluent was monitored by UV absorbance at 215 and 254 nm simultaneously. Identification was carried out by ESI-MS (MSQ Plus Mass Spectrometer, Thermo). The peptides were analysed by analytical HPLC (Dionex Ultimate 3000 system equipped with a PDA UV detector or Dionex P580 pump equipped with Waters 996 PDA and Waters 717plus autosampler) using 'orthogonal' eluent systems, first A–B (solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA, as above), then eluent system C–D (solvent C, 10 mM NH_4OAc; solvent D, 10% 100 mM NH_4OAc in AcN). First analysis; eluent system A–B was applied on a C4 analytical column (Phenomenex, Jupiter, 300 Å 5 μm C4 particles, 3.9 × 150 mm) where a B gradient elution (0–14 min: 5–100%) was applied at a flow rate of 1.0 ml min^{-1}. Second analysis; eluent system C–D was applied on a C18 analytical column (Phenomenex, Gemini, 110 Å 3 μm C18 particles, 4.60 × 50 mm) where a C gradient elution (0–14 min: 5–100%) was applied at a flow rate of 1.0 ml min^{-1}. Quantification and characterisation data are given in Table 2.

Journal of PeptideScience

Table 2. Quantification and characterisation data of PYY3-36 and H-IKPEAPGEDASPEELNRYYASLRHYLNLVTRQR^{36}**X**-NH_2 analogs

No	^{36}X	ESI-MS (m/z)[a] Calculated MS	Found	Assigned	Purity (%) Eluent system A-B[b]	Eluent system C-D[c]
PYY3-36	Tyr	4049.46	1012.76	$[M + 4H]^{4+}$	98	99
			810.21	$[M + 5H]^{5+}$		
1	*homo*-Tyr	4063.49	1016.87	$[M + 4H]^{4+}$	99	99
			813.36	$[M + 5H]^{5+}$		
2	*D*-Tyr	4049.46	1013.03	$[M + 4H]^{4+}$	97	97
			810.40	$[M + 5H]^{5+}$		
3	*p*-fluoro-Phe	4052.44	1013.51	$[M + 4H]^{4+}$	99	99
			810.83	$[M + 5H]^{5+}$		
4	*p*-chloro-Phe	4061.91	1017.19	$[M + 4H]^{4+}$	99	99
			814.17	$[M + 5H]^{5+}$		
5	*p*-bromo-Phe	4112.36	1028.66	$[M + 4H]^{4+}$	99	100
			823.19	$[M + 5H]^{5+}$		
6	*p*-iodo-Phe	4160.35	1040.09	$[M + 4H]^{4+}$	95	95
			832.35	$[M + 5H]^{5+}$		
7	*p*-nitro-Phe	4078.46	1019.81	$[M + 4H]^{4+}$	96	95
			816.18	$[M + 5H]^{5+}$		
8	*p*-amino-Phe	4048.48	1013.12	$[M + 4H]^{4+}$	99	96
			810.16	$[M + 5H]^{5+}$		
9	*m*-nitro-Tyr	4094.16	1024.15	$[M + 4H]^{4+}$	98	98
			819.20	$[M + 5H]^{5+}$		

[a] Identified by ESI-MS on an MSQ Plus Mass Spectrometer (Dionex).
[b] Quantified by RP-HPLC at 215 nm. Eluent A: 0.1% TFA in H_2O. Eluent B: 0.1% TFA in AcN.
[c] Quantified by RP-HPLC at 215 nm. Eluent C: 10 mM NH_4OAc. Eluent D: 10% 100mM NH_4OAc in AcN.

