0022-3565/07/3232-692–700$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 323:692–700, 2007

Vol. 323, No. 2
125211/3262427
Printed in U.S.A.

The Journal of PHARMACOLOGY And Experimental Therapeutics

A Novel Long-Acting Selective Neuropeptide Y2 Receptor Polyethylene Glycol-Conjugated Peptide Agonist Reduces Food Intake and Body Weight and Improves Glucose Metabolism in Rodents

Astrid A. Ortiz, Lucinda F. Milardo, Lynn B. DeCarr, Thomas M. Buckholz, Michelle R. Mays, Thomas H. Claus, James N. Livingston, Cathy D. Mahle, and Kevin J. Lumb

Departments of Metabolic Disorders Research (A.A.O., L.F.M., L.B.D., M.R.M., T.H.C., J.N.L., C.D.M.) and Research Technologies (T.M.B., K.J.L.), Bayer Pharmaceuticals Corporation, West Haven, Connecticut

Received May 11, 2007; accepted July 31, 2007

ABSTRACT

Selective activation of the neuropeptide Y (NPY)2 receptor to suppress appetite provides a promising approach to obesity management. A selective NPY2 polyethylene glycol-conjugated (PEGylated) peptide agonist is described that consists of a peptide core corresponding to residues 13 to 36 of human peptide YY (PYY) and a nonpeptidic moiety (2-mercaptonicotinic acid) at the peptide N terminus that is derivatized with 20-kDa monomethoxypolyethylene glycol. The PEGylated peptide elicits a dose-dependent reduction in food intake in lean C57BL/6 mice and Wistar rats that persists for 72 and 48 h, respectively. The effect on food intake in lean C57BL/6 mice is blocked by the selective NPY2 antagonist BIIE0246 (*N*-[(1*S*)-4-[(aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino]carbonyl]butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5*H*-dibenz[*b,e*]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentaneacetamide formate). A dose-dependent reduction in body weight in diet-induced obese (DIO) mice is seen following daily dosing for 14 days. The reduction in body weight is sustained following dosing for 40 days, and it is accompanied by an increase in plasma adiponectin. Improvements in glucose disposal and in plasma insulin and glucose levels that are risk factors for type II diabetes are observed following once-daily subcutaneous dosing in DIO mice. The results provide evidence from two animal species that the long-acting selective NPY2 peptide agonist has potential for obesity management.

Obesity presents an increasing public health burden that is associated with several cancers, hypertension, osteoarthritis, type II diabetes, and other illnesses, and with reduced life expectancy. Current therapeutic options are limited, and the unmet medical need for new, effective treatments is high. Several neurological pathways contribute to food intake and energy homeostasis that moderate appetite, weight maintenance and weight gain. One family of G protein-coupled receptors involved in the regulation of food intake is the NPY family, made up of the NPY2 and NPY4 receptors involved in satiety and the NPY1 and NPY5 receptors implicated in feeding.

The naturally occurring gut hormone PYY(3-36) is a nonselective agonist of the NPY receptor family (Small and Bloom, 2005). PYY(3-36) reduces acute food intake in lean, DIO, ob/ob, and db/db mice, and in rats, rabbits, and monkeys, and daily administration of PYY(3-36) causes body weight loss in DIO and ob/ob mice, rats, and rabbits (Batterham et al., 2002; Challis et al., 2003; Cox and Randich, 2004; Halatchev et al., 2004; Pittner et al., 2004; Abbott et al., 2005; Chelikani et al., 2005; Koegler et al., 2005; Moran et al., 2005; Neary et al., 2005; Adams et al., 2006; Sileno et al., 2006; Vrang et al., 2006). PYY(3-36) does not reduce food

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.107.125211.

ABBREVIATIONS: NPY, neuropeptide Y; PYY, peptide YY; DIO, diet-induced obese; PEGylated, polyethylene glycol-conjugated; PEGylation, polyethylene glycol-conjugation; HPLC, high-performance liquid chromatography; USP, United States Pharmacopeia; mPEG, monomethoxypolyethylene glycol; PEG, polyethylene glycol; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; IPGTT, intraperitoneal glucose tolerance test; L-152,804, 5,5-dimethyl-2-(2,3,4,9-tetrahydro-3,3-dimethyl-1-oxo-1*H*-xanthen-9-yl)-1,3-cyclohexanedione; SR141716, 5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide; BIIE0246, *N*-[(1*S*)-4-[(aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino]carbonyl]butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5*H*-dibenz[*b,e*]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentaneacetamide formate.

intake in NPY2 knockout mice, or when it is coadministered with the NPY2 antagonist BIIE0246 in wild-type rats, suggesting that the anorexigenic effect of PYY(3-36) is mediated specifically through the NPY2 receptor (Batterham et al., 2002; Abbott et al., 2005). In addition to the anorexigenic effect associated with NPY2 receptor activation, PYY(3-36) also activates the NPY1 and NPY5 receptors. The nonselective nature of PYY(3-36) is undesirable, because NPY1 and NPY5 agonists stimulate feeding in rodents (Hu et al., 1996; Kanatani et al., 2000b; Mullins et al., 2001).

The properties of PYY(3-36) in animal models of feeding and obesity make NPY2 receptor modulation an attractive mechanism for the therapeutic management of obesity. Indeed, intravenous and nasal administration of PYY(3-36) to humans reduces caloric intake (Batterham et al., 2002, 2003; Degen et al., 2005). However, the effect of PYY(3-36) is short-lived, and it may be as short as 4 h in humans (Brandt et al., 2004; http: www.nastech.com). Therefore, PYY(3-36) suffers from a short physiological duration of action and the potential for NPY1 and NPY5 receptor activation to undermine the reduction of food intake. It is desirable, then, to discover a selective, long-acting NPY2 receptor agonist of potential superior therapeutic benefit to PYY(3-36). In particular, a NPY2-selective mechanism of action will avoid potential appetite stimulation due to NPY1 and NPY5 activation as well as the possibility of other potential effects associated with the activation of the NPY1 and NPY5 receptors, and an extended duration of action will reduce dosing frequency and potentially improve patient compliance.

We have described previously an approach to attain long-acting, selective NPY2 peptide agonists using PYY as a peptide scaffold (DeCarr et al., 2007a; Lumb et al., 2007). NPY1 and NPY5 receptor affinity was abrogated at the expense of NPY2 affinity by N-terminal deletions of residues from PYY to arrive at a peptide core corresponding to residues 25 to 36 of human PYY. NPY2 affinity was then restored with N-terminal nonpeptidic modifications while maintaining selectivity against the NPY1 and NPY5 receptors. Members of the resulting series of peptides exhibit greater selectivity for the NPY2 receptor than previously described peptide agonists (DeCarr et al., 2007a). Use of N-terminal nonpeptidic modifications that incorporate a thiol for site-specific PEGylation led to the identification of a second series of NPY2 agonists that contains peptide **1** (Table 1) (Lumb et al., 2007). Once-daily subcutaneous dosing of peptide **1** over 7 days in DIO mice results in a significant body-weight reduction of 7% (Lumb et al., 2007).

Here, we present a substantially more efficacious peptide resulting from structure-activity relationship studies of the peptide core of peptide **1**. Peptide **2** reduces food intake in both lean mice and rats. The effect is mitigated in lean mice by coadministration with the selective NPY2 antagonist BIIE0246, suggesting that peptide **2** functions via activation of the NPY2 receptor. Once-daily dosing for 40 days results in a significant and sustained reduction in body weight in DIO mice. In addition, metabolic risk factors associated with glucose metabolism are improved following once-daily dosing in DIO mice. Therefore, peptide **2** constitutes a promising therapeutic for the management of obesity.

