Research Article

Journal of Peptide Science

Received: 6 January 2012 Revised: 2 March 2012 Accepted: 5 March 2012 Published online in Wiley Online Library: 4 May 2012

(wileyonlinelibrary.com) DOI 10.1002/psc.2408

Restoration of leptin responsiveness in diet-induced obese mice using an optimized leptin analog in combination with exendin-4 or FGF21

Timo D. Müller,[a,b‡] Lorraine M. Sullivan,[c‡] Kirk Habegger,[a‡] Chun-Xia Yi,[a] Dhiraj Kabra,[a] Erin Grant,[a] Nickki Ottaway,[a] Radha Krishna,[a] Jenna Holland,[a] Jazzminn Hembree,[a] Diego Perez-Tilve,[a] Paul T. Pfluger,[a,b] Michael J. DeGuzman,[c] Marc E. Siladi,[c] Vadim S. Kraynov,[c] Douglas W. Axelrod,[c] Richard DiMarchi,[d] Jason K. Pinkstaff [c*] and Matthias H. Tschöp[a,b*]

The identification of leptin as a mediator of body weight regulation provided much initial excitement for the treatment of obesity. Unfortunately, leptin monotherapy is insufficient in reversing obesity in rodents or humans. Recent findings suggest that amylin is able to restore leptin sensitivity and when used in combination with leptin enhances body weight loss in obese rodents and humans. However, as the uniqueness of this combination therapy remains unclear, we assessed whether co-administration of leptin with other weight loss-inducing hormones equally restores leptin responsiveness in diet-induced obese (DIO) mice. Accordingly, we report here the design and characterization of a series of site-specifically enhanced leptin analogs of high potency and sustained action that, when administered in combination with exendin-4 or fibroblast growth factor 21 (FGF21), restores leptin responsiveness in DIO mice after an initial body weight loss of 30%. Using either combination, body weight loss was enhanced compared with either exendin-4 or FGF21 monotherapy, and leptin alone was sufficient to maintain the reduced body weight. In contrast, leptin monotherapy proved ineffective when identical weight loss was induced by caloric restriction alone over a comparable time. Accordingly, we find that a hypothalamic counter-regulatory response to weight loss, assessed using changes in hypothalamic agouti related peptide (AgRP) levels, is triggered by caloric restriction, but blunted by treatment with exendin-4. We conclude that leptin re-sensitization requires pharmacotherapy but does not appear to be restricted to a unique signaling pathway. Our findings provide preclinical evidence that high activity, long-acting leptin analogs are additively efficacious when used in combination with other weight-lowering agents. **Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.**

Supporting information can be found in the online version of this article.

Keywords: leptin; FGF21; Ex4; DIO; HFD; AgRP

Introduction

The regulation of energy homeostasis is controlled by complex central and peripheral signaling systems and depends on constant signal integration. This integrative system uses afferent hormonal and nutrient signals to modulate food intake and energy utilization [1]. Among the many regulators of energy homeostasis, the adipocyte hormone leptin is considered to be a key modulator. Since its discovery in 1994 [2], leptin has been intensely studied in the treatment of obesity [3–5]. Leptin is effective in inducing weight loss in obese congenitally leptin-deficient mice [6] and humans [4,5]. However, in rodent models of diet-induced obesity [7–9] or in obese humans [3], leptin has little efficacy [10]. Administration of higher doses of native leptin or analogs with sustained pharmacokinetics failed to enhance weight loss and increased adverse effects [11]. Recently, co-administration of leptin and the pancreatic hormone amylin

* *Correspondence to: Matthias H. Tschöp, Institute for Diabetes and Obesity, Helmholtz Center Munich, German Research Center for Environmental Health (GmbH), 85764 Munich-Neuherberg, Germany. E-mail: matthias.tschoep@helmholtz-muenchen.de*

* *Jason Pinkstaff, Ambrx, Inc., San Diego, CA 92037, USA. E-mail: jason.pinkstaff@ambrx.com*

‡ *Timo D. Müller, Lorraine M. Sullivan and Kirk Habegger contributed equally to this work.*

a *Metabolic Disease Institute, Division of Endocrinology, Diabetes & Metabolism, Department of Internal Medicine, College of Medicine, , University of Cincinnati, Cincinnati, OH 45230, USA*

b *Institute of Diabetes and Obesity, , Helmholtz Center Munich, German Research Center for Environmental Health (GmbH), Munich, Germany*

c *Ambrx, Inc., San Diego, CA 92037, USA*

d *Department of Chemistry, , Indiana University, Bloomington, IN 47405, USA*

Abbreviation used: *PEG, polyethylene glycol; Lep, leptin; Ex4, exendin-4; FGF21, fibroblast growth factor 21.*

 Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

was shown to synergistically decrease body weight in rodents and humans, implying that the latter had unique ability to restore leptin sensitivity [12–14]. However, as the uniqueness of this combination therapy remains unclear, we here assessed whether co-administration of leptin with other weight loss-inducing hormones, such as exendin-4 (Ex4) or fibroblast growth factor 21 (FGF21), equally restores leptin responsiveness in diet-induced obese (DIO) mice. To further investigate combination leptin therapy, an analog of superior pharmaceutical properties, relative to native leptin, was pursued. The duration of native leptin *in vivo* action is limited, and the molecule is biophysically prone to self-association. It was expected that both of these limitations could be addressed through site-specific PEGylation of the molecule, as this form of chemical modification is well recognized to enhance biophysical characteristics while extending the time action of native proteins [15]. We utilized knowledge of the chemical structure of leptin [16] to identify structural sites suitable for PEGylation, with the goal of enhancing biophysical characteristics while preserving biological function. The site-directed placement of a novel amino acid, termed ReCode™ (reconstituting chemically orthogonal directed engineering) utilizes a tRNA that has been modified to recognize the UAG (amber) codon and an evolved aminoacyl tRNA synthetase (O-RS). This synthetase activates the cognate tRNA (O-tRNA) with a novel amino acid that has unique chemical attributes, as compared with those found in the naturally occurring 20 amino acids. In this way, the placement site of the UAG codon determines the specific location of the novel amino acid in the protein [17]. The O-tRNA and O-RS are designed to function in the context of a living cell without cross-talk with the endogenous tRNAs, aminoacyl tRNA synthetases [18]. The protein containing the site-specific novel amino acid can then be modified with complete chemical specificity [19]. A set of leptin analogs containing single *para*-acetylphenylalanine (*p*AF) substitutions was selected based on the crystal structure of the native hormone. These analogs were then conjugated to an amino-oxy functionalized PEG polymer, resulting in stable, site-specific, and monosubstituted PEGylated protein analogs.

These PEGylated leptin analogs demonstrated differential *in vitro* and *in vivo* pharmacology when evaluated as single agents. Furthermore, this pharmacology was dependent on the site of PEGylation. We next explored the pharmacology of the best PEGylated leptin analog in combination with the pharmacologic agents Ex4 and FGF21, and with caloric restriction. We have chosen Ex4 and FGF21 for the combination with PEG-leptin, as both hormones effectively promote a major body weight loss in rodent models of DIO [20–22] and as, at present, there is no known clear overlap in signaling between these hormones and amylin.

Methods

Generation of Novel PEG-Leptin Variants

Amber-codon containing leptin variants were grown in lysogeny broth (LB) medium, cells were lysed, and inclusion bodies (IBs) were purified using successive washes with IB buffer 1 (50 mM Tris, pH 8.0; 100 mM NaCl; 1 mM EDTA; 1% Triton X-100) and IB buffer 2 (IB buffer 1 without Triton X-100). The washed IBs were solubilized with 20 mM Tris, pH 8.0, containing 8 M urea and 10 mM ß-mercaptoethanol (15 ml/g of IBs). The solubilized IBs were diluted 1 : 20 with 50 mM glycine, pH 9.0; 1 M urea; 0.2 mM cysteine; and 2.0 mM cystine to refold at 4 °C for 1–3 days. The refolded leptin was loaded onto a Super Q650M column (Tosoh Bioscience, South San Francisco, CA, USA) equilibrated with 20 mM Tris, pH 8.0. Protein was eluted using 0–250 mM NaCl gradient in the running buffer over a 20 column volume (CV). Monomeric leptin was loaded onto MEP column (Pall Corporation, Covina, CA, USA) equilibrated with 10 mM Tris, pH 8.0. Elution was performed with 50 mM sodium acetate, pH 4.0. Eluate was concentrated to 2 mg/ml using 5 kDa MWCO membrane filter (Millipore, Bellerica, MA, USA) and pH adjusted to 4.0 with 10% acetic acid. PEG was added to the protein sample at 12 : 1 PEG-to-protein ratio, and the reaction was allowed to continue for 3 days at 28 °C. The PEGylation reaction was diluted 1 : 5 with water, clarified using 0.22 μm filter (Millipore) and loaded onto an SP HP column (GE Healthcare, Pittsburgh, PA, USA) equilibrated with 10 mM sodium acetate buffer, pH 4.0. PEGylated leptin was eluted with a liner gradient of 0–250 mM NaCl in 10 mM sodium acetate, pH 4.0, over 20 CV. Main peak was pooled and diafiltered using 10 kDa MWCO (Millipore) into phosphate-buffered saline (PBS) at a final concentration of ~1 mg/ml. The purity of PEGylated leptin variants was analyzed using reverse phase HPLC (RP-HPLC) and size exclusion-HPLC (SEC-HPLC)(Figure S1).