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) 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 HEK-derived (293 Flp-In T-Rex) cell line was cultured in D-MEM, containing 10% (v/v) 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, six 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 homogenisation 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 (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 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 every concentration point was performed as triplicates. 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)], radioligand solution (10 µl; Y1R: ^{125}I-[^{31}Leu,^{34}Pro]-pNPY; Y2R: ^{125}I-PYY3-36; and Y4R: ^{125}I-PP; specific activity of 800–1000 Ci/mol). After 1–2 h of incubation, 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 % (w/w) BSA fraction V. Bound radioactivity was determined as counts per minute (Wallac 1470 Wizard™ Automated Gamma Counter). Binding data were analysed with GraphPad Prism 5.0 (GraphPad Software, Inc. San Diego, CA, USA). The displacement experiments were replicated until the standard error of mean (S.E.M.) of $-\log IC_{50}$ was below 0.1 ($n = 2–5$).

Functional Assay

Transfections and tissue culture: COS-7 cells were grown in D-MEM 1885 supplemented with 10% FCS, 2 mM glutamine and 0.01 mg/ml gentamicin. Cells were transfected with 10 µg cDNA

of wild type Y1 or Y2 receptors and 10 μg cDNA of a Gα Δ6qi4myr [39], using the calcium phosphate precipitation method with chloroquine addition. The chimeric G-protein allow 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

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 analogs 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 (except PYY3-36 which was replicated four times).

Results and Discussion

Peptide Synthesis

All peptides were assembled using the Fmoc/*t*-Bu strategy by automated SPPS on Rink Amide TentaGel resin. The N^{α}-Fmoc-protected amino acids were coupled using HBTU as coupling reagent, DIEA as base and HOBt as additive, in DMF. Coupling times were generally 45 min, except for the unnatural amino acid derivatives which were coupled for 2 h. The peptides were side-chain deprotected and simultaneously cleaved from the solid support by a TFA cocktail containing TES and water as scavengers. Finally, the peptides were purified by RP-HPLC and characterized orthogonally by analytical HPLC and mass spectrometry. The final products were obtained with >95% purity (Table 2).

Affinity and Activity Evaluation

To characterize the influence of substituting Tyr-36 with unnatural amino acid analogs, the binding affinity of the novel PYY3-36 analogs were tested using a radioligand displacement assay (competition binding assay) based on membranes from SK-N-MC and HEK293 Flp-In T-Rex cell lines expressing the human Y1, Y2 and Y4 receptor subtypes, 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 analogs with the highest affinity and potency for the Y2 receptor were additionally tested for their ability to activate the Y1 and Y2 receptors. Binding affinities (IC_{50} values) and activation potencies (EC_{50} values) for the [^{36}X]-PYY3-36 analogs as well as native PYY3-36 are summarized in Table 3.

Structure–Affinity Relationship

PYY3-36 is highly potent but only moderately selective, whereas all the novel peptides in this series had an increased Y receptor selectivity. First, extending the length of the Tyr-36 side-chain by an extra methylene moiety moves the phenol group further away from the peptide backbone, which potentially influences the aromatic $\pi-\pi$ interaction and the hydroxyl hydrogen bonding interactions between the receptor and the ligand in a positive manner. [^{36}*homo*Tyr]-PYY3-36 (Table 3, peptide **1**) lowered the affinity more than 100-fold for the Y1 receptor, whereas only a modest threefold drop in affinity to the Y2 receptor was observed. These results could indicate steric restrictions in the Tyr-36 binding pocket of the Y1 receptor, compared to the Y2 receptor. Other explanations for the decreased Y1 receptor affinity could be due to the side-chain of *homo*-Tyr having one more rotatable bond, compared to Tyr, and hence the possibility that it binds to another region of the Y1 receptor binding pocket.

