GLP-1/glucagon co-agonism restores leptin responsiveness in obese mice chronically maintained on an obesogenic diet

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Running tittle: GLP-1/glucagon restores leptin responsiveness in DIO mice

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ABSTRACT

We recently reported restoration of leptin responsiveness in diet-induced obese (DIO) mice using a pharmacologically-optimized, PEGylated (PEG) leptin analog in combination with exendin-4 or FGF21. However, return of leptin action required discontinuation of high-fat diet (HFD) exposure. Here we assess whether a single peptide possessing balanced co-agonism at the GLP-1 and glucagon receptors can restore leptin responsiveness in DIO mice maintained on HFD. DIO mice were treated with PEG-GLP-1/glucagon (30 nmol/kg every fourth day) to induce a ~15% body weight loss, once upon they were randomized to continue PEG-GLP-1/glucagon therapy or reassigned to receive supplemental daily PEG-leptin (185 nmol/kg/day). The addition of PEG-leptin to PEG-GLP-1/glucagon resulted in an ~18% greater weight loss as compared to PEG-GLP-1/glucagon alone, and was accompanied by further decreases in food intake and improved glucose and lipid metabolism. The beneficial effect of PEG-leptin supplementation occurred after an initial body weight loss similar to what we previously reported following reduced dietary fat along with PEG-leptin and exendin-4 or FGF21 co-treatment. In summary, we report that GLP-1/glucagon co-agonism restores leptin responsiveness in mice maintained on a HFD, thus emphasizing the translational value this polypharmacotherapy for the treatment of obesity and diabetes.

INTRODUCTION

Pharmacological approaches to combat obesity are hampered by limited efficacy or considerable adverse effects. Therefore the development of safe and efficient pharmacotherapies is an increasing global priority. The adipocyte hormone leptin plays a pivotal role in energy metabolism due to its ability to decrease body weight by inhibiting food intake and increasing energy expenditure. Since its discovery (1), leptin has been extensively studied for its potential in weight management. However, whereas leptin replacement promotes weight loss in congenitally leptin deficient obese rodents (2; 3) and humans (4-6), its potential as a stand-alone therapy under conditions of diet-induced obesity is limited due to leptin resistance (7; 8). However, leptin responsiveness can be restored using pharmacology (9-11) and suggests that leptin administration may hold promise as a valuable adjunct in novel combinational pharmacotherapies (12; 13). Accordingly, co-administration of leptin with the pancreatic peptide amylin restores leptin responsiveness and synergistically decreases body weight in DIO prone rats and in calorie-restricted obese humans (9). Similar results were observed in DIO mice by co-administration of PEG-leptin with exendin-4 or FGF21 (10). In both studies, adjunctive administration of the respective peptides with leptin synergistically improved weight loss relative to treatment with the peptides alone. However, both studies required a moderately low content of dietary lipids to observe a return of leptin responsiveness. These data align with a growing number of reports indicating that dietary macronutrients, especially fat and sugar, are detrimental to leptin sensitivity even before the onset of obesity or hyperleptinemia (14-19). Restoration of leptin responsiveness in the context of a chronic obesogenic environment has yet to be demonstrated. This challenge has led us to explore the potential of GLP-1/glucagon co-agonism,

which normalizes adiposity without the necessity of dietary change (20), as a complement to leptin-based therapy.

Our results show that PEG-GLP-1/glucagon restores leptin responsiveness and, when combined with PEG-leptin, synergistically improves body weight loss in DIO mice continuously exposed to a HFD. The regain of leptin action occurred after an initial weigh loss that is similar to previous reports (10) following reduced dietary fat. The return of leptin responsiveness was reflected by enhanced weight loss induced by adjunctive PEG-leptin treatment, which was mediated by decreased food intake and accompanied by improved glucose and lipid metabolism.

RESEARCH DESIGN AND METHODS

PEG-leptin and PEG-GLP-1/glucagon co-agonist. The synthesis, purification and characterization of PEG-leptin and PEG-GLP-1/glucagon were described previously (10; 20), and both compounds were used without any further chemical modification or change in formulation.