Materials and Methods

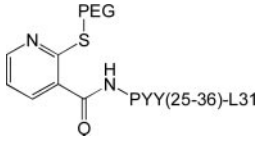
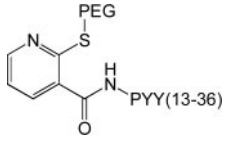
Reference Compounds. Human PYY(3-36) was purchased from American Co., Inc. (Sunnyvale, CA) (catalog number 48-0-33). (Pro^{30},Tyr^{32},Leu^{34})-Neuropeptide Y(28-36) (Leban et al., 1995) was purchased from Bachem Biosciences (King of Prussia, PA) (catalog number H-3546). BIIE0246 (Doods et al., 1999) and L-152,804 (Kanatani et al., 2000a) were purchased from Tocris Cookson Inc. (Ellisville, MO) (catalog numbers 1700 and 1382, respectively).

Peptide Synthesis. Peptides were synthesized with solid-phase, 9-fluorenylmethoxycarbonyl chemistry on Rink Amide resin with an ABI 433A synthesizer (Applied Biosystems, Foster City, CA) as described previously (Lumb et al., 2007). The N terminus was derivatized with 2-mercaptonicotinic acid (Aldrich Chemical Co., Milwaukee, WI) (catalog number 419702) in peptides **1** and **2**, or with 2-methythionicotinic acid (Aldrich Chemical Co.) (catalog number 523127) to generate peptide **3**. Purification was by reversed phase

TABLE 1

In vitro receptor activation and binding profile

All peptides are amidated at the C terminus. PYY(25-36)-L31 denotes the peptide sequence RHYLNLLTRQRY-NH_2, corresponding to residues 25 to 36 of human PYY with the amino acid change of Val31 to Leu. PYY(13-36) denotes the peptide sequence SPEELNRYYASLRHYLNLVTRQRY-NH_2, corresponding to residues 13 to 36 of human PYY. The mouse and rat PYY sequences are the same as the human PYY sequence over residues 13 to 36, except for the single amino acid change N18S. PEG denotes that the peptides are derivatized with 20-kDa mPEG. EC_{50} values were determined with a [^{35}S]GTPγS accumulation assay using KAN-TS cells, and K_i values were determined using a ^{125}I-PYY-displacement assay. Values are means of experiments performed in triplicate ± S.E.M. Three independent experiments were performed with equivalent results.

	Peptide	EC_{50} Human NPY2	K_i				
			Human NPY2	Human NPY1	Human NPY5	Mouse NPY2	Rat NPY2
			nM				
PYY(3-36)		0.3 ± 0.1	0.4 ± 0.1	21 ± 2	20 ± 2	0.5 ± 0.2	0.4 ± 0.1
1		25 ± 6	41 ± 8	4000	1600	N.D.	N.D.
2		6.5 ± 1.6	9.1 ± 2.2	760 ± 70	630 ± 30	21 ± 2	31 ± 6

N.D., not determined.

C18 HPLC and a linear water/acetonitrile gradient containing 0.1% trifluoroacetic acid. Purity (>98%) was confirmed with analytical C18 and cation-exchange HPLC. Identity was confirmed with electrospray mass spectrometry, and in each case, the expected and observed masses agreed to within 1 Da. The peptide sequence was confirmed with electrospray tandem mass spectrometry.

Polyethylene Glycol-Conjugation. Peptides were cross-linked to mPEG conjugated to maleimide via the thiol group of the N-cap. Peptides were incubated with a 2-fold molar excess of 20-kDa mPEG derivatized with α-methoxy-ω-(3-[3-maleimido-1-oxopropyl]amino propyl polyoxyethylene) (catalog number ME-200MA; NOF Corporation, Tokyo, Japan) in 100 mM Tris, pH 8, for 2 h. The PEGylated peptide was purified with cation-exchange HPLC using a SP-5PW column (TosaHaas, Philadelphia, PA) equilibrated in 10 mM HCl, 20% methanol using a linear NaCl gradient. The product was dialyzed against water and lyophilized. Purity (>97%), including the absence of free peptide, was confirmed with analytical C18 and cation-exchange HPLC.

Preparation of PEG-Cys. Cys was used to block the maleimide moiety of the mPEG reagent for use as a control for in vivo studies. A 2-fold molar excess of Cys and 20-kDa mPEG (NOF ME-200MA) were incubated overnight in Tris, pH 7.4, at room temperature, and then they were dialyzed against water and lyophilized.

Peptide Concentration. Peptide amount was determined with amino acid analysis performed by the W. M. Keck Foundation Biotechnology Facility, Yale University (New Haven, CT).

Receptor Binding and Activation Assays. Assays using human receptors were performed as described previously (DeCarr et al., 2007a). NPY2 receptor [^{35}S]GTPγS incorporation assays and ^{125}I-PYY displacement assays were performed in a scintillation proximity assay format with membrane prepared from KAN-TS cells as a source of endogenous human NPY2 receptor. NPY1 and NPY5 receptor ^{125}I-PYY displacement assays were performed in a filter-binding format using membranes prepared from SK-N-MC cells as a source of endogenous human NPY1 receptor or human embryonic kidney 293 cells expressing recombinant human NPY5 receptor.

Mouse and rat NPY2 receptor ^{125}I-PYY competition-binding assays were performed in a filter-binding format with membrane pellets obtained from whole mouse brain homogenates and rat hippocampus. Endogenous NPY1 and NPY4 receptors were blocked with 1 μM (Pro^{30},Tyr^{32},Leu^{34})-neuropeptide Y(28-36), and the NPY5 receptor was blocked with 1 μM L-152,804 (Leban et al., 1995; Kanatani et al., 2000a). Membrane pellets were suspended in ice-cold buffer A (50 mM HEPES, pH 7.4, 10 mM $CaCl_2$, and 5 mM $MgCl_2$) and immediately used for determining protein content with the Bradford assay (Bio-Rad, Hercules, CA) and for binding assays. Increasing concentrations of peptide were incubated with 75 pM (rat) or 200 pM ^{125}I-PYY (mouse) (PerkinElmer Life and Analytical Sciences, Boston, MA) and membrane (35 μg of rat and 50 μg of mouse protein) in buffer A containing 0.1% bovine serum albumin at a final volume of 200 μl for 2 h at room temperature. Nonspecific binding was defined with 1 μM PYY. The binding reactions were terminated by filtration through pretreated (0.05% bovine serum albumin in buffer A) Millipore HV filter plates (Millipore Corporation, Billerica, MA) using a vacuum manifold. Filters were washed three times with 200 μl of ice-cold buffer A, and filters dried overnight. MicroScint-O (20–30 μl; PerkinElmer Life and Analytical Sciences) was added to each well, and radioactivity bound to the filters was measured using a Wallac 1450 MicroBeta Trilux liquid scintillation counter (PerkinElmer Wallac, Gaithersburg, MD). Data were fit to a single-site binding model with Prism 3.0.3 (GraphPad Software Inc., San Diego, CA).

Fasted-Refed Feeding Studies in C57BL/6 Lean Mice. Lean C57BL/6 male mice (Taconic Farms, Germantown, NY) were acclimated for a minimum of a week with controlled temperature and humidity on a 12-h light/dark cycle. Mice were housed in pairs in cages with a grid floor with water and food (standard chow pellet diet) continuously available. A study included 20 mice per treatment group with an average body weight of 22 g. The mice were fasted overnight (18 h) with water available during the dark phase, and they were dosed subcutaneously with peptide in USP saline. Control groups were dosed with USP saline or PEG-Cys in USP saline. Preweighed food was provided 30 min after dosing.

