Ejarque Miriam (Orcid ID: 0000-0003-0545-9817)

Sabadell-Basallote Joan (Orcid ID: 0000-0003-3608-1837)

Adipose tissue is a key organ for the beneficial effects of GLP-2 on glucose metabolism

Miriam Ejarque[a,b], Joan Sabadell-Basallote[a,b], Daniel Beiroa[c,d], Enrique Calvo[a,b], Noelia Keiran[a,b], Catalina Nuñez-Roa[a,b], Mª del Mar Rodríguez[a,b], Fatima Sabench[a,e,f], Daniel del Castillo[a,e,f], Veronica Jimenez[b,g], Fatima Bosch[b,g], Ruben Nogueiras[c,d], Joan Vendrell[a,b,e,#], Sonia Fernández-Veledo[a,b,#]

[a] Unitat de Recerca. Hospital Universitari de Tarragona Joan XXIII. Institut d´Investigació Sanitària Pere Virgili. Tarragona, Spain

[b] CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, Madrid, Spain

[c] Department of Physiology, CIMUS, University of Santiago de Compostela-Instituto de Investigación Sanitaria, Santiago de Compostela, Spain

[d] CIBER Fisiopatología de la Obesidad y Nutrición (CIBERobn), Instituto de Salud Carlos III, Madrid, Spain.

[e] Facultat de Medicina i Ciències de la Salut de Reus, Universitat Rovira Virgili, Spain

[f] Surgery Service, Hospital Sant Joan de Reus. Reus, Spain

[g] Center of Animal Biotechnology and Gene Therapy and Department of Biochemistry and Molecular Biology, School of Veterinary Medicine, Universitat Autònoma de Barcelona, Bellaterra,

#co-corresponding and senior authors: J Vendrell or S Fernández-Veledo, Endocrinology,

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Pere Virgili, Research Unit, University Hospital of Tarragona Joan XXIII, c/o Dr Mallafré Guasch, 4, Tarragona 43007, Spain. Tel: +34 977 29 58 00 Ext 3401; Fax: +34 977 29 58 23; E-mail: jvo@comt.es or sonia.fernandezveledo@gmail.com

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Running title: Glucagon-like peptide-2 ameliorates obesity-induced adipose tissue inflammation

ABSTRACT

Background and purpose: Glucagon-like peptide-2 (GLP-2) is a gastrointestinal hormone released in response to nutritional intake that promotes a wide range of effects by activating the GLP-2 receptor. In addition to its intestinotrophic effects, GLP-2 has been shown to positively influence glucose metabolism under conditions of obesity, but the mechanisms behind this remain unclear. Here we investigate the molecular role of the GLP-2/GLP-2R axis in energetic metabolism, focusing on its potential modulatory effects on adipose tissue (AT). **Experimental approach:** Physiological measurements (body weight, food intake, locomotor activity and energy expenditure) and metabolic studies (glucose and insulin tolerance tests) were performed on lean and obese mice treated with the protease-resistant GLP-2 analog teduglutide. **Key Results:** Acute but not chronic centrally administered teduglutide decreased food intake and weight-gain. By contrast, chronic activation of peripheral GLP-2R increased body weight-independent glucose tolerance and had anti-inflammatory effects on visceral AT. Using a gene silencing approach, we found that AT is

necessary for the beneficial effects of teduglutide. Finally, teduglutide regulates the inflammatory state and acts as an anabolic signal in human adipocytes. **Conclusion and Implications:** Overall, our data identify AT as a new clinical target for GLP-2 activity in obesity.

Abbreviations

AT- Adipose tissue

WAT- White adipose tissue

SAT- Subcutaneous adipose tissue

VAT- Visceral adipose tissue

CD- Chow diet

HFD- High fat diet

AAV- Adeno-associated viral vector

ICV- Intracerebroventricular

IP- Intraperitoneal

IV-Intravenous

DIO- Diet-induced obesity

What is already known

-GLP-2 has been reported to act as a protective factor against glucose dysregulation in the setting of obesity

What does this study add

-Adipose tissue underlies the beneficial effects of systemic GLP-2 on glucose metabolism

-GLP-2 exerts anti-inflammatory effects in visceral adipose tissue

-GLP-2 is a pleiotropic hormone with anti-inflammatory effects and behaves as an anabolic factor

What is the clinical significance

-Understanding the role of GLP-2 in the metabolic events in adipose tissue might help to define new clinical indications for GLP-2 agonists in obesity

INTRODUCTION

Obesity is a global public health concern and a major risk factor for several diseases, including type 2 diabetes, cardiovascular disease and some forms of cancer. The worldwide increase in obesity and associated comorbidities points to an urgent need for new pharmacological approaches for the treatment of these conditions. Obesity develops as a result of altered energy homeostasis, leading to maladaptive adipose tissue (AT) expansion and metabolic dysregulation. AT not only plays a crucial role in fuel storage and maintenance of energy balance, but it is also a major endocrine organ that secretes multiple adipokines involved in metabolism and inflammation at local and systemic levels (Vegiopoulos, Rohm, & Herzig, 2017).