Animals and diet. Six to eight week-old male C57BL/6 mice were maintained at 23 ± 1°C, constant humidity and a 12h light-dark cycle. Mice had free access to water and were fed *ad libitum* with a HFD comprising 58% of calories from fat (D12331; Research Diets, New Brunswick, NJ). All procedures were approved by the Animal Use and Care Committee of Bavaria, Germany.

In vivo evaluation of PEG-GLP-1/glucagon and PEG-leptin co-treatment. Mice were treated via subcutaneous (sc) injection (5µl/g body weight) as indicated. Body composition was analyzed using a magnetic resonance whole-body composition analyzer (EchoMRI, Houston, TX, USA). For glucose tolerance, levels of blood glucose were sampled from 6h fasted mice following intraperitoneal (ip) administration of 1.5 g glucose per kg body weight.

Biochemical analysis. For tissue collection, mice were fasted for 4h and treated with the compounds 2h prior to sample collection. Plasma levels of insulin (Crystal Chem, Inc., Dowers Grove, IL, USA), cholesterol (Wako Chemicals, Neuss, Germany), leptin (ALPCO Diagnostics, Salem, NH, USA) and adiponectin (Millipore, Schwalbach, Germany) were measured using commercially available kits according to the manufacturer's instructions. Levels of liver triglycerides were assessed as previously described (21).

Gene expression analysis. Gene expression was profiled with quantitative real time RT-PCR-based techniques using TaqMan single probes (n = 6-7 mice per group), or TaqMan low density arrays (n = 4-6 mice per group) (Applied Biosystems, Germany). The relative expression of the selected genes was normalized to the housekeeping gene *Hprt* or *Rpl27*.

Statistical analysis. Differences between treatment groups were assessed by 1-way or 2-way ANOVA followed by post-hoc comparison or Student's 2-tailed unpaired *t*-test. All results are given as mean ± SEM. $P<0.05$ was considered statistically significant.

RESULTS

GLP-1/glucagon co-agonism restores leptin responsiveness during chronic HFD exposure. Male 24 week-old DIO mice (52.69 ± 0.68g) were treated for 9 days with the PEG-GLP-1/glucagon co-agonist (30nmol/kg/ every fourth day) or vehicle control. Similar to previous reports (20), treatment with PEG-GLP-1/glucagon resulted in decreased body weight relative to mice treated with vehicle control (-14.46 ± 1.26% vs. -2.07 ± 0.72%, $p<0.001$; Fig. 1*A*). The decreased body weight was the result of a decrease in body fat and lean tissue mass, and was accompanied by lower food intake and improved glucose tolerance relative to mice treated with vehicle control (all $p<0.001$; Fig. 1B-E).

At study day 9, mice treated with the co-agonist were continued on either PEG-GLP-1/glucagon or assigned to receive additional daily PEG-leptin (Fig. 2). As negative controls, mice that were initially treated with vehicle were re-randomized to receive either daily vehicle or PEG-leptin (185nmol/kg/day) alone. Daily adjunctive administration of PEG-leptin and PEG-GLP-1/glucagon led to a significantly greater reduction in body weight as compared to mice treated with PEG-GLP-1/glucagon alone (Δ body weight at study day 33 relative to day 0: -44.05 ± 2.83% (-23.68 ± 2.17g) vs. -26.49 ± 4.93% (-13.93 ± 2.67g); $p<0.05$; Fig. 2*A*). The enhanced weight loss in mice treated with PEG-GLP-1/glucagon and PEG-leptin was associated with decreased body fat mass, reduced food intake, and improved glucose tolerance and insulin sensitivity relative to mice maintained on PEG-GLP-1/glucagon alone (all $p<0.05$; Figure 2B-F). Together, these data indicate that GLP-1/glucagon co-agonism restores leptin responsiveness under persistent HFD exposure. Of note, the beneficial effect of PEG-leptin occurred after an initial body weight loss of ~15% relative to day 0 (Fig. 2*A*). The return of leptin responsiveness at this degree of weight loss is similar to what we previously reported in DIO mice treated with

the combination of PEG-leptin and exendin-4, or FGF21 and following switch to conventional chow diet (10).