Biacore Assessment of Affinity

The leptin receptor was immobilized on CM5 to a density of 1200 response units (RUs) according to the manufacturer instructions (Biacore Inc./GE Healthcare, Pittsburgh, PA, USA). Solutions of leptin (0.1–300 nM) in HBS-EP (0.1M HEPES, pH 7.4, 0.15M NaCl, 3mM EDTA, 0.005% surfactant p20 buffer (Biacore Inc.) were injected over both flow cells at 25 °C and flow rate of 50 μl/min for 180 sec, followed by a 12-min dissociation phase. Receptor-binding surface was regenerated with 20 μl glycine, pH 2.0, and analyzed using the BiaEvaluation 4.0 software (Biacore Inc.).

In Vitro Activity Assay

A luciferase stable reporter cell line was developed to assess the biological activity of the PEG-leptin molecules. The human leptin receptor cDNA clone (Origene, Rockville, MD, USA) was inserted into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). HEK 293 cells were stably transfected with two plasmids: one containing the firefly luciferase reporter gene under control of a STAT3 response element and the other encoding OB-Rb, which was expressed on the cell surface. The biological activity was based on the half maximal effective concentration (EC50) of a 4-PL sigmoidal curve relative to the EC50 of a recombinant human leptin reference standard. Assays were repeated 2–4 times to ensure reproducibility.

Immunohistochemistry of Hypothalamic AgRP

Mice were treated for 4 days via once daily subcutaneous (sc) injections of either saline or Ex4 (0.5 mg/kg/day). Mice were perfused 8 h after treatment with saline and 4% paraformaldehyde. Brains were sunk in 30% sucrose and cut into 30 μm thickness. Hypothalamic sections were incubated overnight with guinea pig anti-AGRP antibody (1 : 1000 dilution; Abcam, Cambridge, MA, USA). After incubations with 1 : 400 biotinylated horse-anti guinea pig secondary antibody, ABC reagent (Vector Laboratories, Burlingame, CA, USA), and visualized using 1% diaminobenzidine, sections were mounted and relative densitometry of AGRP-immunoreactivity of cells and fibers were measured using ImageJ (NIH,Bethesda, Maryland, USA).

Immunohistochemistry of Hypothalamic pSTAT3

Mice were fasted for 6 h and administered a sc injection of 0.5 mg/kg 40K PEG G111 or saline. After 30 min, mice were anesthetized and perfused with saline and 4% paraformaldehyde.

 Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Brains were fixed in 30% sucrose, and hypothalamic and brainstem sections were incubated overnight with rabbit anti-pSTAT3 antibody (1 : 1000 dilution; Cell Signaling Technologies, Danvers, MA, USA). After incubations with 1 : 400 biotinylated goat-anti rabbit secondary antibody, ABC reagent (Vector Laboratories), and 1% diaminobenzidine, pSTAT3 was visualized using light microscopy.

In Vivo Efficacy Studies

Nine-week-old C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME, USA) were kept at ambient temperature (23 °C) with constant humidity and a 12 h light–dark cycle. Mice had free access to water and were fed *ad libitum* with either a regular chow diet (Teklad LM-485, 5.6% fat) or a high-fat diet (HFD) (58% kcal fat; Research Diets Inc., New Brunswick, NJ, USA). All procedures were approved by the Institutional Animal Care and Use Committee of Ambrx, Inc. or the University of Cincinnati and were performed according to the 'Principles of laboratory animal care' (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm).

In Vivo Evaluation of PEG-Leptin Variants

Eight-week-old to 10-week-old female *ob/ob* mice (Jackson Laboratories) were treated with a single sc injection of a PEG-leptin variant, recombinant human leptin (R&D Systems, Minneapolis, MN, USA) or vehicle control as indicated. Notably, indicated concentrations for PEG-leptin variants refer to the leptin protein only and does not include the molecular weight of the PEG. For pharmacokinetic evaluation, jugular vein-catheterized, male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were treated with a single sc injection of a PEG-leptin variant or recombinant human leptin (0.1 mg/kg). Serum concentrations of PEG-leptin analogs were analyzed by a bridging ELISA. Pharmacokinetic evaluations were performed using non-compartmental modeling and WinNonlin (v 5.0.1; Pharsight Corporation Sunnyvale, CA, USA).

Results

Leptin Structural Modeling

A leptin protein model was constructed based upon its known crystal structure, with putative receptor-binding sites deduced from meta-analysis of published mutagenesis studies [16] (Figure 1A). The resulting model disclosed putative binding surfaces for the leptin receptor, as well as areas distal to these critical surfaces. Based upon this model, sites were selected that would minimize disruption of secondary structure and would not interfere with binding (H46, W100, E105, D108, and G111), as well as one that was anticipated to disrupt receptor binding (S120).

Production of PEG-Leptin Variants

On the basis of the aforementioned modeling, a set of six leptin analogs, plus wild-type recombinant human leptin ('rh leptin'), was produced with the novel amino acid, *p*AF site-specifically positioned by application of ReCode™ methodology. The synthetic chemistry for coupling the amino-oxy functionalized PEGylated polymers to the *p*AF has previously been reported for analogous derivatization of two other unrelated proteins: growth hormone [19] and FGF21 [23] (Figure 1B). The analytical

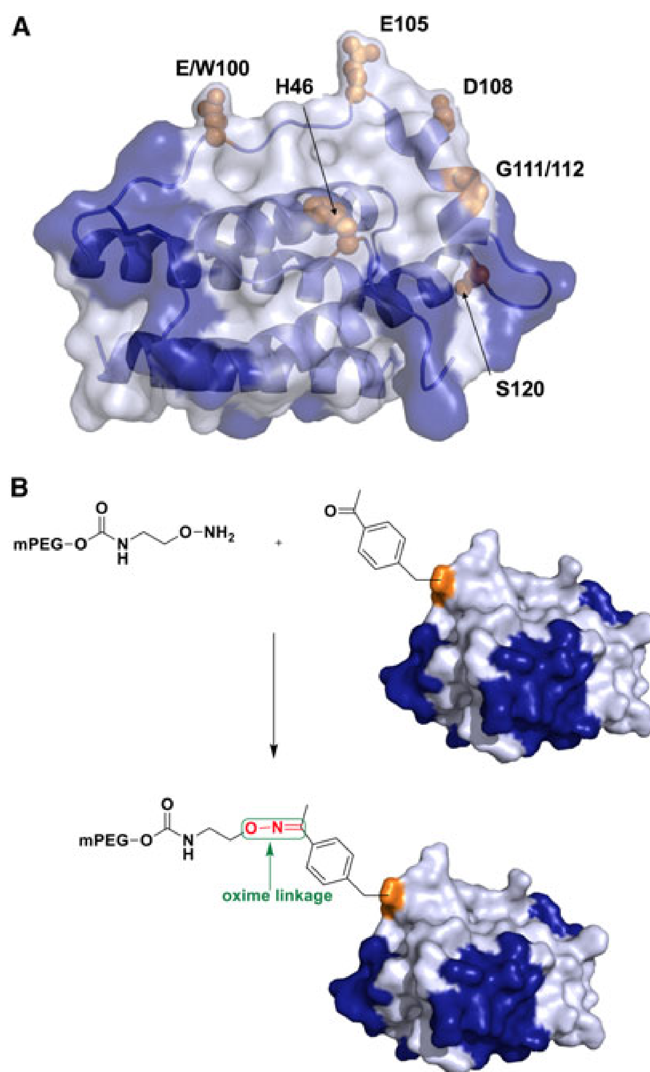

Figure 1. A model of leptin depicted as a ribbon and semitransparent surface diagram. The structure of the protein is based upon the crystal structure A1X8 [16]. The dark blue surface areas are theoretical receptor-binding regions. The specific pAF sites are represented as orange spheres. (A) The figure was generated using the PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC, Portland, OR, USA. (B) Leptin (D108pAF) contains a biosynthetically incorporated, chemically reactive, carbonyl group at amino acid 108 (orange) that provides a single, unique covalent site for attachment of an oxyamino-derivatized poly(ethylene) glycol (PEG).

characterization of the reaction products as purified PEGylated leptin analogs is shown in Figure S1. These proteins once chromatographically purified were mono-PEGylated and characterized by *in vitro* biochemical methods and animal studies [19].

In Vitro Assessment of PEG-Leptin Activity

Receptor-binding potency for all of the *p*AF-leptin variants, before selective conjugation with a single 30K PEG, was preserved (Table 1). As expected, mono-conjugation with 30K PEG caused a reduction in binding affinity of approximately 10-fold to 40-fold, indicating that site selection is of vital importance and function must be assessed in the final PEGylated form.