Glucagon-like peptide-2 (GLP-2) is a peptide hormone produced by enteroendocrine L-cells of the intestine, and is co-secreted with the incretin hormone GLP-1 in response to nutrient ingestion. Both peptides are post-translational products of the proglucagon precursor protein, which is processed by prohormone convertase-1/3 (Rowland & Brubaker, 2011). GLP-2 functions peripherally to slow gastric emptying, enhance digestion and absorption, and regulate intestinal hexose transport (Drucker, 2001; Guan et al., 2012). Interestingly, GLP-2 has been demonstrated to also have anti-inflammatory and trophic effects in the gastrointestinal tract in pre-clinical models, and this has been confirmed in several clinical trials for the treatment of short bowel syndrome and Crohn's disease (Buchman et al., 2010; Jeppesen et al., 2011; Jeppesen et al., 2012). In its native form, GLP-2 has a short circulating

half-life (7 minutes in humans) as a result of dipeptidylpeptidase-IV (DPP-IV)-mediated proteolysis and renal clearance (Tavares, Drucker, & Brubaker, 2000). Thus, for its effective therapeutic use several modifications have been made to address proteolysis; for example, teduglutide ([Gly^2]-GLP-2) is a modified analog of GLP-2 resistant to DPP-IV and has a prolonged half-life and improved efficacy in the context of pharmacological delivery (Tavares et al., 2000).

The integrative responses to GLP-2 are mediated by the GLP-2 receptor (GLP-2R), a G protein-coupled receptor (GPCR) superfamily member closely related to the glucagon receptor (Munroe et al., 1999). The general view that GLP-2R expression is restricted mainly to the intestinal tract and the central nervous system (CNS) (Lovshin, Estall, Yusta, Brown, & Drucker, 2001; Yusta et al., 2000) has been recently challenged by several observations of its expression in various other tissues, including liver and mesenteric, gonadal and epicardial white adipose tissue (WAT) (El-Jamal et al., 2014; Iacobellis, Camarena, Sant, & Wang, 2017; Yusta et al., 2019). Interestingly, GLP-2R signaling in proopiomelanocortin (POMC) neurons is essential for suppressing feeding behavior, and for gastrointestinal motility and hepatic glucose production in mice (Guan, 2014), suggesting that GLP-2R in the CNS has a physiological role in the control of food intake and glucose homeostasis.

Although GLP-2 seems to be dispensable for glucose homeostasis regulation under normal conditions, peripherally administered GLP-2 has been shown to positively influence glucose metabolism in the setting of obesity (Baldassano, Amato, Caldara, & Mule, 2016; Baldassano, Rappa, Amato, Cappello, & Mule, 2015; Cani et al., 2009). In the present study, we sought to examine the central and peripheral effects of GLP-2 under normal diet conditions and during overnutrition, and to address whether the protective effects of teduglutide on glucose metabolism specifically involved its action on adipose tissue.

METHODS

Mice

Wild-type male C57BL/6 mice were purchased from Charles River Laboratories (Barcelona, Spain). Mice were housed under conditions of controlled temperature (23°C) and illumination (12-h light/dark cycle) with *ad libitum* access to water and standard chow (SAFE diets, A04: 8.3-kcal% fat; 19.3% protein; 72.4% carbohydrates; 3.3 kcal/g) or high-fat diet (HFD) (Research diets, D12451: 45-kcal% fat; 20% protein; 35% carbohydrates; 4.7 kcal/g) (starting at 8 weeks of age). Animal studies adhered to the institutional animal care committee guidelines, and all procedures were reviewed and approved by the Ethics Committee of the University of Santiago de Compostela and Universitat Rovira i Virgili, in accordance with European Union normative for the use of experimental animals. At the end of the experiments, mice (not food deprived) were killed by cervical dislocation, tissues were removed rapidly, frozen immediately on dry ice, and kept at -80 °C until analysis. Blood samples were also collected for biochemical analyses. The authors declare that this work followed BJP guidelines and animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010) and BJP recommendations.

Animals were assigned to different experimental groups of equal size in a blinded and random fashion according to BJP guidelines. Animals that did not respond to anesthesia or in whom the cannula was not attached correctly during the entire experiment were not used for further study.

Sample size calculations for the long-term experiments were based on the food intake differences observed in our pilot study (short-term teduglutide treatments) between drug and vehicle group, at a power of 0.8 and a level of significance α=0.05. A sample size of at least 6 per group was calculated using Ene 3.0 software (Autonomous University of Barcelona, Spain).

Physiological measurements

Whole-body composition was measured using NMR imaging (Whole Body Composition Analyzer; EchoMRI, Houston, TX). Whole-body metabolic analysis was performed in a custom 12-cage indirect calorimetry, food intake, and locomotor activity monitoring system (TSE LabMaster, TSE Systems, Bad Homburg, Germany), as described (Czyzyk et al., 2012; Folgueira C, 2019; Nogueiras et al., 2009). Mice were acclimatized to the test chambers for 48 h and were monitored for an additional 48 h. Data collected from the last 48 h was used to calculate all parameters for which results are reported. Heat production was visualized using a high-resolution infrared camera (FLIR Systems Inc., Boston, MA), as described (Czyzyk et al., 2012). Insulin sensitivity and plasma glucose tolerance tests were performed in mice deprived of food for 4 and 6 h, respectively. Glucose (2 g kg^{-1}) and insulin (0.75 IU kg^{-1}) were injected intraperitoneally (i.p.) and blood glucose was determined at the indicated time points. Blood samples were collected from the tail vein and glucose was measured with a glucometer (Arkray, Kyoto, Japan).