Consistent with the observation that leptin decreases body weight and food intake when added to the PEG-GLP-1/glucagon co-agonist, we also found improved measures of glucose and lipid metabolism in these mice. Mice treated with PEG-GLP-1/glucagon alone, as compared to vehicle-treated controls, showed lower levels of liver triglycerides and decreased plasma levels cholesterol and endogenous leptin, whereas plasma levels of insulin and adiponectin were unchanged (Suppl. Fig. 1A-E). Except for adiponectin, all of these biochemical measures, including insulin, were improved by the addition of PEG-leptin (Suppl. Fig. 1A-E). The reduced cholesterol levels were also accompanied by a trend for decreased apolipoprotein B48 (Suppl. Fig. 1F), a profound decrease in leptin mRNA levels in the epidymal white adipose tissue (eWAT), and a decrease in hypothalamic expression of the leptin receptor (Suppl. Fig. 1G-H). No differences in leptin or its receptor mRNA levels were observed in mice treated with PEG-GLP-1/glucagon alone. Similarly, no difference in any mRNA measurements were observed in mice pre-treated with vehicle and subsequently continued on PEG-leptin monotherapy, which is consistent with the lack of improvements in body weight or hormonal status in these mice. Notably, plasma levels of endogenous leptin, as well as mRNA levels of leptin and its receptor, showed a strong correlation with both body weight and body fat mass (Suppl. Fig. 2A-F), suggesting that the decrease in body fat mass accounts for the observed changes of leptin and its receptor.

Metabolic effects of leptin monotherapy after terminating adjunctive therapy with GLP-1/glucagon. To assess whether PEG-leptin sustains the improved metabolic benefits after adjunctive GLP-1/glucagon therapy, a subset of mice co-treated with PEG-GLP-1/glucagon and

PEG-leptin were continued on PEG-leptin alone starting on study day 21. At day 33, mice continued on PEG-leptin alone showed no statistical difference in body weight relative to mice treated with the combination therapy but showed a trend of increased weight gain (Fig. 3A). However, mice that were treated with PEG-leptin alone showed an increased lean tissue mass ($p<0.01$) without any difference in body fat mass or food intake as compared to mice continued on the combination therapy (Fig. 3B-D). Mice randomized to PEG-leptin monotherapy were unable to sustain the improved glucose tolerance and insulin sensitivity observed by adjunctive administration with PEG-GLP-1/glucagon (Fig. 3E,F), corroborating the profound effect of the co-agonist on glucose metabolism. Despite no overall changes in body weight, fat mass and food intake, levels of liver triglycerides, and plasma levels of insulin, cholesterol and endogenous leptin all increased in mice continued on PEG-leptin monotherapy relative to mice continued on the combination therapy (Suppl. Fig. 1A-D). Collectively, these data suggest that leptin monotherapy is incapable of sustaining the improved metabolic state once GLP-1/glucagon is removed. Our observations, however, warrant further studies over a longer time period as a means to increase success in translation to clinical studies. Of specific interest is the change in body composition during leptin monotherapy following combination therapy.

DISCUSSION

Leptin resistance impairs the efficacy of leptin pharmacology when administered as stand-alone therapy. Treatment with amylin, exendin-4, or FGF21 was shown to restore leptin responsiveness and when combined with leptin synergistically lowers body weight in obese rodents (9; 10). These observations encourage further consideration of leptin-based poly-pharmacy. A central limitation of these studies, however, is that return of leptin action required a moderately low content of dietary lipids. The observation that co-administration of PEG-leptin with exendin-4 or FGF21 failed to restore leptin responsiveness when mice were maintained on a HFD (10) correlates with recent reports demonstrating that leptin resistance occurs immediately following HFD exposure, and prior to an increase in adiposity (14; 18; 19; 22; 23). These findings collectively demonstrate that HFD is a detrimental factor contributing to the development of leptin resistance and underscore the importance of addressing the obesogenic environment to unleash the beneficial aspects inherent to leptin therapy.