Fasted-Refed Feeding Studies in Wistar Rats. Lean Wistar male rats (Harlan, Indianapolis, IN) were acclimated for a minimum of a week with controlled temperature and humidity on a 12-h light/dark cycle. Rats were housed singly in cages with a grid floor with water and food (standard chow powder diet) continuously available. A study included 10 rats per treatment group with an average body weight of 264 g (SD 14 g). The rats were fasted overnight (18 h) with water available during the dark phase, and they were dosed subcutaneously with peptide in USP saline. Control groups were dosed with USP saline or PEG-Cys in USP saline. Preweighed food was provided 6 h after dosing (reflecting the longer plasma T_{max} of peptide **2** in the rat compared with the mouse).

Body-Weight Studies in DIO Mice. Male C57BL/6 mice (Taconic Farms) were fed a high-fat diet containing 45% calories from fat (rodent diet D12451; Research Diets, Inc., New Brunswick, NJ) for 20 to 22 weeks, and they had an average body weight over 5 SDs greater than mice fed a standard chow pellet diet (5% calories from fat). A study included 10 mice per treatment group. The mice used for the 14-day dose-response study had an average initial body weight of 46.9 g (SD 0.9 g), and the mice used for the 40-day study had an average initial body weight of 48.5 g (SD 0.6 g). Mice were kept in standard animal rooms under controlled temperature and humidity and a 12/12-h light dark cycle. Water and food were continuously available. Animals were adapted to the grid floors for 4 days, and they were dosed with vehicle (either USP saline or PEG-Cys in USP saline) for a further 4 days before recording of 2 days of baseline body weight and 24-h food and water consumption. Mice were assigned to treatment groups based upon their body weight such that the initial mean and standard error of the mean of body weight were similar. Animals were administered peptide in USP saline subcutaneously every day before the dark phase, and body weight and food and water consumption were measured.

Glucose Metabolism Parameters. Male DIO mice were dosed for 11 days with 0.94 μmol/kg peptide **2** or PEG-Cys as described above. The mice had an average initial body weight of 45.4 g (SD 0.2 g). Blood samples were collected from the tail of fed mice and analyzed with an Ascensia glucose meter (Bayer Healthcare, Tarrytown, NY) on day 10 of treatment. Food was removed 5 h into the feeding phase, and the animals were fasted for 14 h before blood collection using retro-orbital bleeding for insulin analysis. The IPGTT was performed 2 h after retro-orbital bleeding following intraperitoneal administration of 2 g/kg glucose, and tail blood glucose was measured at 30-min intervals for 3 h. Insulin was measured by enzyme-linked immunosorbent assay (Alpco Diagnostics, Windham, NH).

Statistics. The significance of differences was evaluated by analysis of variance followed by Fisher's protected least significant difference test post hoc analysis (StatView; SAS Institute, Cary, NC).

Regulatory Approvals. All procedures involving animals were reviewed and approved by the Bayer Animal Use and Care Committee, and all experiments were performed in accordance with relevant guidelines and regulations.

Results

In Vitro Pharmacology. Peptide **1** contains a peptide core made up of residues 25 to 36 of human PYY that is modified with N-terminal 2-mercaptonicotinic acid and 20-kDa mPEG. Peptide **1** binds and activates the human NPY2 receptor, with K_i and EC_{50} values of 41 $\pm$ 8 and 25 $\pm$ 6 nM, respectively, and peptide **1** is selective against the NPY1 and NPY5 receptors (K_i = 4.0 and 1.6 μM, respectively; Table 1)

(Lumb et al., 2007). Use of a longer peptide core corresponding to residues 13 to 36 of human PYY to generate peptide **2** increases the in vitro potency 4-fold compared with peptide **1**, with K_i and EC_{50} values of 9.1 ± 2.2 and 6.5 ± 1.6 nM, respectively (Fig. 1, A and B). Peptide **2** maintains selectivity against the human NPY1 and NPY5 receptors (K_i = 760 ± 70 and 630 ± 30 nM, respectively; Table 1). Peptide **2** binds rodent NPY2 receptors with similar affinity to the human receptor, with K_i values of 21 ± 2 and 31 ± 6 nM for the mouse (whole brain) and rat (hippocampus) NPY2 receptors, respectively (Table 1). Therefore, increasing the length of the peptide core improves in vitro receptor affinity while maintaining a high degree of specificity for the NPY2 receptor.

Acute Anorexigenic Activity in Lean Mice. The in vivo activity of peptide **2** was monitored in lean fasted-refed C57BL/6 mice and compared with PYY(3-36). In all feeding studies, the change in cumulative food intake was measured relative to control groups dosed subcutaneously with USP saline for PYY(3-36) or with PEG-Cys in USP saline for the PEGylated peptides. No statistically significant difference in food intake was observed for mice treated with saline or PEG-Cys, in accordance with previous results (DeCarr et al., 2007b; Lumb et al., 2007).

PYY(3-36) administered to lean mice at 0.94 μmol/kg elicits a significant reduction in cumulative food intake of 42% at 4 h ($p < 0.05$; Fig. 2). However, the anorexigenic effect of PYY(3-36) is not sustained, and cumulative food intake reduction after 24 h is only 9% ($p > 0.05$; Fig. 2). The results are in accordance with reports that PYY(3-36) reduces food intake in mice at doses over 25 nmol/kg (Batterham et al., 2002; Challis et al., 2003; Halatchev et al., 2004; Pittner et al., 2004; Neary et al., 2005; Adams et al., 2006; Vrang et al., 2006; DeCarr et al., 2007b; Lumb et al., 2007).

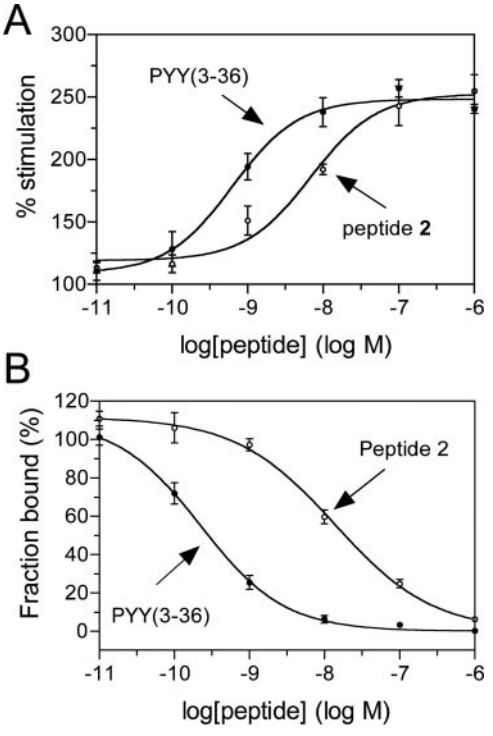


Fig. 1. Human NPY2 receptor activation and binding by human PYY(3-36) and peptide **2**. A, NPY2 receptor activation by PYY(3-36) and peptide **2**, measured by [^{35}S]GTPγS accumulation in KAN-TS cells. B, NPY2 receptor binding by PYY(3-36) and peptide **2**, measured by competitive displacement of ^{125}I-labeled PYY from KAN-TS cells. Values are means of experiments performed in triplicate ± S.E.M. Three independent experiments were performed with equivalent results.