Treatments

Intracerebroventricular: Mice were anesthetized with a mixture of ketamine (15 mg kg^{-1}) and xylazine (3 mg kg^{-1}) and intracerebroventricular (ICV) cannulae were implanted stereotaxically (David Kopf Instruments, Tujunga, CA) into the lateral ventricle (Imbernon et

al., 2013). The stereotaxic coordinates were -0.6 mm posterior to bregma, ±1.2 mm lateral from midline, and -2.0 mm ventral from the brain surface. After surgery, the animals were singly-housed and given at least 4 days to recover. **Single-injection:** Lean mice (10 weeks of age) were infused with 2 µl of either vehicle (saline) or teduglutide (2.5, 5, or 10 µg per mouse) (Creative Peptides, Shirley, NY) for 1 day. Food intake was recorded at 2, 4, 6, 8, 12 and 24 h after the single injection and body weight was recorded at 24 h. **Chronic treatment:** Lean (10 weeks of age) or obese (20 weeks of age) mice were infused with 2 µl of either vehicle (saline) or teduglutide (10 µg per mouse) for 14 consecutive days using osmotic pumps (ALZET Osmotic Pumps, DURECT Corp., Cupertino, CA) that deliver the compound at a continuous and controlled rate, as described (Quinones et al., 2018). Food intake and body weight were recorded daily.

Intraperitoneal: After 12 weeks of HFD, a subgroup of mice was injected i.p. once daily (9:00 am) with 100 µl teduglutide (4 µg per mouse) or GLP-2 (3–33) (60 ng per mouse) (CASLO Laboratory ApS, Lyngby, Denmark) or liraglutide (50 µg kg^{-1}) (Peprotech, London, UK) or PBS (vehicle control) for 2 weeks.

Recombinant AAV vectors

Single-stranded adeno-associated viral (AAV) vectors were produced as reported (Jimenez et al., 2013). The transgene included the short version of the adipocyte protein 2 (mini/aP2) promoter with the addition of four tandem repeats of the mirGLP-2R sequence (5' CCCATTCTGCTTTGTGTAA 3') cloned into the 3' untranslated region of the expression cassette (termed AAV8-mini/aP2-miRGLP2R) or an miRNA negative control (AAV8-mini/aP2-miRNegative).

Administration of AAV vectors

Obese mice (10 weeks on HFD) were anesthetized with ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) for intraepididymal WAT delivery by a laparotomy procedure. AAV vectors were resuspended in PBS with 0.001% Pluronic® F68 (Invitrogen Gibco, Carlsbad, CA). Mice received two injections of 50 µl. The abdomen was rinsed with sterile saline solution and closed with a two-layer suture. After allowing two weeks for recovery (12 weeks on HFD), the intraperitoneal treatment was performed as described above.

Histology and immunohistochemistry

WAT and intestine samples were fixed in 10% formalin buffer for 24 h and then dehydrated and embedded in paraffin by a standard procedure. Sections (3 µm) were prepared with a microtome and stained using a standard hematoxylin-eosin (Bio-Optica, Milan, Italy) procedure. Other paraffin sections were used for immunohistochemistry detection of F4/80, as described (Keiran et al., 2019), using a rabbit anti-F4/80 antibody (1:1000, #MCA497R; RRID: AB_323279; BioRad, Hercules, CA). Two tissue sections were selected from each mouse, and 20 random non-overlapping fields at 10× magnification were imaged. Crown-like structures of F4/80 staining were analyzed using ImageJ software (RRID: SCR_003070; NIH).

Enzyme-linked immunosorbent assay (ELISA)

Cytokine analysis in serum samples was performed using a mouse inflammatory cytokine multi-analyte ELISArray Kit (Qiagen, Hilden, Germany, #MEM-004A), and evaluated using a Varioskan™ LUX multimode plate reader (Thermo Fisher Scientific, Waltham, MA).

qPCR

RNA of collected tissues and cells was extracted using Trizol® Reagent (Invitrogen) and was treated with RNase-free DNase I to reduce the risk of genomic DNA contamination. RNA quantification was performed by the photometric measurement device μDrop plate (Thermo Fisher Scientific). A total of 2 μg of RNA was transcribed to complementary DNA with random primers using dNTP Mix (100 mM), MultiScribe Reverse Transcriptase (50 U/μL) and RNase Inhibitor using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carslbad, CA) as described (Ejarque et al., 2018). Thermal Cycler Conditions were: 25°C 10 minutes, 37°C 120 minutes, 85°C 5 minutes according to the manufacturer's protocol. Real-time PCR (qPCR) was conducted on a 7900HT Fast Real-Time PCR System using TaqMan Gene Expression Assays hydrolysis probes (Applied Biosystems) (Supplementary Table S1). Each qPCR reaction was performed in duplicate, and the analysis of the results was performed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) and expressed relative to the expression of the reference gene (beta-2 microglobulin [B2M] for mouse studies and 18s ribosomal RNA [18s] for human studies). Taqman Fast Advanced Master Mix (Applied Biosystems) was used and the protocol used in 7900HT Fast Real-Time PCR System was as follows: 50ºC 2 min, 95ºC 10 min and 95ºC 15 sec, 60ºC 1 min x 40 cycles. Taqman assays qPCR guarantee program (Applied Biosystems) certifies all information necessary to ensure MIQE compliance guidelines.