PEG-GLP-1/glucagon co-agonism reverses obesity and the associated metabolic syndrome in rodents maintained on HFD (20). We report here that treatment with the same PEG-GLP-1/glucagon co-agonist restores leptin responsiveness in mice chronically exposed to a HFD. Co-administration of PEG-GLP-1/glucagon and PEG-leptin resulted in ~18% greater reduction in body weight as compared to PEG-GLP-1/glucagon alone, whereas leptin monotherapy without prior PEG-GLP-1/glucagon treatment had no effect on body weight. The beneficial effect of co-therapy with PEG-leptin was associated with decreased food intake, improved glucose tolerance and insulin sensitivity, decreased liver triglycerides and lower plasma levels of insulin and cholesterol. The pronounced weight loss observed by PEG-GLP/glucagon and PEG-leptin co-therapy was associated with reduction of endogenous, circulating leptin that was paralleled by a

substantial decrease in leptin mRNA levels in eWAT and hypothalamic leptin receptor expression. The alteration in leptin homeostasis mirrors the marked decrease in adipose tissue as mRNA levels of leptin and its receptor strongly correlate with the amount of body fat mass. Hypothalamic expression of *Npy* and *Agrp* were increased in these mice as compared to vehicle controls, potentially reflecting the negative energy balance caused by the combination therapy.

Of particular importance are the observations made following the discontinuation of combination therapy. In the group randomized to PEG-leptin monotherapy following co-treatment with PEG-GLP-1/glucagon, we observed a trend for regaining body weight that was associated with an increase in lean but not fat tissue mass. Despite no change in body weight or fat mass, these mice showed increased plasma levels of leptin, insulin, cholesterol, and liver triglycerides as well as a reduction in hypothalamic expression of *Npy* and *Agrp* following removal of PEG-GLP-1/glucagon. These biochemical measures indicate that leptin monotherapy following successful co-therapy with PEG-GLP-1/glucagon is failing to maintain the beneficial effects on glucose and lipid metabolism in the sustained obesogenic environment.

In summary, a series of evidence indicates that the abundance of dietary fat and sugar contribute to the development of leptin resistance and constitute a major impediment to successful treatment of obesity. Consequently, our results are of sizable, potential therapeutic importance as GLP-1/glucagon co-agonism restores leptin responsiveness in obese mice without dietary change, a dietary set-up where exendin-4 and FGF21 failed to improve leptin responsiveness. Ideally, lifestyle modifications should be employed as central tools when it comes to lowering excessive weight and preventing the progression of obesity. Nonetheless, the epidemic nature of the disease and its heterogeneity requires diverse and complementary approaches. Identification of a means to unlock the beneficial effects that endogenously reside in leptin but are suppressed

in conventional forms of obesity remains one of the more promising approaches to successfully address the obesity epidemic. These results advance the prospect that leptin pharmacology in combination with other medicinal agents, most notable GLP-1/glucagon co-agonists, might be effective in a clinical setting associated with less intensive lifestyle modification, rendering it more suitable to a broader population requiring therapy.

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Author contributions

CC performed the experiments, evaluated the data and drafted the manuscript. BF, KF, DK, LS and TDM helped with the in vivo experiments, evaluated the data and helped draft the manuscript. PTP and SS helped with the in vivo experiments and revised the manuscript critically. TO and SH performed the lipid profiling and revised the manuscript critically. LS, JC, JP and RD made substantial contributions in compound development, evaluated the data and helped draft the manuscript. JP, LS, MHT, RD and TDM further made substantial contributions in the study design and interpretation of data and helped edit the manuscript.