The *in vitro* biological character of the PEGylated variants was further assessed using a luciferase reporter assay (Table 2). The remaining 30K PEGylated proteins were less active than native

 Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Table 1. Biacore-derived affinities (*Kd*) of wild-type and 30K PEG-leptin variants

Variant	*Kd* of variant	*Kd* of variant with 30K PEG
Wild type	0.7	Not applicable
30K PEG H46	0.5	11
30K PEG W100	0.5	12
30K PEG E105	0.9	10
30K PEG D108	0.6	Not applicable
30K PEG G111	0.7	27

Table 2. *In vitro* activity of wild-type leptin, 30K PEG-leptin variants, and selected 40K PEGl-leptin variants

Compound	*In vitro* potency; EC50 (ng/ml)
Wild-type leptin	1.8
30K PEG H46	20
30K PEG W100	3.2
30K PEG E105	34
30K PEG D108	20
30K PEG G111	6.4
30K PEG S120/T121A	Not active[a]
40K PEG H46	62
40K PEG G111	13

[a]Activity was assessed using a luciferase reporting system using a HEK 293 background.

leptin (unPEGylated) by 1.3-fold to 22-fold, indicating substantial differences in biological activity based solely on the site of PEGylation. As expected from the molecular model, cell signaling demonstrated the S120 analog to be distinctly different and inactive because of its placement in the binding site of leptin's receptor. The H46 and G111 variants were further evaluated as 40K PEG conjugates. Both of these leptin analogs demonstrated a twofold to threefold further loss in activity than observed for the homologous 30K variants, indicating that PEG size is also an important factor in determining *in vitro* potency.

Pharmacokinetic Evaluation of PEG-Leptin Variants

To evaluate the pharmacokinetic properties of these PEG-leptin variants, male Sprague Dawley rats were treated with a single sc injection of rh leptin or a 30K PEG-leptin variant at an identical protein dose of 0.1 mg/kg. All 30K PEG-leptin variants showed comparable serum concentrations and a significantly greater systemic exposure as compared with native hormone (Figure 2A).

In Vivo Evaluation of PEG-Leptin Molecules

Biological activity in a leptin-sensitive animal model was assessed using a single sc administration of PEG-leptin variants (0.5 mg/kg) to leptin deficient *ob/ob* mice. The rh leptin and the pre PEG H46 analog had no appreciable effect on animal weight as compared with vehicle control. In contrast, single sc doses of 30K PEG variants produced significant decreases in body weight,

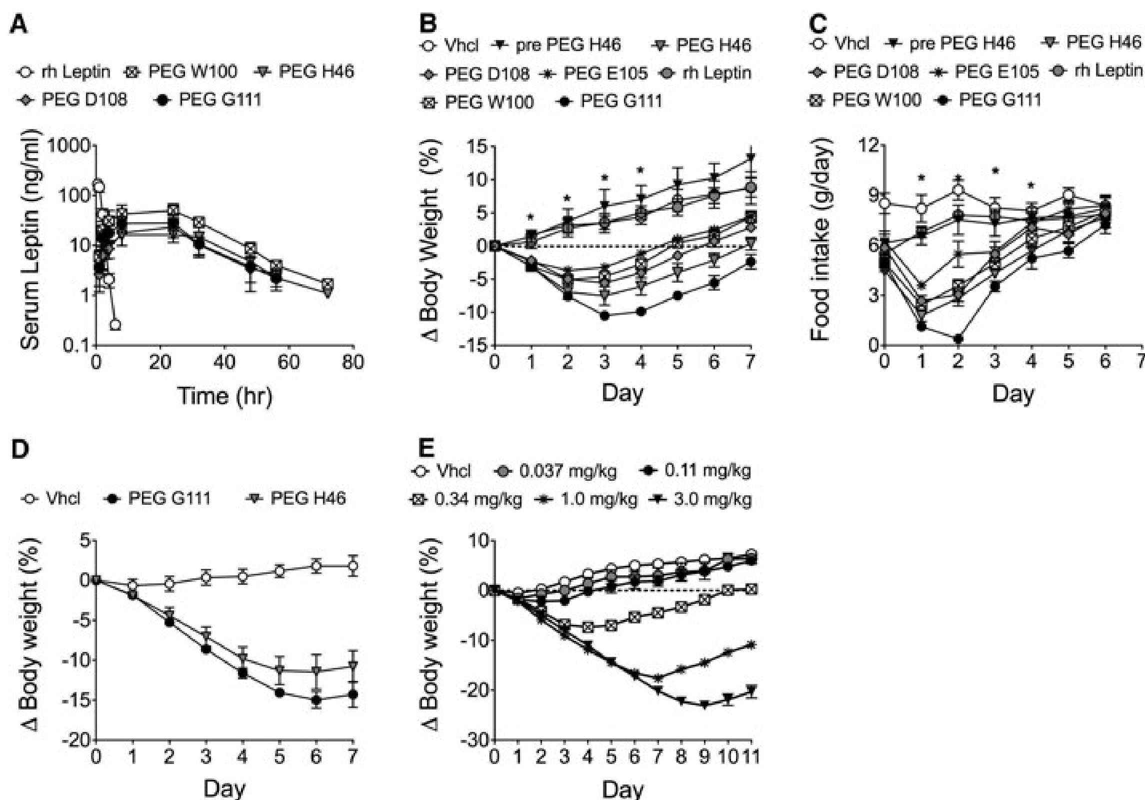

Figure 2. Evaluation of systemic exposure and weight loss efficacy of 40K or 30K PEGylated leptin variants. (A) Serum concentrations of 30K PEGylated leptin variants and rh leptin in male Sprague Dawley rats. Change in (B) body weight (% to baseline) and (C) food intake of *ob/ob* mice treated with a single sc injection (0.5 mg/kg) of rh leptin, 30K PEG-leptin variants, or vehicle control (PBS, $n = 5$ per group). (D) Change in body weight (% to baseline) of *ob/ob* mice treated with a single sc injection (0.5 mg/kg) of 40K PEG G111, 40K PEG H46, or vehicle control (PBS, $n = 5$ per group). (E) Change in body (% to baseline) of *ob/ob* mice treated with single sc injection (0.5 mg/kg) of 40K PEG G111, ranging from 0.037 to 3.0 mg/kg *versus* control (PBS, $n = 5$ per group). Values represent means ± standard error of the mean.

 Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Journal of PeptideScience

with a maximum decrease observed at 3 days post-treatment (Figure 2B). The greatest weight loss was observed in mice treated with the 30K PEG G111 and H46, and the loss appeared to be mediated by a decrease in food consumption (Figure 2C). To evaluate the *in vivo* site of action, a single sc dose of 40K PEG G111 (0.5 mg/kg) was administered to C57/BL6 mice. Immunohistochemical analysis showed increased pSTAT3 immunoreactivity in the ventromedial arcuate nucleus when compared with vehicle controls (Figure S2), suggesting *in vivo* activity at key target sites.

Evaluation of PEG Size and Optimal Concentration of PEG-Leptin Molecules

The two most potent variants as determined by both *in vivo* and *in vitro* activity, PEG G111 and H46, were similarly tested as 40K PEG analogs to assess the impact of additional PEG size on weight loss in *ob/ob* mice. The 40K PEG G111 maintained superiority relative to PEG H46, but the magnitude of the weight loss after 3 days was similar to that seen in the homologous 30K PEG conjugate (Figure 2D). However, both 40K PEG variants caused further weight loss over 7 days, probably because of the increased terminal half-life and systemic exposure. Pharmacokinetics of the PEG H46 and G111 mutants were comparably tested as 40K PEG conjugates. We observed extended duration of action with calculated terminal half-lives of 45.5 ± 4.0 and 41.1 ± 3.2 h, respectively, as compared with native hormone having a terminal half-life of 0.5 h. As a result, the 40K PEG G111 variant was deemed most efficacious and utilized in subsequent pharmacology studies. Dose dependency was demonstrated in a range from 0.037 to 3.0 mg/kg for the 40K PEG G111 variant (Figure 2E). Increasing efficacy was observed with increased dose, and the greatest effect was recorded at 7–9 days post-treatment.

Long-Acting Leptin Analogs in Lean Animals

Single sc injections (0.5 mg/kg) of five PEG-leptin analogs in lean, chow-fed C57BL/6 J mice also caused significant decreases of body weight relative to vehicle treatment within the first 3 days after the injection (Figure 3). The greatest weight loss was observed in mice treated with 40K PEG H46, W100, or G111 (Figure 3). Noteworthy, no changes in *ad libitum* levels of blood glucose were observed between any of the treatment groups (Figure S3).

We also tested whether 40K PEG G111 could prevent diet-induced weight gain in lean mice when switched from a regular chow to a HFD, coincident with initiation of treatment. Neither once weekly treatment with 40K PEG G111 (0.5 mg/kg) nor daily treatment with rh leptin (1 mg/kg/day) prevented weight gain when mice were fed a HFD, confirming the widely reported, rapid, and powerful leptin resistance-inducing effect of HFD exposure [24].

Exendin-4, but not Matched Caloric Restriction, Restores Leptin Sensitivity in Diet-Induced Obese Mice

As several lines of evidence indicates that HFD exposure by itself, even in lean animals, rapidly induces leptin resistance independent of body weight change [24,25] and caloric intake [26], we assessed the effect of combined treatment with Ex4 (0.5 mg/kg/day) and 40K PEG G111 (3 mg/kg/day) in DIO mice that had either been switched from HFD to chow at the beginning of the therapeutic intervention (Figure 4), or which have been maintained on HFD throughout the study (Figure S4). The intention of this experimental design was to eliminate the leptin resistance-promoting effect of high dietary lipids.