Western blot analysis

The immune-related procedures used comply with the recommendations made by the BJP (Alexander et al., 2018) and was performed as described previously (Keiran et al., 2019). Tissue samples were lysed and homogenized in RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich, St louis, MO), electrotransferred on a polyvinylidene difluoride membrane, and probed with an anti-GLP-2R (sc-99092; Santa Cruz Biotechnology Inc.,

Santa Cruz, CA), or an anti-Actin (loading control; #A1978; Sigma-Aldrich). Bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Dako Denmark, Glostrup, Denmark) and chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific).

Human adipose tissue samples

Subjects were recruited by the Endocrinology and Surgery departments at the University Hospital Joan XXIII (Tarragona, Spain). The hospital ethics committee approved the study and informed consent for biobanking surgically removed tissue was obtained from all participants in accordance with the Declaration of Helsinki. All patients had fasted overnight before adipose tissue collection. Adipose tissue samples were obtained during scheduled non-acute surgical procedures including laparoscopic surgery for hiatus hernia repair or cholecystectomies. Donors were classified as lean or obese based on body mass index (BMI) following World Health Organization criteria: lean subjects included 6 males and 1 female, age (mean±sem) 50.9±3.9 years, BMI 21.8±0.5, fasting blood glucose 4.08±0.22 mmol l^{-1}; obese subjects included 4 females and 3 males, age 58.8±2.0 years, BMI 31.6±0.3, glucose 5.54±0.17 (for mRNA expression).

Ex vivo VAT explants: Samples were washed in PBS, diced into small pieces (100 mg) and incubated in DMEM/F12 medium containing 10% fetal bovine serum and 1% antibiotic/antimycotic (penicillin, streptomycin and fungizone) at 37ºC in a humidified incubator (21% O_2 and 5% CO_2) for 24 h in the presence or absence of teduglutide (10 nM). The following day the adipose tissue samples were collected and stored at -80ºC for RNA analysis. Donors for adipose tissue included 1 female and 5 males; age (mean±sem) 50.8±2.2 years, BMI 31±1.2, glucose 5.4±0.28 mmol l^{-1}.

In vitro cell culture: Human adipose-derived stem cells (hASCs) were isolated from the subcutaneous AT (SAT) of lean patients and differentiated to mature adipocytes following a published protocol (Pachon-Pena et al., 2016). Donors for SAT included 5 females; age (mean±sem) 45±3.2 years, BMI 22.3±0.42, glucose 5.13±0.3 mmol l^{-1}. The cells were stored in a tissue biobank registered at the National Register of Biobanks (registration number #C.0003609). After differentiation, cells were treated for 24 h in the presence or absence of teduglutide (10 nM) prior to analysis.

Lipid staining

Accumulation of lipid droplets was visualized by staining cells with Oil Red O (Sigma-Aldrich). Cells were fixed in 4% paraformaldehyde for 15 min and stained for 30 min. After staining, dishes were washed and photographed with a Zeiss Primovert inverted microscope (Zeiss, Jena, Germany).

Glucose uptake

Cells were pretreated or not with teduglutide (10 nM) for 16 h and were then incubated in Krebs-Ringer bicarbonate buffer with or without insulin (100 nM) for 40 min at 37ºC; 2-NBDG (2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose) (100 μM) (Invitrogen) was added for the last 20 min. After incubation, cells were washed twice with cold Krebs Ringer Phosphate buffer and lysed in RIPA buffer. Fluorescence intensity was measured at 466/550 nm on a Varioskan™ LUX multimode plate reader (Thermo Fisher Scientific). Values were normalized to the corresponding protein content and expressed relative to control without insulin treatment, which was set as 1.

Data and statistical analysis

The data and statistical analysis comply with the recommendations of the BJP on experimental design and analysis in pharmacology (Curtis et al., 2018). Data are given as mean ± standard error (SEM) and were tested for normality using the Shapiro-Wilk test; outliers were included in the data analyses. To determine differences in means between two groups Students's *t*-test was conducted, and two-way ANOVA was performed when more than two groups were compared. Bonferroni post hoc test was performed only if the F-value in analysis of variance achieved the necessary level of statistical significance ($P<0.05$), and there was no significant variance in homogeneity. A significance level of 0.05 was accepted for all tests. mRNA data were expressed in relation to controls (vehicle-treated normalization set at 1). All statistical analyses were performed using GraphPad Prism software version 6.00 (RRID: SCR_002798; GraphPad Prism Inc., La Jolla, CA).