FIGURE LEGENDS

Figure 1: Effect of PEG-GLP-1/glucagon monotherapy on energy and glucose metabolism in DIO mice. Percent body weight (**A**), change of body composition (**B,C**), cumulative food intake (**D**) and glucose tolerance (**E**) of DIO mice treated chronically every 4 days with either vehicle (PBS; N=14) or PEG-GLP-1/glucagon (30nmol/kg/4days; N=21). Injections were given s.c. at study days 0, 4, and 8. Glucose tolerance (i.p.; 1.5 g glucose/kg body weight) was measured at study day 8. Changes in body composition reflect changes from study day 0 to 8. Asterisks indicate * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data represent means ± SEM.

Figure 2: Effect of adjunctive treatment of DIO mice with PEG-GLP-1/glucagon and PEG-leptin following initial body weight loss induced by PEG-GLP-1/glucagon monotherapy. Percent body weight (**A**), change of body composition (**B,C**), cumulative food intake (**D**), glucose tolerance (**E**) and HOMA-IR (**F**) of DIO mice treated chronically (s.c.) from day 9 to 33 with either Vehicle (N=7), PEG-leptin (3mg/kg/day; N=7), PEG-GLP-1/glucagon (30nmol/kg/4days; N=6) or the combination of PEG-GLP-1/glucagon and PEG-leptin (N=15). Injections of vehicle and PEG-leptin were given daily, injections of PEG-GLP-1/glucagon was given at study days 12, 16, 20, 24, 28, and 32. Changes in body composition (**B,C**) reflect changes from day 0 to 32. Glucose tolerance (i.p.; 1.5 g glucose/kg body weight) was assessed at study day 32. Dashed line at study day 9 indicates start of the PEG-leptin – PEG-GLP-1/glucagon combination-therapy. Asterisks indicate * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data represent means ± SEM.

Figure 3: Effect of PEG-leptin monotherapy following pre-treatment with PEG-GLP-1/glucagon and PEG-leptin. Percent body weight (**A**), change of body composition (**B,C**), cumulative food intake (**D**), glucose tolerance (**E**), and HOMA-IR (**F**) of DIO mice treated chronically (s.c.) from day 9 - 21 with PEG-GLP-1/glucagon and PEG-leptin and which have then been continued on either the combination of PEG-leptin and PEG-GLP-1/glucagon (N=6) or PEG-leptin alone (3mg/kg/day; N=7). Injections of PEG-leptin were given daily, injections of PEG-GLP-1/glucagon was given at study days 24, 28, and 32. Percent changes in body weight (**A**) reflect changes from day 0 to 33. Changes in body composition (**B,C**) reflect changes from day 20 to 32. Glucose tolerance (i.p.; 1.5 g glucose/kg body weight) was assessed at study day 32. Asterisks indicate * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data represent means ± SEM.

Suppl. Figure 1: Liver triglycerides, plasma analysis and gene expression profiling in eWAT and hypothalamus of mice treated with GLP-1/glucagon and PEG-leptin. Liver triglyceride levels (**A**), plasma analysis of insulin (**B**), cholesterol (**C**), leptin (**D**), adiponectin (**E**), and apolipoprotein B48 (ApoB48) (**F**), as well as mRNA levels of leptin in eWAT (**G**) and hypothalamic expression of the leptin receptor (**H**), AgRP (**I**), Npy (**J**), Pomc (**K**), Socs3 (**L**) and N=6-7 mice each group. Asterisks indicate * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data represent means ± SEM.

Suppl. Figure 2: Correlation analysis of plasma leptin, and mRNA levels of leptin and the leptin receptor with body weight and body fat mass. Correlation of plasma leptin, and expression of leptin and lepr with body weight **(A-C)** and body fat mass **(D-F).**