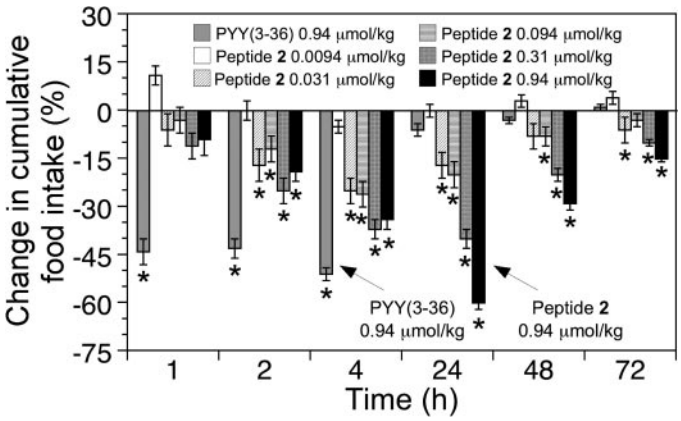


Fig. 2. Peptide **2** reduces food intake in fasted (18 h) lean mice with a longer duration of action than PYY(3-36). Mice were administered a single dose of PYY(3-36) (0.94 μmol/kg s.c.) or peptide **2** (0.0094–0.94 μmol/kg s.c.) in USP saline. Animals were dosed 30 min before food return and cumulative food intake measured over 72 h. *, $p < 0.05$ compared with the USP saline treatment group for PYY(3-36) or the PEG-Cys treatment group for peptide **2**.

Peptide **2** administered to lean mice at 0.94 μmol/kg exhibits a slower onset of food intake reduction and induces a substantial reduction in food intake of 60 ± 2% at 24 h (Fig. 2). In contrast to PYY(3-36), the reduction in food intake caused by peptide **2** is sustained and significant at 48 h (29 ± 2%) and 72 h (15 ± 1%). The reduction in food intake induced by peptide **2** is dependent on dose, and the lowest dose tested (0.0094 μmol/kg) is not efficacious (Fig. 2). Therefore, peptide **2** imparts superior in vivo anorexigenic efficacy with a longer duration of action than PYY(3-36) at a lower dose.

PEGylation Enhances In Vivo Activity. The contribution of PEGylation to in vivo efficacy and duration of action was evaluated using a non-PEGylated analog of peptide **2** in which the thiol group of the N-cap is modified with a methyl group (peptide **3**) instead of PEG. In lean C57BL/6 mice, peptide **3** induces a significant reduction of food intake at 1 h at the highest tested dose of 3.1 μmol/kg that is mitigated at 4 h and does not persist to 24 h (Fig. 3). This low efficacy is in contrast to the potent PEGylated analog **2** (Fig. 3), which differs only in the modification with mPEG, indicating that PEGylation is required to impart an extended in vivo duration of action.

Abrogation of Activity in Mice by the NPY2 Antagonist BIIE0246. BIIE0246 is a highly selective NPY2 receptor antagonist (K_i = 15 nM for human NPY2; data not shown) with weak affinity for the NPY1, NPY4, and NPY5 receptors (K_i >1 μM for the human receptors; data not shown) that blocks the anorexigenic activity of PYY(3-36) in rats (Abbott et al., 2005). In the fasted-refed lean mouse model, subcutaneous administration of BIIE0246 at 11 μmol/kg (10 mg/kg) causes a statistically significant increase in food intake of 10% at 4 h (Fig. 4), as expected for a NPY2 antagonist. When the mice are pretreated with BIIE0246 for 15 min before peptide administration, the acute anorexigenic effects of both PYY(3-36) at 0.74 μmol/kg and peptide **2** at 0.94 μmol/kg are abrogated (Fig. 4). This result suggests that the mechanism of action of peptide **2** is mediated via NPY2 receptor stimulation.

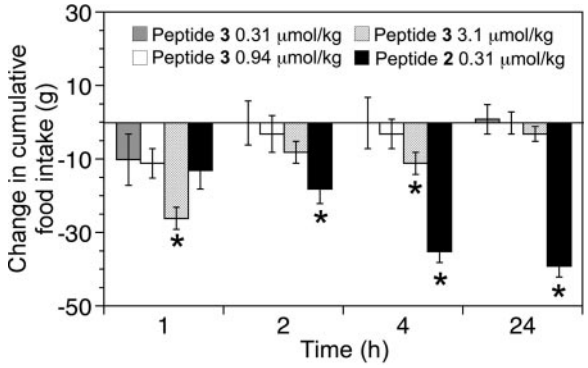


Fig. 3. PEGylation is required for in vivo efficacy. Peptide **3** is the non-PEGylated analog of peptide **2**. Peptide **3** exhibits short-lived efficacy in lean mice. The effects of peptide **3** seem dose-dependent, with no efficacy observed after 2 h at the lowest dose tested (0.31 μmol/kg s.c.). In contrast, the PEGylated peptide **2** at 0.31 μmol/kg exhibits robust activity at 24 h. Mice were administered a single dose of the non-PEGylated analog peptide **3** (0.31–3.1 μmol/kg s.c.), or the PEGylated peptide **2** (0.31 μmol/kg s.c.) in USP saline. Animals were dosed 30 min before food return, and cumulative food intake was measured over 24 h. *, $p < 0.05$ compared with the USP saline treatment group for peptide **3** or the PEG-Cys treatment group for peptide **2**.

Acute Anorexigenic Activity in Lean Rats. The effects of peptide **2** and PYY(3-36) on feeding in the rat were evaluated with fasted-refed lean Wistar rats. Peptide **2** induces a dose-dependent reduction in food intake in the rat that persists to at least 48 h (Fig. 5). The reduction in food intake is lower in the rat than seen in the lean C57BL/6 mouse, with a 0.74-μmol/kg dose in the rat eliciting a 23 ± 4% reduction in food intake at 24 h compared with 40 ± 3% at 0.31 μmol/kg in the mouse (cf. Figs. 2 and 5). PYY(3-36) dosed at 2.74 μmol/kg also elicits a statistically significant reduction in food intake in the rat of 13 ± 4% at 4 h, although as in the

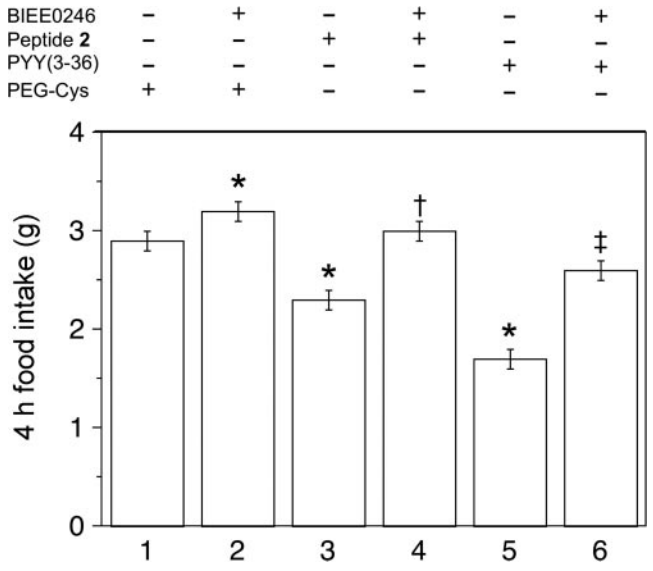


Fig. 4. The anorexigenic effects of peptide **2** and PYY(3-36) are blocked by the small-molecule NPY2 antagonist BIIE0246 in lean mice. BIIE0246 (11 μmol/kg s.c.) causes an increase in food intake (column 2), whereas peptide **2** (0.94 μmol/kg s.c.) and PYY(3-36) (0.74 μmol/kg s.c.) elicit an anorexigenic response (columns 3 and 5, respectively) compared with PEG-Cys (0.94 μmol/kg s.c.). Coadministration of BIIE0246 mitigates the efficacy of both peptide **2** and PYY(3-36) (columns 4 and 6, respectively). Animals were dosed with BIIE0246 45 min before food return and with peptide **2** 30 min before food return and cumulative food intake measured over 24 h. All compounds were formulated in PEG400/H_2O (90:10). *, †, and ‡, $p < 0.05$ compared with columns 1, 3, and 5, respectively.