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

RESULTS

Centrally administered GLP-2 has only transient effects on body weight regulation but impairs glucose tolerance in DIO mice

GLP-2 has been claimed to exert anorectic effects in rodents when centrally administered by ICV injection into the lateral ventricle of the brain (Guan et al., 2012; Lovshin et al., 2001;

Tang-Christensen, Larsen, Thulesen, Romer, & Vrang, 2000), a procedure that bypasses the blood-brain barrier. Based on previous dose-response studies (Dalvi & Belsham, 2012; Guan et al., 2012), chow diet-fed mice were administered a single ICV injection of teduglutide or vehicle, and cumulative food intake and body weight were examined over 24 h in an exploratory study to establish a dose for chronic studies. In agreement with earlier studies (Guan et al., 2012; Tang-Christensen et al., 2000), ICV administration of the GLP-2R agonist resulted in a short-term decrease in both food intake and weight gain (supplementary Figure S1). Using an independent cohort of mice on a chow diet, we next performed a chronic teduglutide ICV infusion study over two weeks using osmotic mini-pumps to deliver a continuous and controlled dose (10 μg per day). Under these conditions, teduglutide failed to have an impact on energy metabolism in terms of body weight change, cumulative food intake, glucose tolerance, energy expenditure, locomotor activity and respiratory quotient, an indicator of fuel selection (Figure 1).

We next addressed whether the chronic central infusion of teduglutide exerts beneficial effects under conditions of overnutrition. To do this, we used a mouse model of diet-induced obesity (DIO) generated by 12 weeks feeding on HFD. After this period, mice were treated with teduglutide (ICV, 10 μg per day) or saline over two weeks using osmotic pumps, as before. Additionally, and in order to validate the specificity of the GLP-2-mediated effects, some mice also received a daily i.p. injection of GLP-2 (3–33), a GLP-2R antagonist (60 ng, once daily). No significant changes were observed in body weight (Figure 2A) or food intake (Figure 2B) between mice treated with saline, teduglutide, GLP-2 (3-33) or the coadministration of teduglutide plus GLP-2 (3-33). However, at the end of the 2-week period, the teduglutide-only treated group seemed to show a slower clearance of glucose in an i.p. glucose tolerance test (Figure 2C), albeit with no significant differences in the area under the curve, suggesting a potential deleterious effect of the GLP-2 analog at the central level on

glucose metabolism. This appeared to be dependent on GLP-2R activation since the simultaneous treatment with teduglutide and GLP-2 (3–33) cancelled this effect. No other significant outcomes were observed for energy expenditure (Figure 2D) locomotor activity (Figure 2E) or respiratory quotient (Figure 2F). Overall, our data show that chronic centrally-administered teduglutide has no beneficial effects on ingestion and glucose metabolism.

Beneficial effects of peripheral GLP-2 activation on glucose tolerance are associated with direct effects on adipose tissue

Given these results and the fact that drugs are usually administered peripherally in a clinical setting, we next evaluated the potential role of GLP-2 administered at the systemic and tissue level. As a positive control for these studies, we used the GLP-1R agonist liraglutide because of its known effects on body weight and glucose homeostasis (Decara et al., 2016). After 12 weeks on HFD, mice received a daily i.p. injection of teduglutide (4 μg) (Baldassano et al., 2016) or liraglutide (50 μg kg^{-1}) during 2 weeks. Liraglutide improved the metabolic phenotype of obese mice by inducing weight loss (Figure 3A), without disrupting food intake (Figure 3B), decreasing fat mass (Figure 3C), improving glucose tolerance (Figure 3D), enhancing insulin sensitivity (Figure 3E), and decreasing the respiratory quotient (Figure 3F), indicating an increase in fat oxidation. By contrast, teduglutide failed to change body weight or fat mass; however, it did significantly improve glucose tolerance and insulin sensitivity (Figure 3D,E). Of note, basal glucose levels were unaffected by long- or short-term treatment with teduglutide (data not shown).

We next focused our attention on WAT as a hypothetical target tissue underlying the beneficial effects of GLP-2 on glucose metabolism in DIO mice, as previously suggested (Amato, Baldassano, & Mule, 2016). Of note, compared with its expression in WAT, *Glpr2* mRNA was barely detectable in brown adipose tissue (supplementary Figure S2). We looked

for putative macroscopic changes in subcutaneous (SAT) and visceral AT (VAT) in DIO mice after teduglutide treatment by hematoxylin and eosin staining. Figure 4A illustrates the enhanced mucosal growth observed in the intestine of teduglutide-treated animals, which served as a positive control of our model (Drucker, Erlich, Asa, & Brubaker, 1996). We found no apparent macroscopic changes in the SAT of DIO mice treated i.p. with saline or with teduglutide over 14 days; however, there was an evident decrease in the density of crown-like structures in the VAT of teduglutide-treated animal (Figure 4A), consisting of dead adipocytes surrounded by macrophages, suggesting that teduglutide has a modifying role in the inflammatory status of this depot. In fact, F4/80 staining of macrophages in VAT sections revealed a lower infiltratory pattern in teduglutide-treated mice than in control mice (Figure 4B). In agreement with the depot-specific changes in the response to the GLP-2R agonist, *Glp2r* expression was significantly higher in VAT than in SAT of mice fed chow diet or HFD (Figure 4C). Teduglutide treatment did not affect the expression of *Glp2r* in WAT or in brown adipose tissue (supplementary Figure S2). A detailed analysis of inflammatory and anti-inflammatory gene expression in isolated SAT and VAT samples of the teduglutide and saline groups showed that the expression of the common anti-inflammatory markers *Il10, Arg1* and *Il4ra* was higher in VAT of teduglutide-treated mice than in equivalent tissue of control mice (Figure 4D). This is in line with the differences in the histology of VAT and suggests that teduglutide has an anti-inflammatory effect in this tissue. Of note, the putative anti-inflammatory effect of teduglutide was also reflected at the systemic level. ELISA analysis of pro- and anti-inflammatory markers in serum from the two groups of mice showed that teduglutide-treated mice had significantly lower levels of the pro-inflammatory cytokine IL-1α and significantly higher levels of the anti-inflammatory cytokines IL-10 and IL-4 (Figure 4E). Likewise, an increase in the levels of IL-6, which can act both as pro- and anti-inflammatory agent (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011), was detected in