After 18 days of treatment, the vehicle control group switched to chow showed a 15% loss of body weight attributable to the

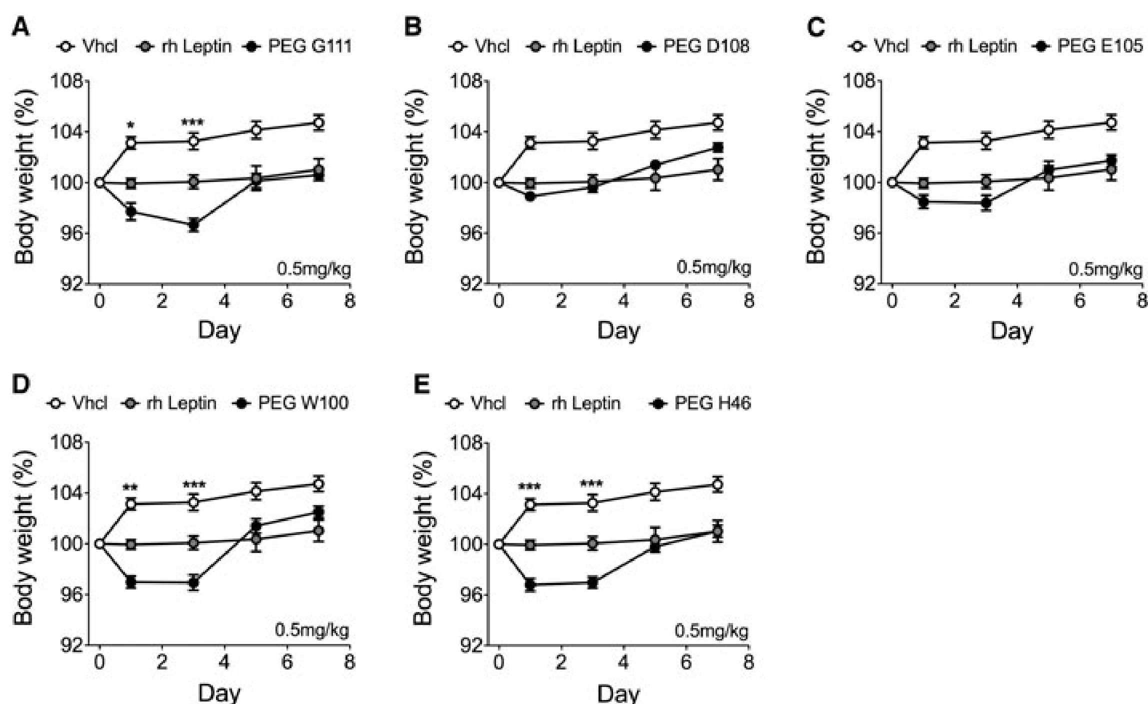

Figure 3. Evaluation of weight loss efficacy of the PEGylated leptin variants in lean C57BL/6 mice. Body weight (% to baseline) of 11-week-old male chow-fed C57BL/6 mice (mean body weight 26.96 ± 0.14 g, $n = 8$ per treatment group) injected with either a single sc injection (0.5 mg/kg) of one of five PEG-leptin analogs or vehicle control (PBS) or which have been injected daily with rh leptin (1 mg/kg). Values represent means ± standard error of the mean. Asterisks indicate significant difference of PEG-leptin from rh leptin; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

 Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

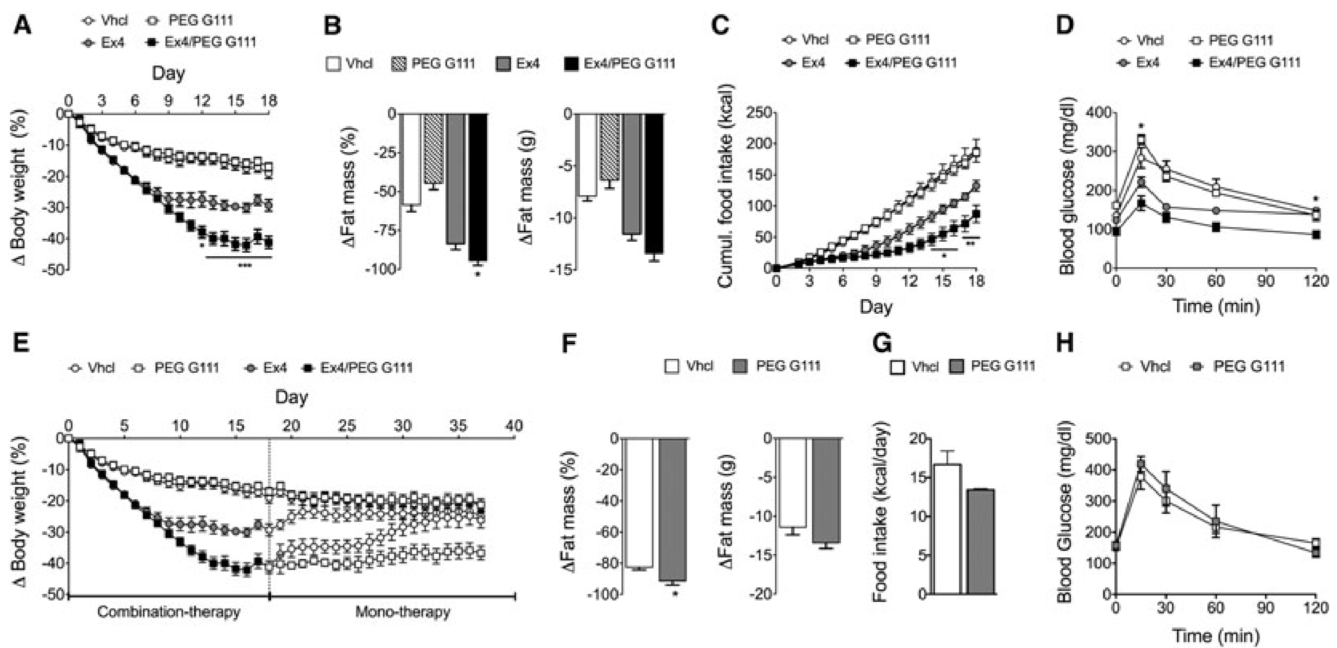

Figure 4. Effects of Ex4 and 40K PEG G111 in obese male C57/BL6 mice switched to regular chow diet at treatment initiation. (A) Body weight, (B) fat mass, (C) cumulative food intake, and (D) glucose tolerance of mice treated for 18 days via twice daily sc injections with either Ex4 (0.5 mg/kg/day, $n = 8$), 40K PEG G111 (3 mg/kg/day, $n = 8$), Ex4/40K PEG G111 ($n = 16$), or vehicle control (PBS, $n = 8$). (E) Body weight, (F) change in fat mass, (G) cumulative food intake, and (H) glucose tolerance of mice that were treated for 18 days with the Ex4/PEG G111 combination and that were continued on vehicle or 40K PEG G111 monotherapy for additional 18 days. Asterisks in panels A–D indicate significant difference of Ex4/40K PEG G111 combination therapy from Ex4 monotherapy; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Values represent means ± standard error of the mean.

change in diet alone (Figure 4A). The PEG-leptin monotherapy had no further beneficial effects on body weight, fat mass, food intake, or glucose tolerance (Figure 4A, B, C, and D). On the contrary, both Ex4 and the Ex4/PEG-leptin combination treatment showed significantly greater decreases in body weight than control or PEG-leptin alone. Ex4/PEG-leptin combination therapy caused a sizable 41.2 ± 2% weight loss after 18 days of treatment, *versus* 29.3 ± 1.9% with Ex4 alone. Weight loss in the combination therapy was accompanied by significantly greater loss of body fat mass (94.5 ± 3.0% vs 83.7 ± 3.7%, $p < 0.05$; Figure 4B) and a significantly lower food intake, as compared with the Ex4 monotherapy ($p < 0.001$; Figure 4C). Interestingly, the additional weight loss caused by the addition of PEG-leptin only emerged after the mice had lost approximately ~28% of their initial body weight. Evaluation of glucose tolerance on day 18 revealed an improved glucose clearance in Ex4-treated mice (compared with vehicle controls) that was further enhanced by combination with PEG-leptin (Figure 4D). To confirm that leptin responsiveness was restored after treatment with the combination of Ex4 and PEG-leptin, we switched the mice that were treated with the Ex4/PEG-leptin combination to either vehicle or PEG-leptin monotherapy for additional 18 days (Figure 4E–H). In this second phase, the mice that were continued on PEG-leptin alone maintained a lower body weight, decreased food intake, and decreased fat mass without any changes in glucose tolerance compared with mice continued on vehicle control, thus indicating that leptin responsiveness was restored in these mice (Figure 4E–H). In accordance to previous data showing that HFD exposure itself rapidly induces leptin resistance, we observed no difference in body weight, fat mass, food intake, or glucose tolerance between mice treated with the Ex4 or the combination with PEG-leptin in mice that were maintained on HFD (Figure S4).

Next, we evaluated whether similar levels of leptin responsiveness could be restored by weight loss resulting from caloric restriction alone. Again, obese male C57/BL6J mice were either switched from HFD to regular chow diet at the start of treatment (Figure 5) or were maintained on HFD throughout the study (Figure S5). Identical levels of weight loss of ~28% were then achieved by either treatment with Ex4 ($n = 16$, 0.5 mg/kg/day, twice daily sc injection) or by caloric restriction ($n = 16$) following similar time courses. After achieving a ~28% weight loss, mice were treated via twice daily sc injections with vehicle control (PBS, $n = 8$) or 40K PEG G111 (3 mg/kg/day, $n = 8$). As with our previous observations, leptin responsiveness was restored after Ex4-induced weight loss. This restoration of sensitivity was marked by a significantly greater decrease of body weight ($p < 0.001$; Figure 5A), fat mass ($p < 0.01$; Figure 5B) and food intake ($p < 0.01$; Figure 5C), and by an improvement of glucose tolerance (Figure 5D) in the PEG-leptin–treated mice as opposed to those randomized to vehicle. In contrast, when weight loss was induced solely by caloric restriction, PEG-leptin did not additionally decrease body weight, fat mass, food intake, or improve glucose tolerance relative to vehicle treatment (Figure 5E–H).