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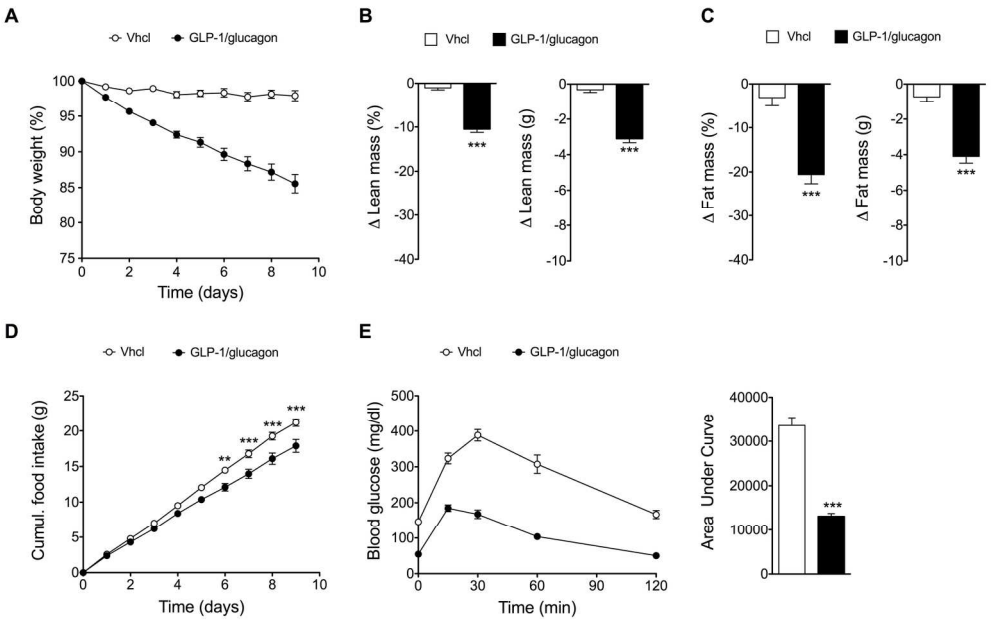

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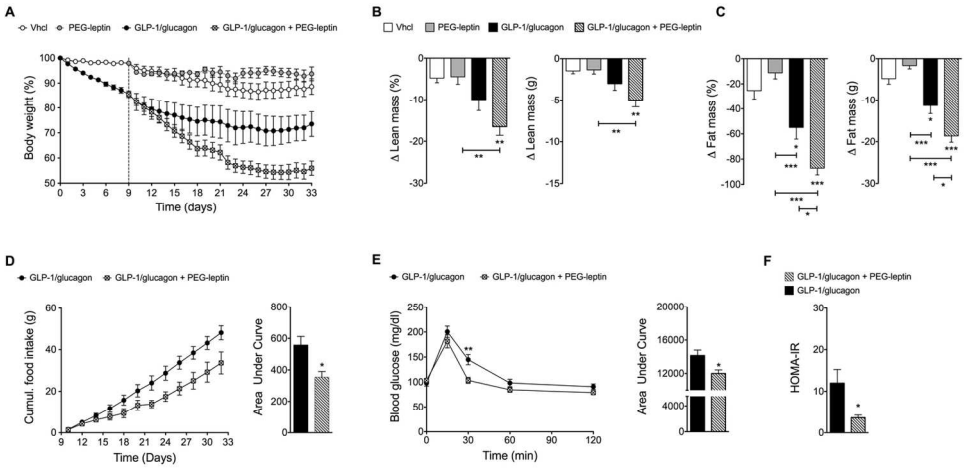