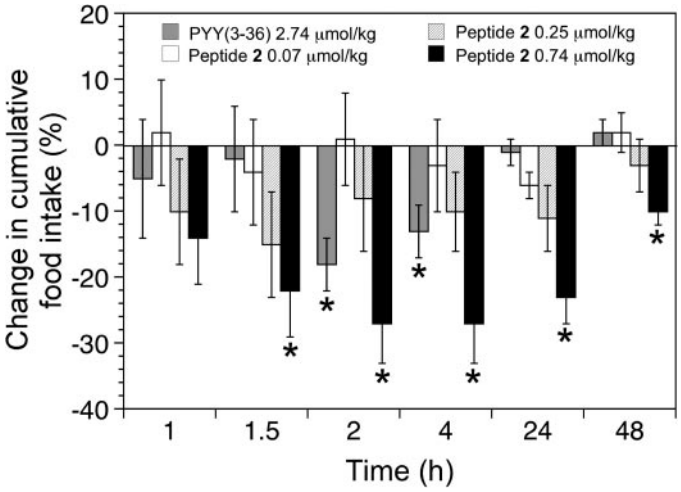


Fig. 5. Peptide **2** reduces food intake in fasted (18 h) lean Wistar rats with a longer duration of action than PYY(3-36). Rats were administered a single dose of PYY(3-36) (2.74 mmol/kg s.c.) or peptide 2 (0.07–0.74 mmol/kg s.c.) in USP saline. Animals were dosed 6 h before food return, and cumulative food intake was measured over 48 h. *, $p < 0.05$ compared with the USP saline treatment group for PYY(3-36) or the PEG-Cys treatment group for peptide **2**.

mouse the anorexigenic effect of PYY(3-36) is short-lived and it does not persistent to 24 h (Fig. 5).

Dose-Dependent Reduction on Body Weight in DIO Mice. The effects of once-daily administration of peptide **2** on body weight were evaluated in the DIO mouse (Fig. 6). Peptide **2** elicits a dose-dependent reduction in body weight, with a statistically significant loss of approximately 9% body weight over 14 days following dosing at 0.94 μmol/kg. In contrast, PYY(3-36) at 0.74 μmol/kg causes a modest, statistically significant 2.6% reduction in body weight over the same period. Efficacy is not observed for peptide **2** at the lowest dose shown (0.0094 μmol/kg).

Chronic Dosing and Drug Withdrawal in DIO Mice. The effects of once-daily administration of peptide **2** were evaluated in a long-term study using a single dose of 0.31 μmol/kg (Fig. 7, A–F). In one part of the study, animals were

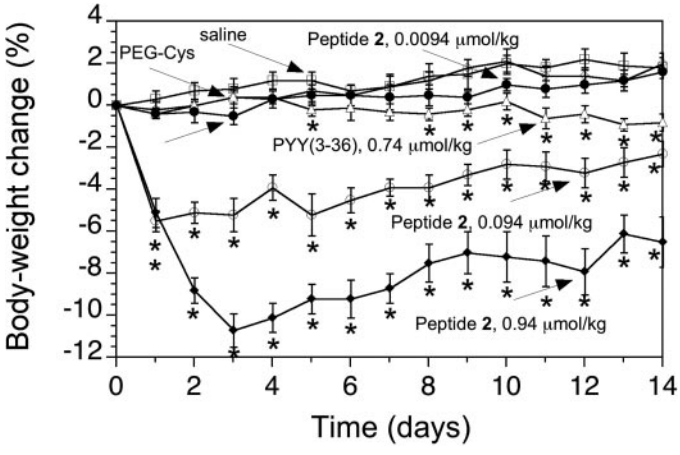


Fig. 6. Dose-dependent reduction in body weight of DIO mice following once-daily administration of peptide **2**. Mice were administered a once-daily dose of peptide 2 (0.0094, 0.31, or 0.94 μmol/kg s.c.) or PYY(3-36) (0.74 μmol/kg s.c.). The highest dose of peptide **2** induces a 9% loss in body weight. In contrast, PYY(3-36) causes a more modest reduction in body weight of 2.6%. *, $p < 0.05$ compared with the USP saline treatment group for PYY(3-36) or the PEG-Cys treatment group for peptide **2**.

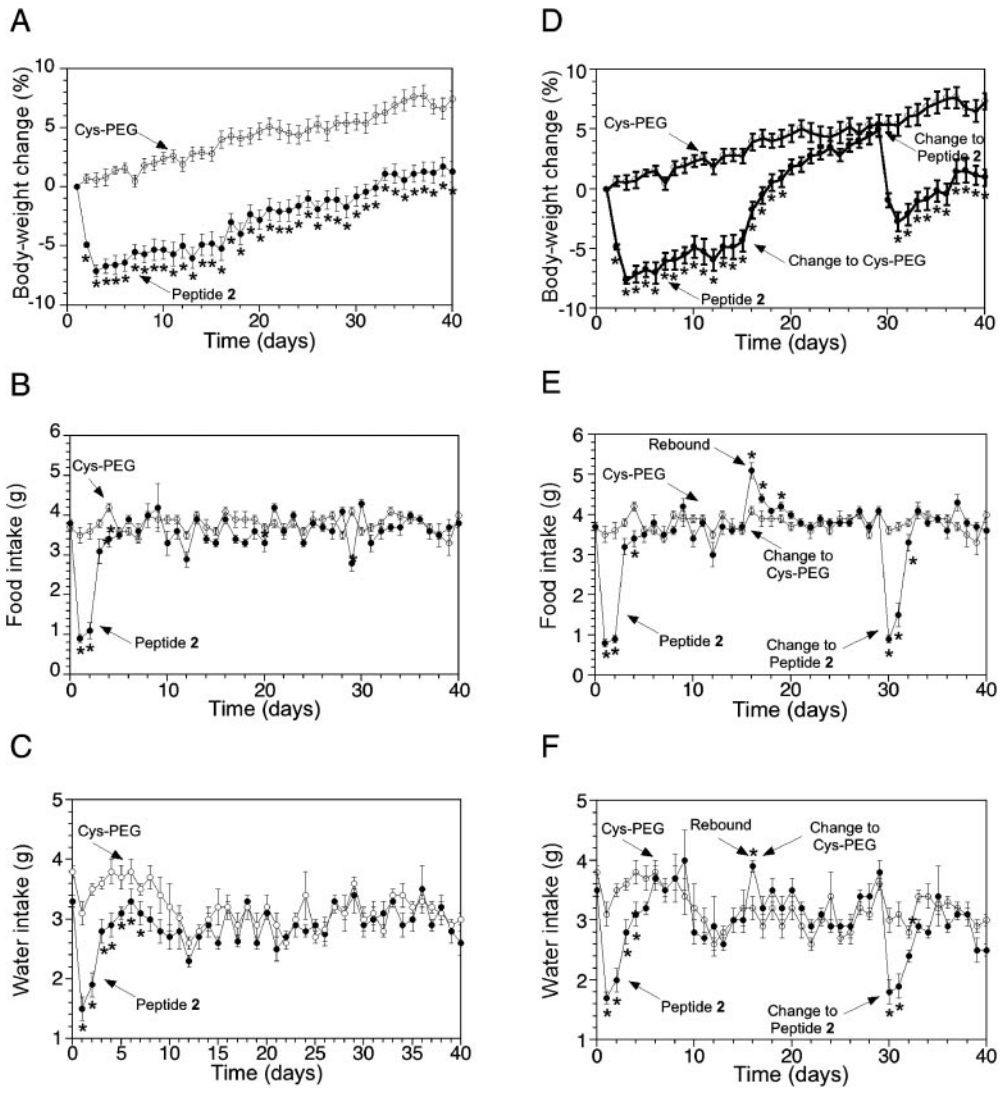


Fig. 7. Sustained reduction in body weight of DIO mice following once-daily administration of peptide **2**. The study was performed in two parts. One set of animals received peptide **2** at 0.31 μmol/kg over the entire study period (A–C) whereas the second set received peptide **2** for 15 days and PEG-Cys for 13 days, and then they were returned to peptide **2** dosing for 12 days (D–F). All agents were dosed at 0.31 μmol/kg s.c. *, $p < 0.05$ compared with the USP saline treatment group.