The binding affinities of the Y-receptors were poorer when the orientation of the side-chain of Tyr-36 was converted to the corresponding D-analog. The binding affinity of [^{36}D-Tyr]-PYY3-36 (Table 3, peptide **2**) to the Y2 receptor decreased sevenfold and basically had no affinity to the Y1 receptor (>100-fold drop). The relative Y2 receptor affinity corresponds to that of [^{36}D-Tyr]-NPY, [38] but [^{36}D-Tyr]-PYY3-36 (Table 3, peptide **2**) was far more

Table 3. PYY3-36[a] and [^{36}X]-PYY3-36 binding to Y receptor subtypes Y1, Y2, and Y4, in addition to functional Y1 and Y2 receptor evaluation of the most potent Y2R agonists

		Binding assay			Functional assay	
Peptide	^{36}X	Y1R[b] IC_{50} [nM]	Y2R[c] IC_{50} [nM]	Y4R[c] IC_{50} [nM]	Y1R[d] EC_{50} [nM]	Y2R[d] EC_{50} [nM]
PYY3-36		7.8 ± 1.1	0.50 ± 0.09	255 ± 29	135 ± 32	7.6 ± 1.6
1	*homo*-Tyr	>1000	1.63 ± 0.44	>1000	>1000	51 ± 6.0
2	*D*-Tyr	>1000	3.50 ± 0.15	>1000	n.d.	36 ± 13
3	*p*-fluoro-Phe	>1000	0.76 ± 0.02	>1000	737 ± 30	12 ± 1.0
4	*p*-chloro-Phe	198 ± 14	1.79 ± 0.22	>1000	>1000	5.2 ± 1.6
5	*p*-bromo-Phe	350 ± 101	1.67 ± 0.06	>1000	>1000	5.4 ± 2.1
6	*p*-iodo-Phe	>1000	1.65 ± 0.51	>1000	>1000	11 ± 4.0
7	*p*-nitro-Phe	>1000	8.27 ± 2.85	>1000	n.d.	55 ± 4.0
8	*p*-amino-Phe	388 ± 175	0.96 ± 0.32	>1000	367 ± 98	11 ± 2.0
9	*m*-nitro-Tyr	246 ± 87	12.0 ± 5.9	>1000	n.d.	50 ± 11

[a] Native peptide.
[b] SK-N-MC cells expressing the Y1R.
[c] Y2R or Y4R transfected HEK293 Flp-In T-Rex cells.
[d] Y1R or Y2R and Gα Δ6qi4myr transfected COS-7 cells.

Journal of PeptideScience

selective. The results above show that the position and orientation of the aromatic side-chain relative to the peptide backbone is very important for binding to the Y1 and Y4 receptors, but the Y2 receptor seems more tolerant of the conformational changes.

Replacing the hydroxyl group with electron-withdrawing substituents at the *para* position of residue Phe-36 (Table 3, peptides **3–7**) resulted in a moderate to significant decrease in the affinity to the Y1 receptor, in the affinity order Cl ≈ Br > F ≈ I ≈ NO_2, but only moderate decreases in the affinity to the Y2 receptor, in the affinity order F ≈ Cl ≈ Br ≈ I ≫ NO_2. The fact that the introduction of electron-withdrawing substituents in the *para* position of Phe-36 negatively affects Y1 receptor binding could be due to steric interactions in the active site as binding affinity decreased with size of the substituents. As [^{36}Phe(*p*-Cl)]-PYY3-36 (Table 3, peptide **4**), [^{36}Phe(*p*-Br)]-PYY3-36 (Figure 1 and Table 3, peptide **5**) and [^{36}Phe(*p*-I)]-PYY3-36 (Table 3, peptide **6**) all bound with nanomolar affinity to the Y2 receptor, and neither chlorine, bromine nor iodine participates in hydrogen bonding, shows that the interaction between residue 36 of PYY3-36 and the Y2 receptor could be influenced by van der Waals forces and $\pi - \pi$ bonding. Interestingly, the fluorinated compound, [^{36}Phe(*p*-F)]-PYY3-36, both increased Y1/Y2 receptor selectivity and maintained a high affinity to the Y2 receptor (Figure 1 and Table 3, peptide **3**). Hydroxyl and fluorine share several properties, in particular polarity. [40,41] Fluorine is not a sterically demanding substituent, given its small van der Waals radius (1.35 Å) which resembles the one of hydrogen (1.20 Å). [42] Fluorine cannot donate a hydrogen bond but may rather, due to its electronegative properties, accept them. [41] Finally, fluorine-containing compounds cause the substituent to be more resistant to metabolic degradation, because of the high carbon-fluorine bond energy. [42] The fact that [^{36}Phe(*p*-F)]-PYY3-36 (Figure 1 and Table 3, peptide **3**) is a good agonist for the Y2 receptor indicates that interactions with the active site resembles that of PYY3-36.