teduglutide-treated mice (Figure 4E) in accord with the gene expression results (Figure 4D). By contrast, the up-regulation of *Tnfa* at the mRNA level (Figure 4D) was not reflected at the systemic level (Figure 4E).

Given the energy absorption function ascribed to GLP-2 (Drucker & Yusta, 2014), we also examined the expression of a panel of glucose and lipid metabolism-related genes in the AT of teduglutide- or saline-treated mice. We observed that teduglutide treatment resulted in higher levels of some typical lipid metabolism genes in SAT, such as hormone sensitive lipase (*Lipe*) and adipose triglyceride lipase (*Atgl*) (Figure 4F), whereas the levels of fatty acid synthase (*Fasn*) and peroxisome proliferator-activated receptor gamma (*Pparg*) were lower in VAT (Figure 4F). Because liver and gut are also important players in blood glucose homeostasis, we examined the expression of inflammatory and metabolic markers in both organs, finding only a lower expression of the glucose transporters *Glut1 and Glut2* in the gut of teduglutide-treated mice (supplementary Figure S3). These latter transcriptional changes likely do not explain the metabolic improvement observed in teduglutide-treated mice. Overall, these data suggest that GLP-2 might have an anti-inflammatory effect in VAT.

As the beneficial effects of peripheral GLP-2R activation could be related to its influence on improving intestinal barrier integrity and function, we used an AAV vector system to express an miRNA against *Glp2r* under the control of the fat-specific *aP2* promoter, to specifically target GLP-2R in AT. We used this approach to determine whether the GLP-2 analog work *via* AT or *via* the gastrointestinal tract. The systemic (i.p. or i.v.) injection of AAV-miRNA was, however, unable to successfully silence *Glp2r* specifically in WAT (data not shown). By contrast, the intra-epididymal injection of the AAV-miRNA construct into the fat pad of DIO mice resulted in long-term, efficient gene transfer to VAT, achieving ~50% knockdown of *Glp2r* expression as compared with the AAV-miRNA negative control (Figure 5A,B). GLP-2R protein expression could not be assessed due to the

impossibility of finding a commercially available selective antibody (Michel, Wieland, & Tsujimoto, 2009) (antibody validation example in supplementary Figure S4). Obese mice were AAV-injected and after a period of recovery, received a two weeks of intraperitoneal tedugludite treatment. We then repeated the gene expression analysis and found that the anti-inflammatory effects of teduglutide on VAT were abolished in *Glp2r*-inhibited VAT, as measured by its failure to upregulate the anti-inflammatory markers *Il10, Arg1 and Fizz1* (Figure 5C). Of note, the enhanced glucose tolerance found in mice treated with teduglutide was completely blocked after the knockdown of *Glp2r* in VAT (Figure 5D), pointing to the importance of VAT GLP-2R in the beneficial effects of the GLP-2 analog in DIO mice.

Effect of GLP-2 effect in human adipose tissue

Finally, we wanted to determine whether the effects of teduglutide GLP-2R engagement in murine AT were recapitulated in human AT in the setting of obesity. Remarkably, we found that GLP-2R is also expressed in human AT (Figure 6A, compare the duodenum positive control to the SAT and VAT depots). Analysis of the heterogeneous stroma-vascular fraction (SVF) revealed that *GLP2R* was expressed mainly in precursors (adipose-derived stem cells [ASCs]) and not in macrophages (Figure 6A). Similar to what we observed in mice, *GLP2R* expression was higher in human VAT than in SAT (Figure 6A,B). Of note, *GLP2R* expression was significantly higher in the SAT of obese subjects than in equivalent lean tissue (Figure 6B), which was different from that observed in the mouse DIO model, where no changes were found (Figure 4C). In contrast to its mitogenic effects in intestine (Jeppesen et al., 2005), *in vitro* analysis revealed that teduglutide had no effect on adipocyte proliferation (supplementary Figure S5). However, and consistent with the function of GLP-2 in energy assimilation (Drucker & Yusta, 2014), teduglutide treatment stimulated lipogenesis in human subcutaneous adipocytes, measured as a significant increase in Oil red O staining