dosed once daily for 40 days. A statistically significant reduction in body weight of 9% is seen (Fig. 7A). There is a sharp decrease in food intake in the first 2 days of dosing, which then increases to the levels seen for the PEG-Cys treatment group (Fig. 7B). Water intake is reduced proportionally with food intake (Fig. 7C), which reflects the normally observed correlation between food and water consumption in rodents (Fitzsimons and Le Magnen, 1969).

In the second part of the study, animals were treated once daily with peptide **2** for 15 days, PEG-Cys for 13 days (i.e., peptide **2** was not administered), and finally, with peptide **2** for 12 days. During the first phase when animals were treated with peptide **2**, essentially identical results were seen for the animals of the first part of the study in terms of body-weight loss and reduction of food and water intake (Fig. 7D). Upon withdrawal of peptide **2**, the animals resumed untreated levels of feeding with a rebound or initial increase in food intake upon drug withdrawal (Fig. 7E). When peptide **2** was reintroduced, the effects on food and water intake and on body weight and feeding were essentially identical to those seen when the peptide was first administered (Fig. 7, D–F).

Adiponectin. An increase in adiponectin is a biomarker for decreased body fat. After 40 days of daily dosing, plasma adiponectin levels are increased significantly by 18% in the treated animals (20 $\pm$ 1 μg/ml) relative to the PEG-Cys control group (17 $\pm$ 1 μg/ml).

Glucose Metabolism. The effects on glucose metabolism were evaluated in DIO mice following 11 days of once-daily dosing at 0.94 μmol/kg. The animals experienced a 9% reduction in body weight (data not shown), consistent with the other two independent body-weight studies in DIO mice (Figs. 6 and 7). In the IPGTT, fasted mice treated with peptide **2** have enhanced glucose disposal compared with untreated mice (Fig. 8A), which translates to a 21% reduction in the glucose area under the curve over the time course of the measurements (Fig. 8B). In addition, fasting plasma insulin levels increased by 18% (Fig. 8C), and nonfasting plasma glucose levels decreased by 35% (Fig. 8D).

Discussion

PYY(3-36) is a clinical candidate for the management of obesity (Small and Bloom, 2005). The peptide functions via activation of the NPY2 receptor to reduce appetite in rodents (Batterham et al., 2002; Abbott et al., 2005), and food intake is reduced in humans following intravenous administration of PYY(3-36) (Batterham et al., 2002, 2003; Degen et al.,

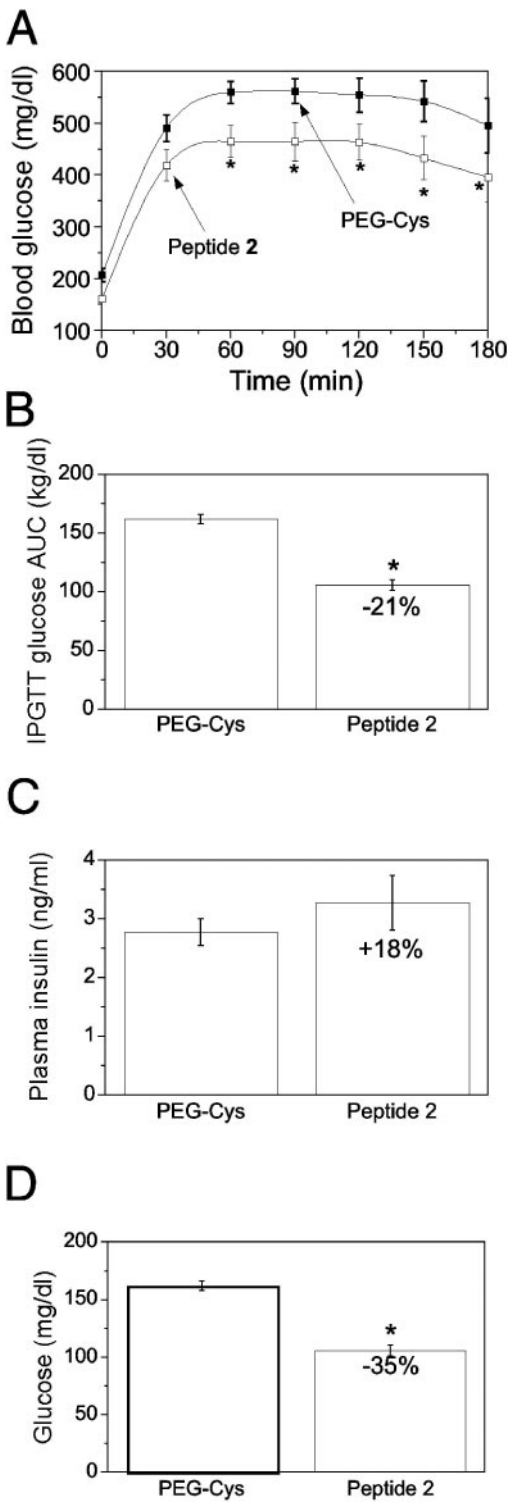


Fig. 8. Peptide **2** improves glucose metabolism parameters DIO mice following 11 days of once-daily dosing (0.94 μmol/kg s.c.). A, glucose disposal following an IPGTT is improved in fasted mice by peptide **2**. B, peptide **2** reduces the glucose area under the curve in fasted mice following an IPGTT by 21%. C, peptide **2** increases plasma insulin in fasted mice by 18%. D, peptide **2** decreases blood glucose in fed mice by 35%. *, $p < 0.05$ compared with the USP saline treatment group.

2005). PYY(3-36) is also an agonist of the NPY1 and NPY5 receptors that induces the undesired effect of feeding stimulation in rodents (Hu et al., 1996; Kanatani et al., 2000a; Mullins et al., 2001). Moreover, the duration of action of PYY(3-36) seems to be short in humans (Brandt et al., 2004). Therefore, a selective, long-acting NPY2 agonist is a desirable alternative to PYY(3-36) for obesity management.

Peptide **1** exemplifies a strategy to attain a series of selective NPY2 receptor peptide agonists (Table 1) (DeCarr et al., 2007a; Lumb et al., 2007). The structural features of **1** include 1) the N-terminal modifying group, which improves in vitro NPY2 receptor affinity; 2) the peptide core corresponding to residues 25 to 36 of human PYY, which provides the scaffold for NPY2 receptor selectivity; and 3) modification with 20-kDa mPEG to improve in vivo efficacy. The resulting peptide **1** induces a 7% reduction in body weight in DIO mice following subcutaneous daily dosing at 2.9 μmol/kg (Lumb et al., 2007).