Exchanging the hydroxyl group of Tyr-36 with an amine, such as that found in [^{36}Phe(*p*-NH_2)]-PYY3-36 (Table 3, peptide **8**), resulted in sub-nanomolar affinity to the Y2 receptor. This amino group is a hydroxyl isostere because the *anilino* amino group of phenylalanine is not protonated at physiological pH. [^{36}Phe(*p*-NH_2)]-PYY3-36 may introduce a minor steric disturbance, as the amino group is slightly larger than the hydroxyl group and a reduced affinity to the Y1 and Y4 receptor subtypes was observed (Table 3, peptide **8**).

None of the peptides in this series showed improved Y4 binding or even an affinity comparable with that of the native PYY3-36. All [^{36}X]-PYY3-36 analogs resulted in poor Y4 affinities above 1000 nM. The receptor sequence homology between the Y1 and Y4 receptor is high (42%) but comparatively it is low between the Y1 and Y2 receptor (31%). [43] Thus, a loss in Y1 receptor affinity is also likely to lead to a decrease in Y4 receptor affinity, which is indeed what is observed (Table 3).

Structure–Activity Relationship

All the PYY3-36 analogs were investigated for Y2 receptor activity and the most potent analogs were additionally tested for Y1 receptor activity (Figure 2 and Table 3). The decreases in affinity observed in competition binding studies for the PYY3-36 analogs, compared to the native PYY3-36, were also mirrored in the functional assay for the Y2 receptor. Only [^{36}*homo*Tyr]-PYY3-36 (Table 3, peptide **1**) was slightly more affected in potency compared to binding, as a sevenfold reduction in Y2 receptor potency was observed compared to a threefold decrease in affinity. This difference could be explained by a steric interference of *homo*-Tyr with the conformational changes to the active state of the receptor independent of the high affinity. [^{36}*homo*Tyr]-PYY3-36 shows a small drop in Y2 receptor activity, however, it also results in a considerable reduction in Y1 receptor activity (Table 3, peptide **1**), which confirms that the introduction of one more rotatable bond increases the Y1/Y2 receptor selectivity considerably.

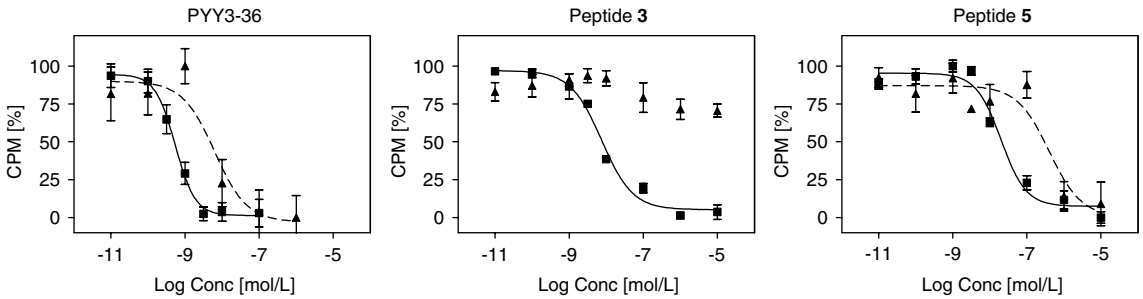

Figure 1. Radioligand binding curves. The binding of PYY3-36, [^{36}Phe(*p*-F)]-PYY3-36 (**3**) and [^{36}Phe(*p*-Br)]-PYY3-36 (**5**) towards the Y1 (▲) and Y2 (■) receptors.