(Figure 6C), and an up-regulation of lipogenic genes (Figure 6D). Interestingly, teduglutide treatment significantly increased basal glucose (but not insulin-stimulated) incorporation into human subcutaneous adipocytes (Figure 6E), suggesting that GLP-2 acts as an anabolic hormone that stimulates glucose and fatty acid uptake into adipose cells. To investigate whether GLP-2R engagement elicited anti-inflammatory effects in human AT, we treated human VAT explants from obese patients overnight with teduglutide and studied the expression of pro-and anti-inflammatory genes. Of note, compared with control-treated explants, teduglutide-treated tissue showed a significant down-regulation of the pro-inflammatory cytokine *IL1b* and a significant up-regulation of the anti-inflammatory genes *ADIPOQ* and *ARG1* (Figure 6F). Overall, these results suggest that GLP-2 also acts as an anti-inflammatory agent in human VAT, and point to its contribution as a protective factor against glucose dysregulation in human obesity.

DISCUSSION

GLP-2 is a proglucagon-derived intestinotrophic hormone released from epithelial intestinal cells in response to dietary nutrients. Notably, GLP-2 has attracted growing interest due to its putative role as a satiety signal (Baldassano, Bellanca, Serio, & Mule, 2012; Guan et al., 2012; Tang-Christensen et al., 2000; Tang-Christensen, Vrang, & Larsen, 2001), and some studies indicate that GLP-2 may inhibit feeding behavior with potential long-term effects on body weight homeostasis. The present study demonstrates that the anorexigenic effect previously described for central GLP-2R activation is lost in the chronic setting. Conversely, its peripheral activation in an obesity context improves glucose tolerance by a mechanism independent of weight loss, as the result of an anti-inflammatory effect in VAT depots.

Accumulating evidence points to GLP-2 as a mediator of energy balance through the gut-brain axis. Acute ICV infusion of GLP-2 increases glucose tolerance and insulin sensitivity and suppresses basal hepatic glucose production mediated by GLP-2R activation in POMC neurons (Shi et al., 2013). Furthermore, mice lacking GLP-2R in POMC neurons develop hyperphagia, food-seeking activity and late-onset obesity (Guan et al., 2012), and acute central GLP-2 administration reduces food intake in rodents (Baldassano et al., 2012; Guan et al., 2012; Tang-Christensen et al., 2000; Tang-Christensen et al., 2001). These findings have led to the view that GLP-2 has a physiological role in the regulation of food intake and body weight (Guan et al., 2012). In the present study, we found that this effect was only transitory, and chronic modulation of central GLP-2R failed to reduce food intake in both standard diet and HFD conditions. By contrast, chronic activation of peripheral GLP-2R provided body weight-independent glucose tolerance and improved insulin sensitivity in DIO mice, as reported previously (Baldassano et al., 2016). Remarkably, we found that this effect was dependent on GLP-2R expression in VAT.

Beyond its enterotrophic role, the mechanisms underlying GLP-2-mediated metabolic actions are unclear (Amato et al., 2016). Here we describe that GLP-2 has a cell-dependent protective role in AT in obesity. We show that the beneficial effects of peripheral teduglutide on glucose homeostasis *in vivo* are dependent on its anti-inflammatory effect on AT, more specifically in visceral depots. Remarkably, we demonstrate that this anti-inflammatory effect on murine VAT (this study and Baldassano et al., 2020), is also detected in human AT explants from subjects with obesity.

Our results suggest that the GLP-2 agonist, by engaging GLP-2R, acts as a protective factor against adipocyte dysfunction in response to overnutrition, a mechanism that might underlie its beneficial impact on glucose metabolism. A similar anti-inflammatory effect has been described in models of enteritis and colitis *via* enteric neural pathways (Drucker, Yusta,

Boushey, DeForest, & Brubaker, 1999; Sigalet et al., 2007) and in the mouse brain (Nuzzo et al., 2019). GLP-2 has also been shown to suppress pro-inflammatory enzymes and cytokines in lipopolysaccharide-primed macrophages by inhibiting NF-kB activity and ERK phosphorylation (Xie et al., 2014). In this line, teduglutide could be acting in a similar manner to the adipocyte-secreted cytokine adiponectin, which exerts anti-inflammatory actions on a number of different cell types (Ouchi & Walsh, 2007). Of note, the beneficial effects of GLP-2 might also be related to its ability to reduce gut permeability and inflammation; however, our gene silencing studies suggest that AT, and not the intestine, is the tissue target involved in the beneficial outcomes of GLP-2.

In the human setting, our results in AT are in agreement with a role for GLP-2 in energy absorption previously reported within the gastrointestinal tract (Amato et al., 2016). In fact, GLP-2 increases the expression of lipogenic genes such as *PPARG* and *FASN*, and glucose and lipid uptake, and so it is not inconceivable that adipocytes contribute to plasma glucose clearance. Additionally, the improvement in glucose tolerance by the anabolic GLP-2 action on adipocytes would also be aided by its modulation of AT inflammation. While the physiological significance of GLP-2 secretion after a meal remains largely unknown, our results are in line with the perception of GLP-2 as a promoter of energy absorption within the gastrointestinal tract (increasing sugar uptake and lipid absorption). Thus, it is possible that GLP-2 acts as a lipogenic factor in AT by regulating energy homeostasis in postprandial states.