We also found in DIO mice that 4 days of similar Ex4 treatment (0.5 mg/kg/day), but not matched caloric restriction (pair-feeding), decreased hypothalamic AgRP expression (Figure 6). These findings are consistent with increased leptin sensitivity and are a hallmark of increased CNS melanocortinergic tone. As a supplementary study, it was observed that, in mice maintained on HFD, after Ex4-induced or caloric restriction-induced weight loss, there was no effect of PEG-leptin monotherapy on body weight, fat mass, food intake, or glucose tolerance. However, combination of PEG-leptin and Ex4 still led to significantly more weight loss than Ex4 treatment alone (Figure S5).

 Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Journal of PeptideScience

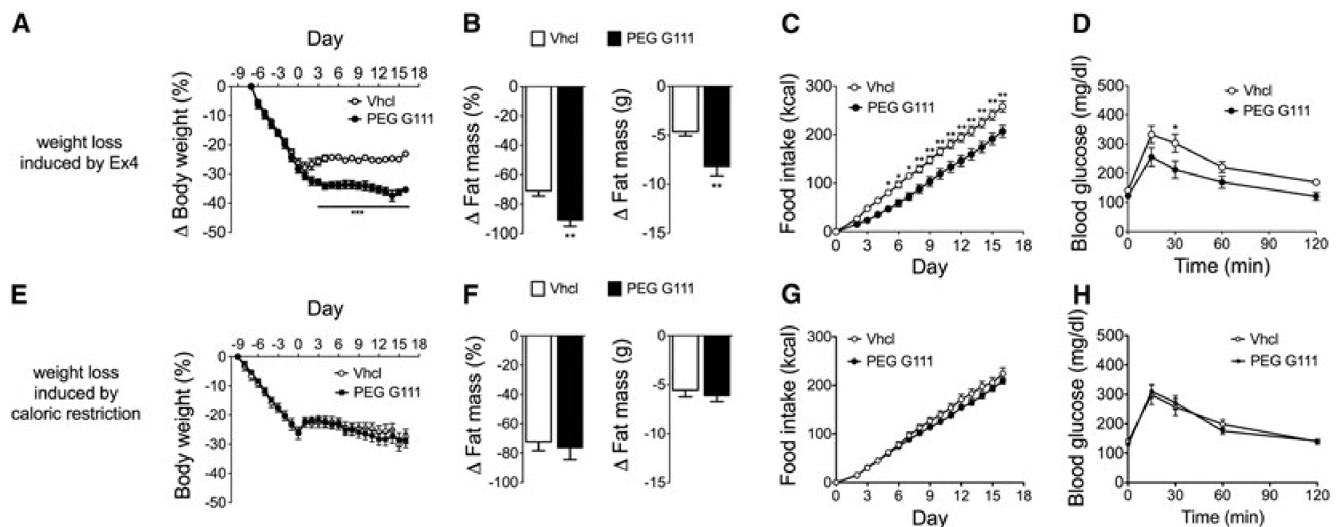

Figure 5. Differential outcome of pharmacologic-induced and caloric restriction-induced weight loss in the effects of Ex4 and 40K PEG G111. Obese male C57/BL6J mice were switched to regular chow diet at treatment initiation. A weight loss of ~28% was then achieved using either treatment with Ex4 ($n = 16$, 0.5 mg/kg/day, twice daily sc injection) or by caloric restriction ($n = 16$). After achieving a ~28% weight loss, mice were treated via twice daily sc injections with vehicle control (PBS, $n = 8$) or 40K PEG G111 (3 mg/kg/day, $n = 8$). Body weight, fat mass, cumulative food intake, and glucose tolerance of mice treated with vehicle (open circle) or 40K PEG G111 (closed circle) following Ex4-induced (A–D) or caloric restriction-induced (E–H) weight loss. Values represent means ± standard error of the mean. Asterisks indicate *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

FGF21 Restores Leptin Sensitivity in Diet-Induced Obese Mice

To explore whether other pharmacologic agents could also enhance leptin efficacy, we assessed the effect of combining FGF21 (2 mg/kg/day) and 40K PEG G111 (3 mg/kg/day) treatments in DIO mice. Again, mice were either switched from HFD to chow at treatment initiation (Figure 7) or were maintained on HFD throughout the study (Figure S6). Similar to the combination with Ex4, the FGF21/PEG-leptin combination therapy elicited greater body weight loss than FGF21 alone (body weight loss at day 18: 39.9 ± 1.82% and 31.8 ± 0.8% compared with 23.4 ± 2.1% in vehicle controls; Figure 7A). Again, this effect was only observed in mice switched from HFD to chow at the beginning of the treatment but not in mice maintained on HFD (Figure S6). Notably, the decreased body weight of the FGF21/PEG-leptin–treated mice was primarily because of loss of fat mass with a lesser degree of lean mass loss (Figure 7B and C) and was associated with a small decrease in food intake (Figure 7D). These findings correlated with improved glucose tolerance after combination therapy as compared with both vehicle treatment and FGF21 monotherapy (Figure 7E). Intriguingly, restoration of leptin responsiveness appeared after a weight loss of ~28%, in a manner apparently identical to that previously found with Ex4/PEG-leptin.

Discussion

Despite its initial promise, leptin has failed to induce significant weight loss or to confer significant beneficial metabolic effects as a therapy for the most common forms of human obesity [3,7–9]. Reports of the co-administration of leptin with amylin have restored interest, but uncertainty remains regarding the uniqueness of this particular combination's efficacy [12,13]. We questioned whether co-administration of leptin with other weight loss-inducing hormones might equally restore leptin responsiveness in DIO mice. Our work simultaneously focused on application of a novel approach in protein engineering to identify a leptin analog more suitable for use in chronic *in vivo* studies.

Starting with a single site mutation that rendered the protein less prone to self-association and more amenable to crystallization, we built a model that informed the selection of point mutations distant from the putative receptor-binding site. Five leptin analogs were biosynthesized using Recode technology [17] to incorporate a single chemically unique amino acid, which was then PEGylated in high yield without modification of any other native sites. The *in vitro* characterization of these leptin analogs demonstrated the power of the model to predict a single modification that would be inactive, as well as five sites that retained high potency as specifically conjugated PEG-analogs. Increasing the PEG-size to 40K on the two 30K-PEG analogs that demonstrated the best *in vivo* potency, reduced the *in vitro* activity comparably while further sustaining the duration of action. Overall, the *in vivo* characterization identified 40K G111 as the preferred analog with much enhanced potency and duration of action relative to the native hormone. Although we have demonstrated hypothalamic penetration and signaling by a select PEGylated leptin analog, it is unclear whether all of these activities are centrally mediated.

The use of the human leptin receptor for *in vitro* study coupled with rodent pharmacology is an imperfect match, despite the high homology and demonstrated activity of human leptin in rodents. A more optimal match between *in vitro* and *in vivo* efficacy is theoretically possible through the use of rodent-sourced leptin receptors. Nonetheless, our intent was twofold: to demonstrate that the site of derivatization can generate differential pharmacology and to select a leptin analog suitable for human study. These results show that the exact site of chemical modification can sizably alter the biological activity, even within a restricted set of structurally preselected analogs. Confirmation that the G111 analog is the optimal analog for use in humans through preclinical animal testing is virtually not possible, and advanced testing in monkeys is a preferred next step to confirming suitability for human study. It should also be noted that there are inconsistencies within the two *in vitro* measurements that we

 Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

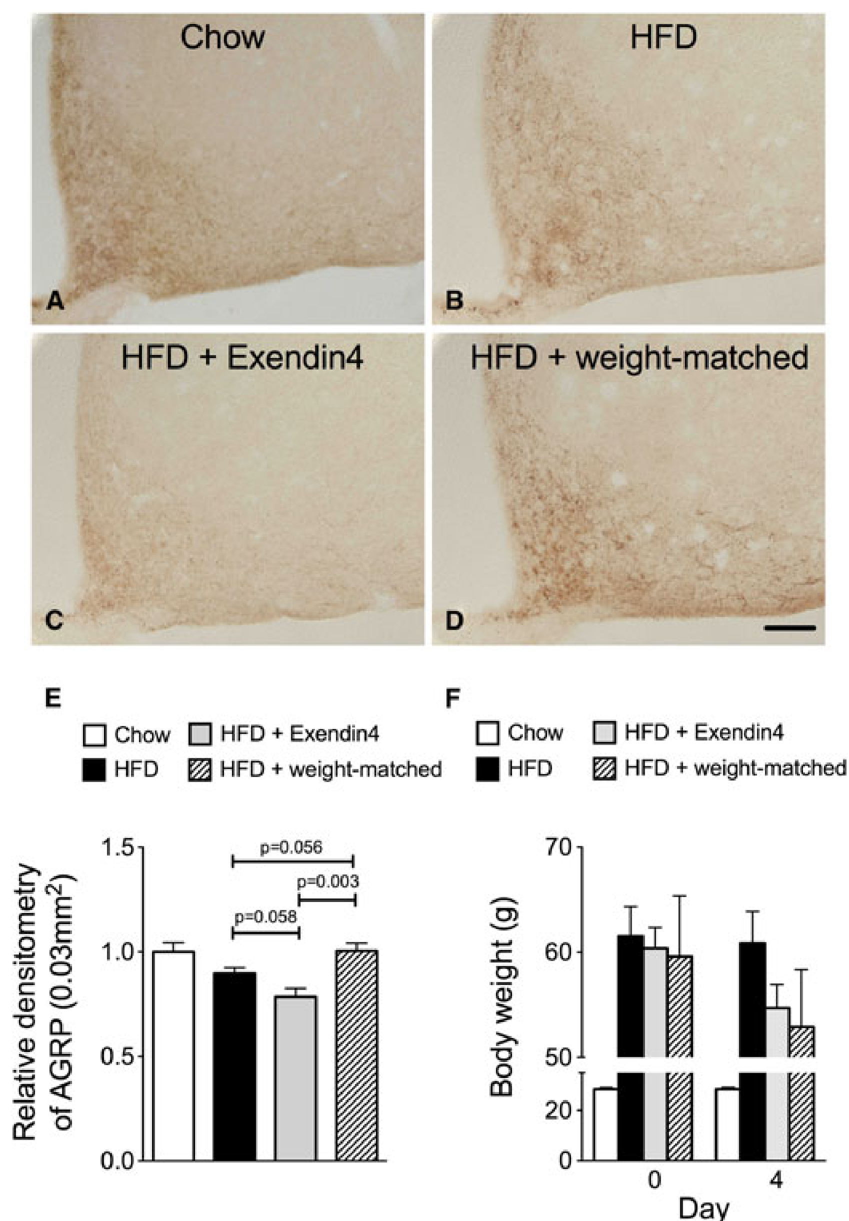

Figure 6. AgRP immunoreactivity in the arcuate nucleus of mice fed for 8 months with either chow or HFD. Mice were treated for 4 days via once daily sc injections of either (A, B, and D) saline or (C) Ex4 (0.5 mg/kg/day). (D) As additional controls, $n = 6$ HFD-fed saline-treated mice were caloric restricted to match the body weight of the Ex4-treated mice ($n = 6$ each group). (E) Densitometry of AgRP immunoreactivity and (F) body weight of mice treated with either saline or Ex4; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Values represent means ± standard error of the mean. Scale bar in panels A–D is 200 μm.

attribute to the differences in the experimental assay conditions. Finally, the protein kinase measurements are one dimension where differences dependent upon PEGylation site were noted, but it should be recognized that systemic concentration may not be consistent with exposure at the CNS receptor sites.

Despite the enhanced pharmaceutical properties of 40K PEG G111, monotherapy still failed to effectively treat diet-induced obesity in mice exposed to HFD. This deepens the established conviction that monotherapy is insufficient to improve leptin responsiveness in common dietary forms of obesity and that success with leptin will depend on an additional agent. Consequently, we directed our attention on the opportunity to evaluate combinatorial therapy with established weight loss-inducing hormones that signal by mechanisms distinct from amylin.

Exendin-4 is a long-acting glucagon-like peptide 1 (GLP-1) receptor agonist, which was originally isolated from the saliva of the Gila monster (*Heloderma suspectum*) [27,28]. Albeit best known for their glucose lowering effects [27,29,30], GLP-1 receptor agonists are well-known to suppress food intake and to increase energy expenditure through centrally regulated mechanisms [31,32]. Although the molecular pathways underlying the effect of GLP-1 on food intake are not yet fully understood, c-FOS expression is increased following GLP-1 receptor activation in the lateral parabrachial nucleus, the nucleus of the solitary tract, the area postrema (AP), and the paraventricular nucleus of the hypothalamus [33,34]. Noteworthy, amylin also increases c-FOS expression in the AP, suggesting a possible shared pathway. However, although lesions of the AP completely

 Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Journal of PeptideScience

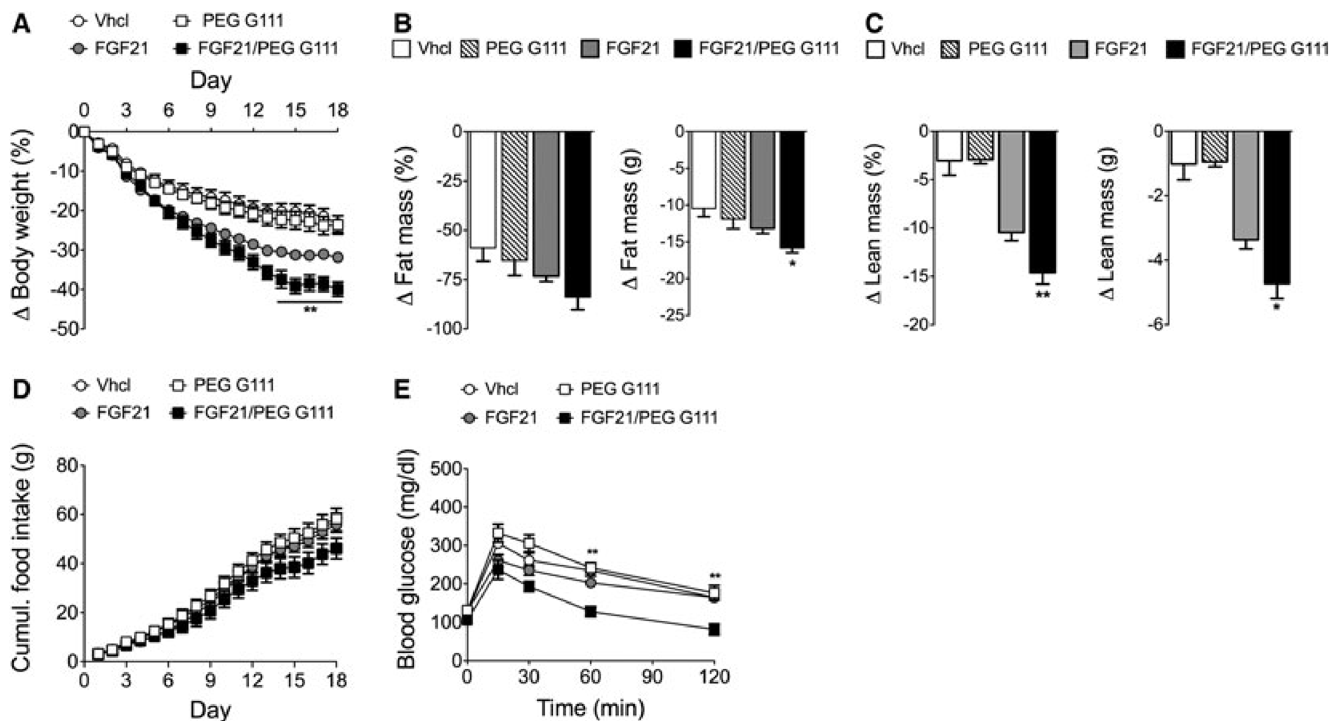

Figure 7. Effects of FGF21 and 40K PEG G111 in obese male C57/BL6 mice switched to regular chow diet at treatment initiation. (A) Body weight, (B) fat mass, (C) lean tissue mass, (D) cumulative food intake, and (E) glucose tolerance of mice treated with vehicle (PBS, $n = 8$), 40K PEG G111 (3 mg/kg/day, $n = 8$), FGF21 (2 mg/kg/day, $n = 8$), or a combination of FGF21 and 40K PEG G111 ($n = 8$). Asterisks indicate significant difference of the FGF21/40K PEG G111 combination therapy from FGF21 monotherapy; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Values represent means ± standard error of the mean.

abolish amylin's effects of food intake [35–39], those of GLP-1 are maintained [40], thus indicating that both hormones modulate food intake through distinct mechanisms.

Like GLP-1, FGF21 is an important modulator of glucose metabolism and energy expenditure [41,42]. FGF21 is member of the endocrine FGF subfamily that is predominantly produced by the liver [43]. FGF21 signaling is mediated via its binding to the FGF receptors upon recruitment of the adaptor molecule, βKlotho [44,45]. This receptor activation results in downstream signaling, leading to activation of ERK1/2 and Akt signaling pathways [46,47]. Study in DIO rat models suggest that, like GLP-1, the effects of FGF21 on energy expenditure and insulin sensitivity are centrally mediated [48]. Furthermore, FGF receptor-1 has been identified in the ventromedial and arcuate nuclei of the hypothalamus [49], areas well-known to regulate peripheral metabolism. Furthermore, these nuclei have been shown to be essential to the metabolic effects of leptin [50], suggesting a possible site of overlap with the FGF21 neurocircuitries. Intriguingly, this increase in energy expenditure and insulin sensitivity is not associated with a decrease in fat mass. Conversely, peripheral infusion of FGF21 has been suggested to increase energy expenditure via AMP-activated protein kinase (AMPK) action in adipose tissue. This study documented an increased activity of AMPK, NAD^+-dependent type III deacetylase sirtuin 1, and peroxisome proliferator-activated receptor-γ coactivator 1α in adipose tissue following FGF21 administration. Furthermore, the effects of FGF21 on energy expenditure were ablated by the deletion of any of the identified signaling nodes. Taken together, it is likely that FGF21 acts on both the CNS and pteripheral tissue to increase energy expenditure and insulin sensitivity.