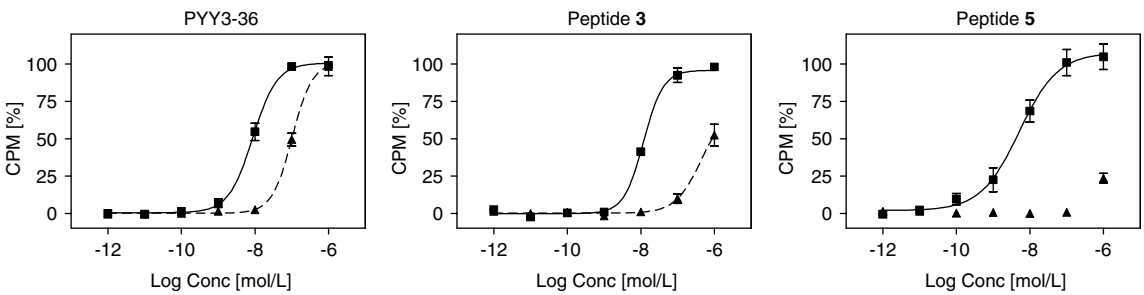

Figure 2. Functional assay. The activity of PYY3-36, [^{36}Phe(*p*-F)]-PYY3-36 (**3**) and [^{36}Phe(*p*-Br)]-PYY3-36 (**5**) towards the Y1 (▲) and Y2 (■) receptors.

[^{36}D-Tyr]-PYY3-36 (Table 3, peptide **2**), [^{36}Phe(*p*-NO_2)]-PYY3-36 (Table 3, peptide **7**) and [^{36}Tyr(*m*-NO_2)]-PYY3-36 (Table 3, peptide **9**) resulted in four- to sevenfold reduction in Y2 receptor activity, as a result these analogs were not investigated for Y1 receptor activity.

Functional data demonstrate that [^{36}Phe(*p*-NH_2)]-PYY3-36 was slightly better accepted by the Y1 receptor than initially shown by the binding assay. The potency of the *anilino* analog was only modestly decreased for the Y1 receptor leading to a small increase in Y1/Y2 receptor selectivity. The halogenated compounds (Figure 2 and Table 3, peptide **3–6**) as well as the *anilino* analog (peptide **8**, Table 3) gave Y2 receptor activities equivalent to PYY3-36, which shows that the Y2 receptor was fairly flexible towards substition in the *para* position of Phe-36. The activity of the Y1 receptor appears to be intolerant to even small changes in the *para* position of Phe-36, as shown by the large drop in affinity for [^{36}Phe(*p*-F)]-PYY3-36 with the small 4-fluoro substituent (Table 3, peptide **3**). The functional activity studies of the chlorine, bromine and iodine analogs (Figure 2, Table 3, peptide **4–6**) confirmed that hydrogen bonding was very important in the ability to activate the Y1 receptor. All halogenated PYY3-36 analogs were poor Y1 receptor activators and resulted in major increases in Y1/Y2 receptor selectivity.

Conclusion

Previously, a significant number of PYY and NPY analogs have been synthesized and analysed, however, the present results show that novel compounds with Y receptor selectivity can be developed by careful modifications of Tyr-36 in the pentapeptide part of PYY3-36, which binds to the receptors. Selectivity was improved using unnatural amino acid derivatives such as *para* substituented phenylalanines. Analogs which were halogenated as well as aminated in the *para* position of Phe-36 of PYY3-36 (**3–6** and **8**) maintained the high Y2 receptor potency shown by native PYY3-36 and all the novel PYY3-36 compounds gave rise to an increased Y1/Y2-receptor selectivity. Most profound were the analogs where the Tyr-36 hydroxyl group was substituted with a halogen (**3–6**), because of their major decreases in Y1 receptor potency, while maintaining an excellent Y2-receptor affinity and activity. *C*-terminal Tyr modified PYY3-36 analogs with sub-nanomolar affinity to the Y2 receptor and simultaneously high Y1/Y2 receptor selectivity have not, to our knowledge, been reported in literature. We believe the insights gained in this study will be useful for the development of potential drug candidates derived from PYY3-36 for the treatment of obesity.