Figure 2: Effect of adjunctive treatment of DIO mice with PEG-GLP-1/glucagon and PEG-leptin following initial body weight loss induced by PEG-GLP-1/glucagon monotherapy. Percent body weight (A), change of body composition (B,C), cumulative food intake (D), glucose tolerance (E) and HOMA-IR (F) of DIO mice treated chronically (s.c.) from day 9 to 33 with either Vehicle (N=7), PEG-leptin (3mg/kg/day; N=7), PEG-GLP-1/glucagon (30nmol/kg/4days; N=6) or the combination of PEG-GLP-1/glucagon and PEG-leptin (N=15). Injections of vehicle and PEG-leptin were given daily, injections of PEG-GLP-1/glucagon was given at study days 12, 16, 20, 24, 28, and 32. Changes in body composition (B,C) reflect changes from day 0 to 32. Glucose tolerance (i.p.; 1.5 g glucose/kg body weight) was assessed at study day 32. Dashed line at study day 9 indicates start of the PEG-leptin – PEG-GLP-1/glucagon combination-therapy. Asterisks indicate * p<0.05; ** p<0.01; *** p<0.001. Data represent means ± SEM.
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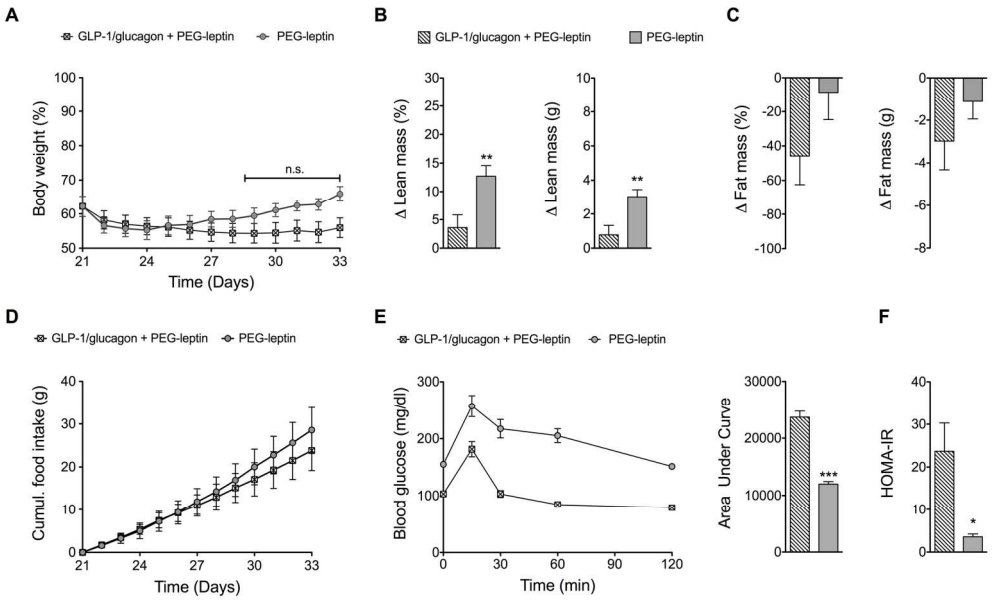