To improve the in vivo efficacy of peptide **1**, the length of the peptide core was increased to create peptide **2**, because a longer peptide core exhibits greater in vitro potency at the NPY2 receptor (DeCarr et al., 2007a) and in vitro potency of a series of peptides related to peptide **1** correlates with in vivo reduction in food intake in mice (Lumb et al., 2007). Peptide **2** contains a peptide core corresponding to residues 13 to 36 of human PYY, the same N-terminal modification 2-mercaptonicotinic acid as peptide **1**, and it exhibits an approximately 4-fold increase of in vitro potency over peptide **1**.

Peptide **2** caused a significant 9% reduction in body weight in DIO mice in three independent studies, and the body-weight loss is sustained after 40 days of dosing. The loss in weight is recovered after a break in dosing in DIO mice, suggesting that tolerance is not developed to the weight-loss effect. Compared with peptide **1**, peptide **2** elicits a higher level of body-weight loss (9% at 0.94 μmol/kg) than peptide **1** (7% at 2.9 μmol/kg; Lumb et al., 2007) at a 3-fold lower dose. Peptide **2** also induces a substantially greater level of body-weight reduction at a lower dose than the clinical candidate PYY(3-36) that acts via the same putative mechanism of NPY2 receptor activation.

Peptide **2** causes a substantial reduction in food intake that persists for at least 72 h in mice and at least 48 h in rats. The extended duration of action is in marked contrast to the short-lived effect of PYY(3-36), which is only 4 h in the mouse and rat. The extended duration of action is attributed to PEGylation, because the non-PEGylated analog peptide **3** exhibits efficacy in reducing food intake in the mouse only for a period of 2 to 4 h. Peptide **2** exhibits a longer half-life, longer T_{max}, and higher C_{max} in rodent plasma than PYY(3-36) (data not shown), suggesting that the improved pharmacodynamic profile of peptide **2** compared with PYY(3-36) is in part related to an extended in vivo lifetime and exposure.

Peptide **2** is envisaged to function through selective NPY2 receptor activation. Indeed, the anorexigenic effects of peptide **2** and PYY(3-36) in mice are blocked by the selective NPY2 antagonist BIIE0246, providing in vivo evidence that the mechanism of action of peptide **2** is via specific NPY2 receptor action.

Peptide **2** induces favorable improvements in metabolic parameters of DIO mice associated with risk for type II diabetes. Glucose disposal, plasma glucose, and insulin levels are all improved to levels expected upon treatment with marketed antidiabetic agents. This probably reflects the expected improvement in metabolic health following weight loss, although PYY(3-36) has been implicated in improving glucose disposal independently of food intake or body weight

in C57BL/6 mice on a high-fat diet (van den Hoek et al., 2004), and it may contribute to glucose homeostasis (Boey et al., 2007).

The importance of PYY(3-36) as an antiobesity agent is controversial. A conglomerate of research groups have concluded that PYY(3-36) is not efficacious in the rat (Tschop et al., 2004; Boggiano et al., 2005). However, these investigators used much lower doses of PYY(3-36) ($\leq$0.1 μmol/kg) than used here (0.74–2.74 μmol/kg) or in other studies in rats in which efficacy was observed. Other factors such as stress may also influence the response to PYY(3-36) (Halatchev et al., 2004; Small and Bloom, 2005), and at least in the studies reported here, animals were acclimated to handling and subcutaneous dosing with either saline or PEG-Cys before dosing. Nonetheless, the simplest explanation for the apparent lack of consistent PYY(3-36) efficacy is the low doses used in the studies that generated negative results. However, human PYY(3-36) is particularly ineffective in the rat. Intravenous administration of PYY(3-36) to humans at 74 pmol/kg resulted in a 16% reduction in calorie intake over 24 h (Batterham et al., 2003), which is a large apparent efficacious dose difference of about 10,000-fold compared with the rat.

Peptide **2** induces a transient reduction in food intake that is accompanied by weight loss in the first 3 days of dosing. After 3 days, food intake returns to untreated levels, and yet the weight loss is maintained. PYY(3-36) also causes a transient reduction in food intake over the first 3 to 7 days that returns to untreated levels while maintaining weight loss in three different 7 to 56 day studies (Pittner et al., 2004; Adams et al., 2006; Vrang et al., 2006). Therefore, it seems possible that NPY2 agonists work initially via an anorexigenic effect that reduces food intake and leads to weight loss. The transient reduction in feeding induced by peptide **2** and PYY(3-36) may reflect NPY2 receptor desensitization, although arguing against this is the observation that the desired physiological effect (weight loss) is maintained. The maintenance of weight loss after the return to normal feeding levels also raises the possibility of an additional role for NPY2 beyond feeding cessation (Adams et al., 2006). In this regard, it is noteworthy that PYY(3-36) administration decreases the respiratory quotient in DIO mice, suggesting a potential role for NPY2 receptors in fat utilization (Adams et al., 2006), and PYY(3-36) may moderate food intake by action in areas other than the arcuate nucleus (Pittner et al., 2004). Other anorexigenic agents such as the small-molecule type 1 cannabinoid receptor antagonist rimonabant (SR141716) and a NPY5 receptor antagonist induce a transient reduction in food intake but a sustained reduction in body weight in rodents (Ravinet Trillou et al., 2003; Mashiko et al., 2007), indicating that the pattern of feeding behavior and weight loss seen is not a specific property of NPY2 agonists.

In conclusion, we have generated a selective NPY2 peptide agonist that outperforms the clinical candidate PYY(3-36) in reducing food intake in both lean mice and leans rats following a single dose, and in reducing body weight in DIO mice following once-a-day subcutaneous dosing. The peptide is long acting, with significant reductions in food intake observed at 48 h in both mice and rats following a single dose. Significant improvements in metabolic parameters representing risk factors for type II diabetes accompany the weight loss in DIO mice. The results provide preclinical evidence in two animal species that peptide **2** has potential for the management of obesity via selective activation of the NPY2 receptor.

Acknowledgments

We thank B. R. Dixon, S. J. Gardell, and P. P. Tamburini for useful discussions, C. M. Pellegrino for mass spectrometry, and H. Brightman, J. Hedde, D. Molstad, Y. Sun, and L. Yang for technical assistance with the animal studies.

References

Abbott CR, Small CJ, Kennedy AR, Neary NM, Sajedi A, Ghatei MA, and Bloom SR (2005) Blockade of the neuropeptide Y Y2 receptor with the specific antagonist BIIE0246 attenuates the effect of endogenous and exogenous peptide YY(3-36) on food intake. *Brain Res* **1043:**139–144.

Adams SH, Lei C, Jodka CM, Nikoulina SE, Hoyt JA, Gedulin B, Mack CM, and Kendall ES (2006) PYY[3-36] administration decreases the respiratory quotient and reduces adiposity in diet-induced obese mice. *J Nutr* **136:**195–201.

Batterham RL, Cohen MA, Ellis SM, Le Roux CW, Withers DJ, Frost GS, Ghatei MA, and Bloom SR (2003) Inhibition of food intake in obese subjects by peptide YY_{3-36}. *N Engl J Med* **349:**941–948.

Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, et al. (2002) Gut hormone PYY_{3-36} physiologically inhibits food intake. *Nature* **418:**650–654.

Boey D, Sainsbury A, and Herzog H (2007) The role of peptide YY in regulating glucose homeostasis. *Peptides* **28:**390–395.

Boggiano MM, Chandler PC, Oswald KD, Rodgers RJ, Blundell JE, Ishii Y, Beattie AH, Holch P, Allison DB, Schindler M, et al. (2005) PYY_{3-36} as an anti-obesity drug target. *Obes Rev* **6:**307–322.