Acknowledgement

We acknowledge the Ministry of Science, Technology and Innovation for co-financing an industrial Ph.D. stipend to SLP. The robotic synthesiser (MultiSynTech Syro II) was acquired through a grant to KJJ from the Villum-Kann-Rasmussen foundation.

References

1 Sahu A. Minireview: A hypothalamic role in energy balance with special emphasis on leptin. *Endocrinology* 2004; **145**(6): 2613–2620.
2 Tatemoto K. Isolation and characterization of peptide YY (PYY), a candidate gut hormone that inhibits pancreatic exocrine secretion. *Proc. Natll. Acad. Sci. USA* 1982; **79**(8): 2514–2518.
3 Neary NM, Small CJ, Druce MR, Park AJ, Ellis SM, Semjonous NM, Dakin CL, Filipsson K, Wang F, Kent AS, Frost GS, Ghatei MA, Bloom SR. Peptide YY_{3-36} and glucagon-like peptide-1_{7-36} inhibit food intake additively. *Endocrinology* 2005; **146**(12): 5120–5127.
4 Fried M, Mayer EA, Jansen JBMJ, Lamers CBHW, Taylor IL, Bloom SR, Meyer JH. Temporal relationships of cholecystokinin release, pancreatobiliary secretion, and gastric emptying of a mixed meal. *Gastroenterology* 1988; **95**(5): 1344–1350.
5 Jin H, Cai L, Lee K, Chang T, Li P, Wagner D, Chey W. A physiological role of peptide YY on exocrine pancreatic secretion in rats. *Gastroenterology* 1993; **105**(1): 208–215.
6 Pappas TN, Debas HT, Taylor IL. Peptide YY: Metabolism and effect on pancreatic secretion in dogs. *Gastroenterology* 1985; **89**(6): 1387–1392.
7 Putnam WS, Liddle RA, Williams JA. Inhibitory regulation of rat exocrine pancreas by peptide YY and pancreatic polypeptide. *Am. J. Physiol. Gastrointest. Liver Physiol.* 1989; **256**(4): G698–G703.
8 Adrian TE, Savage AP, Bacarese-Hamilton AJ, Wolfe K, Besterman HS, Bloom SR. Peptide YY abnormalities in gastrointestinal diseases. *Gastroenterology* 1986; **90**(2): 379–384.
9 Pappas TN, Debas HT, Chang AM, Taylor IL. Peptide YY release by fatty acids is sufficient to inhibit gastric emptying in dogs. *Gastroenterology* 1986; **91**(6): 1386–1389.
10 Pironi L, Stanghellini V, Miglioli M, Corinaldesi R, Giorgio RD, Ruggeri E, Tosetti C, Poggioli G, Labate AM, Monetti N, Gozzetti G, Barbara L, Go V. Fat-induced ileal brake in humans: A dose-dependent phenomenon correlated to the plasma levels of peptide YY. *Gastroenterology* 1993; **105**(3): 733–739.
11 Wiley J, Lu Y, Chung O. Mechanism of action of peptide YY to inhibit gastric motility. *Gastroenterology* 1991; **100**(4): 865–872.