Ex4 and FGF21 proved highly effective in lowering body weight and improving metabolic control, especially in concert with the change in diet from high fat to normal chow. Importantly, the combined administration of leptin with either of the two other agents proved additively beneficial. FGF21 and Ex4 differed only subtly when used in concert with leptin therapy, and the benefit was not apparent until appreciable body weight had been lost. In both studies, leptin responsiveness was achieved after ~28% weight loss, which seemed to indicate a threshold for the restoration of leptin sensitivity. Noteworthy, switch of DIO mice from HFD to chow increased weight loss in all treatment groups. However, switch from HFD to chow alone did not improve leptin sensitivity in any of our studies, even when a weight loss comparable with pharmacologic treatment with Ex4, was achieved by caloric restriction. It is further noteworthy that we observed no adverse side effects of pharmacologic weight loss in any of our studies. Instead, as also shown in Figures 3, 4, and 6, we observed that glucose tolerance was improved in mice treated with the combination therapy compared with all other groups.

The efficacy of leptin in combination with FGF21 or Ex4 was quite similar to that reported for amylin supplementation, and it is striking that it appeared for both Ex4 and FGF21 after ~28% body weight loss. To assess the mechanism of this additional efficacy derived from leptin, we analyzed the effect of PEG-leptin after ~28% weight loss achieved over a comparable period by either caloric restriction or Ex4. In contrast to mice treated with Ex4, weight loss exclusively induced by caloric restriction did not restore responsiveness to leptin therapy. Our observation that pharmacologic-induced, but not caloric restriction-induced, weight loss restores leptin responsiveness suggests a cooperativity between leptin action and the mechanistic effects of other hormones that is otherwise absent. From a mechanistic point of view, the increased hypothalamic AGRP expression we observed

 Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

with caloric restriction, and not with Ex4 treatment, validates biochemical differences that could reset counter-regulatory circuits that maintain leptin resistance. Interestingly, weight reduction by sleeve gastrectomy has also been reported to be incapable of improving leptin sensitivity in previously obese rats [51].

At present, there is no clear overlap in signaling between leptin, Ex4, FGF21, or amylin to offer a plausible mechanistic hypothesis for the observed pharmacology. However, recent data report an increased expression of the leptin receptor in the liver and white adipose tissue following treatment with FGF21 [42]. Additionally, hindbrain leptin receptors and GLP-1 receptors were recently reported to interact to control food intake in an additive manner [52].

Additional studies are warranted to understand these interactions and to further explore additional combinations that might yield greater therapeutic utility. Given the heterogeneity of human disease, coupled to prior disappointments with leptin and other monotherapies, it is uncertain how well these preclinical observations might translate. Nonetheless, the reported comparative performance of amylin/leptin cotherapy in rodents and humans, as well as the proven clinical efficacy of GLP-1, both provide some basis for future exploratory clinical studies.

In summary, we used rational protein design coupled with novel methods in biosynthesis to discover site-specific leptin analogs that once PEGylated, demonstrate *in vivo* efficacy and duration of action that far exceeds that of native leptin in treatment of mice genetically deficient in leptin. Our results validate the belief that even best in class leptin monotherapy is insufficient to treat common dietary forms of obesity. However, combination therapy with either Ex4 or FGF21 demonstrates that pharmacology approaches are capable of restoring leptin responsiveness in ways that diet restriction alone cannot. It is highly plausible that other agents, including conventional small molecules with proven efficacy, may also perform similarly. These findings corroborate the observations previously made with amylin co-therapy but importantly establish the basis for this being more than a singular route to restore leptin action. For this reason, these observations encourage the broad search of additional combinations with the potential to replicate the efficacy in obesity and adult-onset diabetes currently achieved only by surgical intervention.

Acknowledgements

We would like to thank Mark Shimazu and Joe Tuchscherer for production of the PEG-leptin variants and Allison Given for development of the bridging ELISA for protein kinase analysis.

References

1 Barsh GS, Schwartz MW. Genetic approaches to studying energy balance: perception and integration. *Nat. Rev. Genet.* 2002; **3**: 589–600.
2 Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; **372**: 425–432.
3 Heymsfield SB, Greenberg AS, Fujioka K, Dixon RM, Kushner R, Hunt T, Lubina JA, Patane, J, Self B, Hunt P, McCamish M. Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. *JAMA* 1999; **282**: 1568–1575.
4 Farooqi IS, Matarese G, Lord GM, Keogh JM, Lawrence E, Agwu C, Sanna V, Jebb SA, Perna F, Fontana S, Lechler RI, DePaoli AM, O'Rahilly S. Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. *J. Clin. Invest.* 2002; **110**: 1093–1103.
5 Licinio J, Caglayan S, Ozata M, Yildiz BO, de Miranda PB, O'Kirwan F, Whitby R, Liang L, Cohen P, Bhasin S, Krauss RM, Veldhuis JD, Wagner AJ, DePaoli AM, McCann SM, Wong ML. Phenotypic effects of leptin replacement on morbid obesity, diabetes mellitus, hypogonadism, and behavior in leptin-deficient adults. *Proc. Natl. Acad. Sci. U. S. A.* 2004; **101**: 4531–4536.
6 Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 1995; **269**: 543–546.
7 Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, Friedman JM. Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proc. Natl. Acad. Sci. U. S. A.* 1997; **94**: 8878–8883.
8 Lin S, Thomas TC, Storlien LH, Huang XF. Development of high-fat diet-induced obesity and leptin resistance in C57Bl/6J mice. *Int. J. Obes. Relat. Metab. Disord.* 2000; **24**: 639–646.
9 Van Heek M, Compton DS, France CF, Tedesco RP, Fawzi AB, Graziano MP, Sybertz EJ, Strader CD, Davis HR Jr. Diet-induced obese mice develop peripheral, but not central, resistance to leptin. *J. Clin. Invest.* 1997; **99**: 385–390.
10 Scarpace PJ, Zhang Y. Leptin resistance: a predisposing factor for diet-induced obesity. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2009; **296**: R493–R500.
11 Hukshorn CJ, Saris WH, Westerterp-Plantenga MS, Farid AR, Smith FJ, Campfield LA. Weekly subcutaneous pegylated recombinant native human leptin (PEG-OB) administration in obese men. *J. Clin. Endocrinol. Metab.* 2000; **85**: 4003–4009.
12 Roth JD, Roland BL, Cole RL, Trevaskis JL, Weyer C, Koda JE, Anderson CM, Parkes DG, Baron AD. Leptin responsiveness restored by amylin agonism in diet-induced obesity: evidence from nonclinical and clinical studies. *Proc. Natl. Acad. Sci. U. S. A.* 2008; **105**: 7257–7262.
13 Trevaskis JL, Coffey T, Cole R, Lei C, Wittmer C, Walsh B, Weyer C, Koda J, Baron AD, Parkes DG, Roth JD. Amylin-mediated restoration of leptin responsiveness in diet-induced obesity: magnitude and mechanisms. *Endocrinology* 2008; **149**: 5679–5687.
14 Moon HS, Chamberland JP, Diakopoulos KN, Fiorenza CG, Ziemke F, Schneider B, Mantzoros CS. Leptin and amylin act in an additive manner to activate overlapping signaling pathways in peripheral tissues: *in vitro* and *ex vivo* studies in humans. *Diabetes Care* 2011; **34**: 132–138.
15 Harris JM, Martin NE, Modi M. Pegylation: a novel process for modifying pharmacokinetics. *Clin. Pharmacokinet.* 2001; **40**: 539–551.
16 Zhang F, Basinski MB, Beals JM, Briggs SL, Churgay LM, Clawson DK, DiMarchi RD, Furman TC, Hale JE, Hsiung HM, Schoner BE, Smith DP, Zhang XY, Wery JP, Schevitz RW. Crystal structure of the obese protein leptin-E100. *Nature* 1997; **387**: 206–209.
17 Deiters A, Cropp TA, Mukherji M, Chin JW, Anderson JC, Schultz PG. Adding amino acids with novel reactivity to the genetic code of *Saccharomyces cerevisiae*. *J. Am. Chem. Soc.* 2003; **125**: 11782–11783.
18 Liu DR, Magliery TJ, Pastrnak M, Schultz PG. Engineering a tRNA and aminoacyl-tRNA synthetase for the site-specific incorporation of unnatural amino acids into proteins in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 1997; **94**: 10092–10097.
19 Cho H, Daniel T, Buechler YJ, Litzinger DC, Maio Z, Putnam AM, Kraynov VS, Sim BC, Bussell S, Javahishvili T, Kaphle S, Viramontes G, Ong M, Chu S, Becky GC, Lieu R, Knudsen N, Castiglioni P, Norman TC, Axelrod DW, Hoffman AR, Schultz PG, DiMarchi RD, Kimmel BE. Optimized clinical performance of growth hormone with an expanded genetic code. *Proc. Natl. Acad. Sci. U. S. A.* 2011; **108**: 9060–9065.
20 Reidelberger RD, Haver AC, Apenteng BA, Anders KL, Steenson SM. Effects of exendin-4 alone and with peptide YY(3–36) on food intake and body weight in diet-induced obese rats. *Obesity (Silver Spring)* 2011; **19**: 121–127.
21 Samson SL, Gonzalez EV, Yechoor V, Bajaj M, Oka K, Chan L. Gene therapy for diabetes: metabolic effects of helper-dependent adenoviral exendin 4 expression in a diet-induced obesity mouse model. *Mol. Ther.* 2008; **16**: 1805–1812.
22 Xu G, Stoffers DA, Habener JF, Bonner-Weir S. Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. *Diabetes* 1999; **48**: 2270–2276.
23 Mu J, Pinkstaff J, Li Z, Skidmore L, Li N, Myler H, Dallas-Yang Q, Putnam AM, Yao J, Bussell S, Wu M, Norman TC, Rodriguez CG, Kimmel B,

 Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Metzger JM, Manibusan A, Lee D, Zaller DM, Zhang BB, DiMarchi RD, Berger JP, Axelrod DW. FGF21 analogs of sustained action enabled by orthogonal biosynthesis demonstrate enhanced antidiabetic pharmacology in rodents. *Diabetes* 2012; **61**: 505–512.