Figure 3: Effect of PEG-leptin monotherapy following pre-treatment with PEG-GLP-1/glucagon and PEG-leptin. Percent body weight (A), change of body composition (B,C), cumulative food intake (D), glucose tolerance (E), and HOMA-IR (F) of DIO mice treated chronically (s.c.) from day 9 - 21 with PEG-GLP-1/glucagon and PEG-leptin and which have then been continued on either the combination of PEG-leptin and PEG-GLP-1/glucagon (N=6) or PEG-leptin alone (3mg/kg/day; N=7). Injections of PEG-leptin were given daily, injections of PEG-GLP-1/glucagon was given at study days 24, 28, and 32. Percent changes in body weight (A) reflect changes from day 0 to 33. Changes in body composition (B,C) reflect changes from day 20 to 32. Glucose tolerance (i.p.; 1.5 g glucose/kg body weight) was assessed at study day 32. Asterisks indicate * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data represent means ± SEM.
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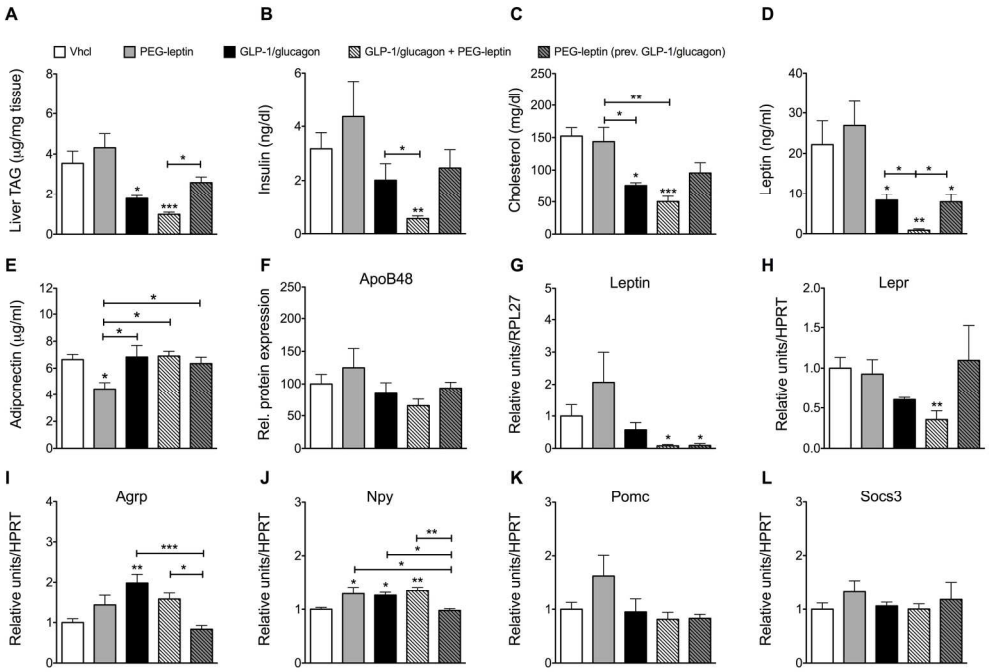
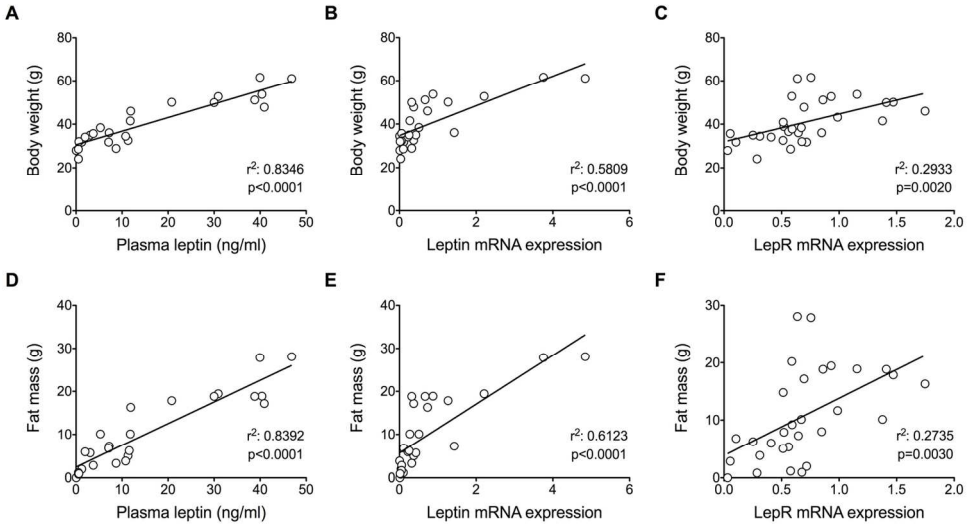


Suppl. Figure 1: Liver triglycerides, plasma analysis and gene expression profiling in eWAT and hypothalamus of mice treated with GLP-1/glucagon and PEG-leptin. Liver triglyceride levels (A), plasma analysis of insulin (B), cholesterol (C), leptin (D), adiponectin (E), and apolipoprotein B48 (ApoB48) (F), as well as mRNA levels of leptin in eWAT (G) and hypothalamic expression of the leptin receptor (H), AgRP (I), Npy (J), Pomc (K), Socs3 (L) and N=6-7 mice each group. Asterisks indicate * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data represent means ± SEM.
186x128mm (300 x 300 DPI)



Suppl. Figure 2: Correlation analysis of plasma leptin, and mRNA levels of leptin and the leptin receptor with body weight and body fat mass. Correlation of plasma leptin, and expression of leptin and lepr with body weight (A-C) and body fat mass (D-F).
146x78mm (300 x 300 DPI)