12 Adrian TE, Savage AP, Sagor GR, Allen JM, Bacarese-Hamilton AJ, Tatemoto K, Polak JM, Bloom SR. Effect of peptide YY on gastric, pancreatic, and biliary function in humans. *Gastroenterology* 1985; **89**(3): 494–499.
13 Guo YS, Fujimura M, Lluis F, Tsong Y, Greeley GH Jr, Thompson JC. Inhibitory action of peptide YY on gastric acid secretion. *Am. J. Physiol. Gastrointest. Liver Physiol.* 1987; **253**(3): G298–G302.
14 Conter RL, Roslyn JJ, Taylor IL. Effects of peptide YY on gallbladder motility. *Am. J. Physiol. Gastrointest. Liver Physiol.* 1987; **252**(6): G736–G741.
15 Batterham RL, Cohen MA, Ellis SM, Le Roux CW, Withers DJ, Frost GS, Ghatei MA, Bloom SR. Inhibition of food intake in obese subjects by peptide YY3-36. *N. Engl. J. Med.* 2003; **349**(10): 941–948.
16 Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, Cone RD, Bloom SR. Gut hormone PYY_{3-36} physiologycally inhibts food intake. *Nature* 2002; **418**(6898): 650–654.
17 Abbott CR, Small CJ, Kennedy AR, Neary NM, Sajedi A, Ghatei MA, Bloom SR. Blockade of the neuropeptide YY2 receptor with the specific antagonist BIIE0246 attenuates the effect of endogenous and exogenous peptide $YY_{(3-36)}$ on food intake. *Brain Res.* 2005; **1043**(1–2): 139–144.
18 Halatchev IG, Ellacott KLJ, Fan W, Cone RD. Peptide YY_{3-36} inhibits food intake in mice through a melanocortin-4 receptor-independent mechanism. *Endocrinology* 2004; **145**(6): 2585–2590.
19 Vrang N, Madsen AN, Tang-Christensen M, Hansen G, Larsen PJ. PYY(3–36) reduces food intake and body weight and improves insulin sensitivity in rodent models of diet-induced obesity. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2006; **291**(2): R367–R375.
20 Chelikani PK, Haver AC, Reidelberger RD. Intravenous infusion of peptide YY(3–36) potently inhibits food intake in rats. *Endocrinology* 2005; **146**(2): 879–888.
21 Scott V, Kimura N, Stark JA, Luckman SM. Intravenous peptide YY_{3-36} and Y_2 receptor antagonism in the rat: Effects on feeding behaviour. *J. Neuroendocrinol.* 2005; **17**(7): 452–457.
22 Pittner RA, Moore CX, Bhavsar SP, Gedulin BR, Smith PA, Jodka CM, Parkes DG, Paterniti JR, Srivastava VP, Young AA. Effects of PYY [3–36] in rodent models of diabetes and obesity. *Int. J. Obes.* 2004; **28**(8): 963–971.
23 Katsuura G, Asakawa A, Inui A. Roles of pancreatic polypeptide in regulation of food intake. *Peptides* 2002; **23**(2): 323–329.
24 McTigue DM, Rogers RC. Pancreatic polypeptide stimulates gastric motility through a vagal-dependent mechanism in rats. *Neurosci. Lett.* 1995; **188**(2): 93–96.