Brandt G, Park A, Wynne K, Sileno A, Jazrawi R, Woods A, Quay S, and Bloom S (2004) Nasal peptide PYY_{3-36}: phase 1 dose ranging and safety studies in healthy human subjects. *ENDO 2004. The 86th Annual Meeting of the Endocrine Society*; 2004 Jun 16–19; New Orleans, LA. The Endocrine Society, Chevy Chase, MD.

Challis BG, Pinnock SB, Coll AP, Carter RN, Dickson SL, and O'Rahilly S (2003) Acute effects of PYY_{3-36} on food intake and hypothalamic neuropeptide expression in the mouse. *Biochem Biophys Res Commun* **311:**915–919.

Chelikani PK, Haver AC, and Reidelberger RD (2005) Intravenous infusion of peptide YY(3-36) potently inhibits food intake in rats. *Endocrinology* **146:**879–888.

Cox JE and Randich A (2004) Enhancement of feeding suppression by PYY(3-36) in rats with area postrema ablations. *Peptides* **25:**985–989.

DeCarr LB, Buckholz TM, Coish PD, Fathi Z, Fisk SE, Mays MR, O'Connor SJ, and Lumb KJ (2007a) Identification of selective neuropeptide Y2 peptide agonists. *Bioorg Med Chem Lett* **17:**538–541.

DeCarr LB, Buckholz TM, Milardo LF, Mays MR, Ortiz A, and Lumb KJ (2007b) A long-acting selective neuropeptide Y2 receptor PEGylated peptide agonist reduces food intake in mice. *Bioorg Med Chem Lett* **17:**1916–1919.

Degen L, Oesch S, Casanova M, Graf S, Ketterer S, Drewe J, and Beglinger C (2005) Effect of peptide YY_{3-36} on food intake in humans. *Gastroenterology* **129:**1430–1436.

Doods H, Gaida W, Wieland HA, Dollinger H, Schnorrenberg G, Esser F, Engel W, Eberlein W, and Rudolf K (1999) BIIE0246: a selective and high affinity neuropeptide Y Y(2) receptor antagonist. *Eur J Pharmacol* **384:**R3–R5.

Fitzsimons TJ and Le Magnen J (1969) Eating as a regulatory control of drinking in the rat. *J Comp Physiol Psychol* **67:**273–283.

Halatchev IG, Ellacott KL, Fan W, and Cone RD (2004) Peptide YY_{3-36} inhibits food intake in mice through a melanocortin-4 receptor-independent mechanism. *Endocrinology* **145:**2585–2590.

Hu Y, Bloomquist BT, Cornfield LJ, DeCarr LB, Flores-Riveros JR, Friedman L, Jiang P, Lewis-Higgins L, Sadlowski Y, Schaefer J, et al. (1996) Identification of a novel hypothalamic neuropeptide Y receptor associated with feeding behavior. *J Biol Chem* **271:**26315–26319.

Kanatani A, Ishihara A, Iwaasa H, Nakamura K, Okamoto O, Hidaka M, Ito J, Fukuroda T, MacNeil DJ, Van der Ploeg LH, et al. (2000a) L-152,804: orally active and selective neuropeptide Y Y5 receptor antagonist. *Biochem Biophys Res Commun* **272:**169–173.

Kanatani A, Mashiko S, Murai N, Sugimoto N, Ito J, Fukuroda T, Fukami T, Morin N, MacNeil DJ, Van der Ploeg LH, et al. (2000b) Role of the Y1 receptor in the regulation of neuropeptide Y-mediated feeding: comparison of wild-type, Y1 receptor-deficient, and Y5 receptor-deficient mice. *Endocrinology* **141:**1011–1016.

Koegler FH, Enriori PJ, Billes SK, Takahashi DL, Martin MS, Clark RL, Evans AE, Grove KL, Cameron JL, and Cowley MA (2005) Peptide YY(3-36) inhibits morning, but not evening, food intake and decreases body weight in rhesus macaques. *Diabetes* **54:**3198–3204.

Leban JJ, Heyer D, Landavazo A, Matthews J, Aulabaugh A, and Daniels AJ (1995) Novel modified carboxy terminal fragments of neuropeptide Y with high affinity for Y2-type receptors and potent functional antagonism at a Y1-type receptor. *J Med Chem* **38:**1150–1157.

Lumb KJ, DeCarr LB, Milardo LF, Mays MR, Buckholz TM, Fisk SE, Pellegrino CM, Ortiz AA, and Mahle CD (2007) Novel selective neuropeptide Y2 receptor PEGylated peptide agonists reduce food intake and body weight in mice. *J Med Chem* **50:**2264–2268.

Mashiko S, Ishihara A, Iwaasa H, Sano H, Ito J, Gomori A, Oda Z, Moriya R, Matsushita H, Jitsuoka M, et al. (2007) A pair-feeding study reveals that a Y5 antagonist causes weight loss in diet-induced obese mice by modulating food intake and energy expenditure. *Mol Pharmacol* **71:**602–608.

Moran TH, Smedh U, Kinzig KP, Scott KA, Knipp S, and Ladenheim EE (2005)

Peptide YY(3-36) inhibits gastric emptying and produces acute reductions in food intake in rhesus monkeys. *Am J Physiol* **288:**R384–R388.
Mullins D, Kirby D, Hwa J, Guzzi M, Rivier J, and Parker E (2001) Identification of potent and selective neuropeptide Y Y_1 receptor agonists with orexigenic activity in vivo. *Mol Pharmacol* **60:**534–540.
Neary NM, Small CJ, Druce MR, Park AJ, Ellis SM, Semjonous NM, Dakin CL, Filipsson K, Wang F, Kent AS, et al. (2005) Peptide YY_{3-36} and glucagon-like peptide-1_{7-36} inhibit food intake additively. *Endocrinology* **146:**5120–5127.
Pittner RA, Moore CX, Bhavsar SP, Gedulin BR, Smith PA, Jodka CM, Parkes DG, Paterniti JR, Srivastava VP, and Young AA (2004) Effects of PYY[3-36] in rodent models of diabetes and obesity. *Int J Obes Relat Metab Disord* **28:**963–971.
Ravinet Trillou C, Arnone M, Delgorge C, Gonalons N, Keane P, Maffrand JP, and Soubrie P (2003) Anti-obesity effect of SR141716, a CB1 receptor antagonist, in diet-induced obese mice. *Am J Physiol Regul Integr Comp Physiol* **284:**R345–R353.
Sileno AP, Brandt GC, Spann BM, and Quay SC (2006) Lower mean weight after 14 days intravenous administration peptide YY_{3-36} (PYY_{3-36}) in rabbits. *Int J Obes (Lond)* **30:**68–72.
Small CJ and Bloom SR (2005) The therapeutic potential of gut hormone peptide YY_{3-36} in the treatment of obesity. *Expert Opin Investig Drugs* **14:**647–653.
Tschop M, Castaneda TR, Joost HG, Thone-Reineke C, Ortmann S, Klaus S, Hagan MM, Chandler PC, Oswald KD, Benoit SC, et al. (2004) Physiology: does gut hormone PYY3-36 decrease food intake in rodents? *Nature* **430:**165–166.
van den Hoek AM, Heijboer AC, Corssmit EP, Voshol PJ, Romijn JA, Havekes LM, and Pijl H (2004) PYY_{3-36} reinforces insulin action on glucose disposal in mice fed a high-fat diet. *Diabetes* **53:**1949–1952.
Vrang N, Madsen AN, Tang-Christensen M, Hansen G, and Larsen PJ (2006) 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* **291:**R367–R375.

Address correspondence to: Dr. Kevin J. Lumb, Merck Research Laboratories, West Point, PA 19486. E-mail: kevin_lumb@merck.com