24 Wang J, Obici S, Morgan K, Barzilai N, Feng Z, Rossetti L. Overfeeding rapidly induces leptin and insulin resistance. *Diabetes* 2001; **50**: 2786–2791.

25 El-Haschimi K, Pierroz DD, Hileman SM, Bjorbaek C, Flier JS. Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. *J. Clin. Invest.* 2000; **105**: 1827–1832.

26 Frederich RC, Lollmann B, Hamann A, Napolitano-Rosen A, Kahn BB, Lowell BB, Flier JS. Expression of *ob* mRNA and its encoded protein in rodents. Impact of nutrition and obesity. *J. Clin. Invest.* 1995; **96**: 1658–1663.

27 Young AA, Gedulin BR, Bhavsar S, Bodkin N, Jodka C, Hansen B, Denaro M. Glucose-lowering and insulin-sensitizing actions of exendin-4: studies in obese diabetic (*ob*/*ob*, *db*/*db*) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (*Macaca mulatta*). *Diabetes* 1999; **48**: 1026–1034.

28 Greig NH, Holloway HW, De Ore KA, Jani D, Wang Y, Zhou J, Garant MJ, Egan JM. Once daily injection of exendin-4 to diabetic mice achieves long-term beneficial effects on blood glucose concentrations. *Diabetologia* 1999; **42**: 45–50.

29 Goke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, Goke B. Exendin-4 is a high potency agonist and truncated exendin-(9–39)-amide an antagonist at the glucagon-like peptide 1-(7–36)-amide receptor of insulin-secreting beta-cells. *J. Biol. Chem.* 1993; **268**: 19650–19655.

30 Kolterman OG, Buse JB, Fineman MS, Gaines E, Heintz S, Bicsak TA, Taylor K, Kim D, Aisporna M, Wang Y, Baron AD. Synthetic exendin-4 (exenatide) significantly reduces postprandial and fasting plasma glucose in subjects with type 2 diabetes. *J. Clin. Endocrinol. Metab.* 2003; **88**: 3082–3089.

31 Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR. A role for glucagon-like peptide-1 in the central regulation of feeding. *Nature* 1996; **379**: 69–72.

32 Tang-Christensen M, Larsen PJ, Goke R, Fink-Jensen A, Jessop DS, Moller M, Sheikh SP. Central administration of GLP-1-(7–36) amide inhibits food and water intake in rats. *Am. J. Physiol.* 1996; **271**: R848–R856.

33 Baggio LL, Kim JG, Drucker DJ. Chronic exposure to GLP-1R agonists promotes homologous GLP-1 receptor desensitization *in vitro* but does not attenuate GLP-1R-dependent glucose homeostasis in vivo. *Diabetes* 2004; **53** Suppl 3: S205–S214.

34 Rowland NE, Crews EC, Gentry RM. Comparison of Fos induced in rat brain by GLP-1 and amylin. *Regul. Pept.* 1997; **71**: 171–174.

35 Becskei C, Grabler V, Edwards GL, Riediger T, Lutz TA. Lesion of the lateral parabrachial nucleus attenuates the anorectic effect of peripheral amylin and CCK. *Brain Res.* 2007; **1162**: 76–84.

36 Riediger T, Zuend D, Becskei C, Lutz TA. The anorectic hormone amylin contributes to feeding-related changes of neuronal activity in key structures of the gut-brain axis. *Am. J. Physiol.* 2004; **286**: R114–R122.

37 Mack CM, Soares CJ, Wilson JK, Athanacio JR, Turek VF, Trevaskis JL, Roth JD, Smith PA, Gedulin B, Jodka CM, Roland BL, Adams SH, Lwin A, Herich J, Laugero KD, Vu C, Pittner R, Paterniti JR Jr, Hanley M, Ghosh S, Parkes DG. Davalintide (AC2307), a novel amylin-mimetic peptide: enhanced pharmacological properties over native amylin to reduce food intake and body weight. *Int. J. Obes. (Lond)* 2010; **34**: 385–395.

38 Rowland NE, Richmond RM. Area postrema and the anorectic actions of dexfenfluramine and amylin. *Brain Res.* 1999; **820**: 86–91.

39 Lutz TA, Senn M, Althaus J, Del Prete E, Ehrensperger F, Scharrer E. Lesion of the area postrema/nucleus of the solitary tract (AP/NTS) attenuates the anorectic effects of amylin and calcitonin gene-related peptide (CGRP) in rats. *Peptides* 1998; **19**: 309–317.

40 Baraboi ED, Smith P, Ferguson AV, Richard D. Lesions of area postrema and subfornical organ alter exendin-4-induced brain activation without preventing the hypophagic effect of the GLP-1 receptor agonist. *Am. J. Physiol.* 2010; **298**: R1098–R1110.

41 Badman MK, Koester A, Flier JS, Kharitonenkov A, Maratos-Flier E. Fibroblast growth factor 21-deficient mice demonstrate impaired adaptation to ketosis. *Endocrinology* 2009; **150**: 4931–4940.

42 Coskun T, Bina HA, Schneider MA, Dunbar JD, Hu CC, Chen Y, Moller DE, Kharitonenkov A. Fibroblast growth factor 21 corrects obesity in mice. *Endocrinology* 2008; **149**: 6018–6027.

43 Badman MK, Pissios P, Kennedy AR, Koukos G, Flier JS, Maratos-Flier E. Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell Metab.* 2007; **5**: 426–437.

44 Kurosu H, Choi M, Ogawa Y, Dickson AS, Goetz R, Eliseenkova AV, Mohammadi M, Rosenblatt KP, Kliewer SA, Kuro-o M. Tissue-specific expression of betaKlotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activity of FGF19 and FGF21. *J. Biol. Chem.* 2007; **282**: 6687–6695.

45 Ogawa Y, Kurosu H, Yamamoto M, Nandi A, Rosenblatt KP, Goetz R, Eliseenkova AV, Mohammadi M, Kuro-o M. BetaKlotho is required for metabolic activity of fibroblast growth factor 21. *Proc. Natl. Acad. Sci. U. S. A.* 2007; **104**: 7432–7437.

46 Izumiya Y, Bina HA, Ouchi N, Akasaki Y, Kharitonenkov A, Walsh K. FGF21 is an Akt-regulated myokine. *FEBS Lett.* 2008; **582**: 3805–3810.

47 Kharitonenkov A, Shiyanova TL, Koester A, Ford AM, Micanovic R, Galbreath EJ, Sandusky GE, Hammond LJ, Moyers JS, Owens RA, Gromada J, Brozinick JT, Hawkins ED, Wroblewski VJ, Li DS, Mehrbod F, Jaskunas SR, Shanafelt AB. FGF-21 as a novel metabolic regulator. *J. Clin. Invest.* 2005; **115**: 1627–1635.

48 Sarruf DA, Thaler JP, Morton GJ, German J, Fischer JD, Ogimoto K, Schwartz MW. Fibroblast growth factor 21 action in the brain increases energy expenditure and insulin sensitivity in obese rats. *Diabetes* 2010; **59**: 1817–1824.

49 Matsuo A, Tooyama I, Isobe S, Oomura Y, Akiguchi I, Hanai K, Kimura J, Kimura H. Immunohistochemical localization in the rat brain of an epitope corresponding to the fibroblast growth factor receptor-1. *Neuroscience* 1994; **60**: 49–66.

50 Coppari R, Ichinose M, Lee CE, Pullen AE, Kenny CD, McGovern RA, Tang V, Liu SM, Ludwig T, Chua SC Jr, Lowell BB, Elmquist JK. The hypothalamic arcuate nucleus: a key site for mediating leptin's effects on glucose homeostasis and locomotor activity. *Cell Metab.* 2005; **1**: 63–72.

51 Stefater MA, Perez-Tilve D, Chambers AP, Wilson-Perez HE, Sandoval DA, Berger J, Toure M, Tschop M, Woods SC, Seeley RJ. Sleeve gastrectomy induces loss of weight and fat mass in obese rats, but does not affect leptin sensitivity. *Gastroenterology* 2010; **138**: 2426–2436.

52 Zhao S, Kanoski SE, Yan J, Grill HJ, Hayes MR. Hindbrain leptin and glucagon-like-peptide-1 receptor signaling interact to suppress food intake in an additive manner. *Int. J. Obes. (Lond)* 2012; DOI: 10.1038/ijo.2011.265.

 Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.