Journal of PeptideScience

25 McTigue DM, Chen CH, Rogers RC, Stephens RL Jr. Intracisternal rat pancreatic polypeptide stimulates gastric emptying in the rat. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 1995; **269**(1): R167–R172.

26 McTigue DM, Edwards NK, Rogers RC. Pancreatic polypeptide in dorsal vagal complex stimulates gastric acid secretion and motility in rats. *Am. J. Physiol. Gastrointest. Liver Physiol.* 1993; **265**(6): G1169–G1176.

27 Allen Y, Adrian T, Allen J, Tatemoto K, Crow T, Bloom S, Polak J. Neuropeptide Y distribution in the rat brain. *Science* 1983; **221**(4613): 877–879.

28 Balasubramaniam A. Neuropeptide Y family of hormones: Receptor subtypes and antagonists. *Peptides* 1997; **18**(3): 445–457.

29 Keire DA, Bowers CW, Solomon TEJR, Reeve J. Structure and receptor binding of PYY analogs. *Peptides* 2002; **23**(2): 305–321.

30 Grandt D, Schimiczek M, Beglinger C, Layer P, Goebell H, Eysselein VE, Reeve JR Jr. Two molecular forms of Peptide YY (PYY) are abundant in human blood: Characterization of a radioimmunoassay recognizing PYY1–36 and PYY3–36. *Regul. Pept.* 1994; **51**(2): 151–159.

31 Gantz I, Erondu N, Mallick M, Musser B, Krishna R, Tanaka WK, Snyder K, Stevens C, Stroh MA, Zhu H, Wagner JA, MacNeil DJ, Heymsfield SB, Amatruda JM. Efficacy and safety of intranasal peptide YY_{3-36} for weight reduction in obese adults. *J. Clin. Endocrinol. Metab.* 2007; **92**(5): 1754–1757.

32 Larhammar D. Evolution of neuropeptide Y, peptide YY and pancreatic polypeptide. *Regul. Pept.* 1996; **62**(1): 1–11.

33 Lindner D, Stichel J, Beck-Sickinger AG. Molecular recognition of the NPY hormone family by their receptors. *Nutrition* 2008; **24**(9): 907–917.

34 Merten N, Lindner D, Rabe N, Rompler H, Morl K, Schoneberg T, Beck-Sickinger AG. Receptor subtype-specific docking of Asp6.59 with *C*-terminal arginine residues in Y receptor ligands. *J. Biol. Chem.* 2007; **282**(10): 7543–7551.

35 Beck-Sickinger AG, Wieland HA, Wittneben H, Willim K-D, Rudolf K, Jung G. Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y_1 and Y_2 receptors with distinguished conformations. *Eur. J. Biochem.* 1994; **225**(3): 187–198.

36 Ahn JS, Nazarbaghi R, D'Souza LJ, Ghosh S, Jodka CM, Lwin AN, Levy OE. Synthesis and biological evalution of PYY(3–36) analogs substituted with alanine. *J. Pept. Sci.* 2007; **88**(4): 570 (Poster abstract, 20th APS).

37 Cabrele C, Beck-Sickinger AG. Molecular characterization of the ligand-receptor interaction of the neuropeptide Y family. *J. Pept. Sci.* 2000; **6**(3): 97–122.

38 Kirby DA, Boublik JH, Rivier JE. Neuropeptide Y: Y_1 and Y_2 affinities of the complete series of analogs with single D-residue substitutions. *J. Med. Chem.* 1993; **36**(24): 3802–3808.

39 Kostenis E. Potentiation of PCR-signaling via menbrane targeting of G protein α subunits. *J. Recept. Signal Transduct. Res.* 2002; **22**(1–4): 267–281.

40 Chapeau M, Frey PA. Synthesis of UDP-4-deoxy-4-fluoroglucose and UDP-4-deoxy-4-fluorogalactose and their interactions with enzymes of nucleotide sugar metabolism. *J. Org. Chem.* 1994; **59**(23): 6994–6998.

41 Howard JAK, Hoy VJ, O'Hagan D, Smith GT. How good is fluorine as a hydrogen bond acceptor? *Tetrahedron* 1996; **52**(38): 12613–12622.

42 Welch JT. Tetrahedron report number 221: Advances in the preparation of biologically active organofluorine compounds. *Tetrahedron* 1987; **43**(14): 3123–3197.

43 Parkinson JRC, Dhillo WS, Small CJ, Chaudhri OB, Bewick GA, Pritchard I, Moore S, Ghatei MA, Bloom SR. PYY3-36 injection in mice produces an acute anorexigenic effect followed by a delayed orexigenic effect not observed with other anorexigenic gut hormones. *Am. J. Physiol. Endocrinol. Metab.* 2008; **294**(4): E698–E708.