Overall, our study identifies adipose tissue as a direct target for GLP-2. Our data suggest that teduglutide regulates the inflammatory state of AT under conditions of overnutrition and also acts as a metabolic signal to improve glucose regulation in adipocytes *via* GLP-2R signaling, particularly in VAT depots. A better understanding of the metabolic

role of GLP-2 in adipose tissue may help to define new clinical indications for teduglutide in obesity.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

ME, SFV, JV and RB conceived and designed the experiments. ME, JSB, DB, EC, NK, CÑR, MMR, DC and FS performed the experiments. ME, SVF, DB and RN analysed the data. VJ and FB designed and created the AAV system. ME and SFV wrote the manuscript.

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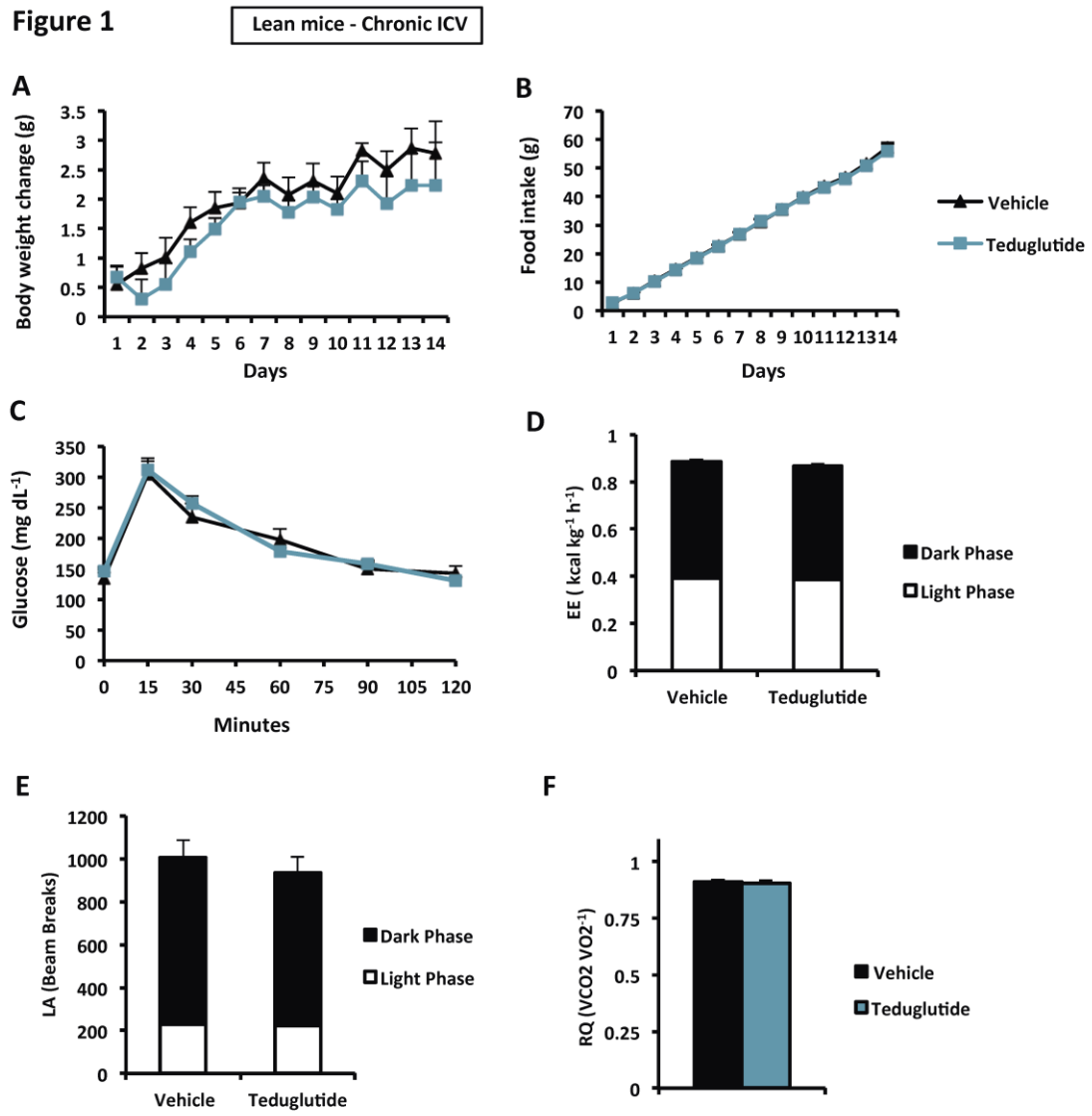

Figure 1. Chronic modulation of central GLP-2R does not affect energy metabolism. Effects of 14-day intracerebroventricular (ICV) infusion of the GLP-2R agonist teduglutide (10 μg/mouse) or saline in lean mice on **(A)** Body weight change, **(B)** Cumulative food intake, **(C)** Intraperitoneal glucose tolerance test, **(D)** Energy expenditure (EE), body mass-corrected, **(E)** Locomotor activity (LA), body mass-corrected, and **(F)** Respiratory quotient (RQ). n=8 saline; n=10 teduglutide.

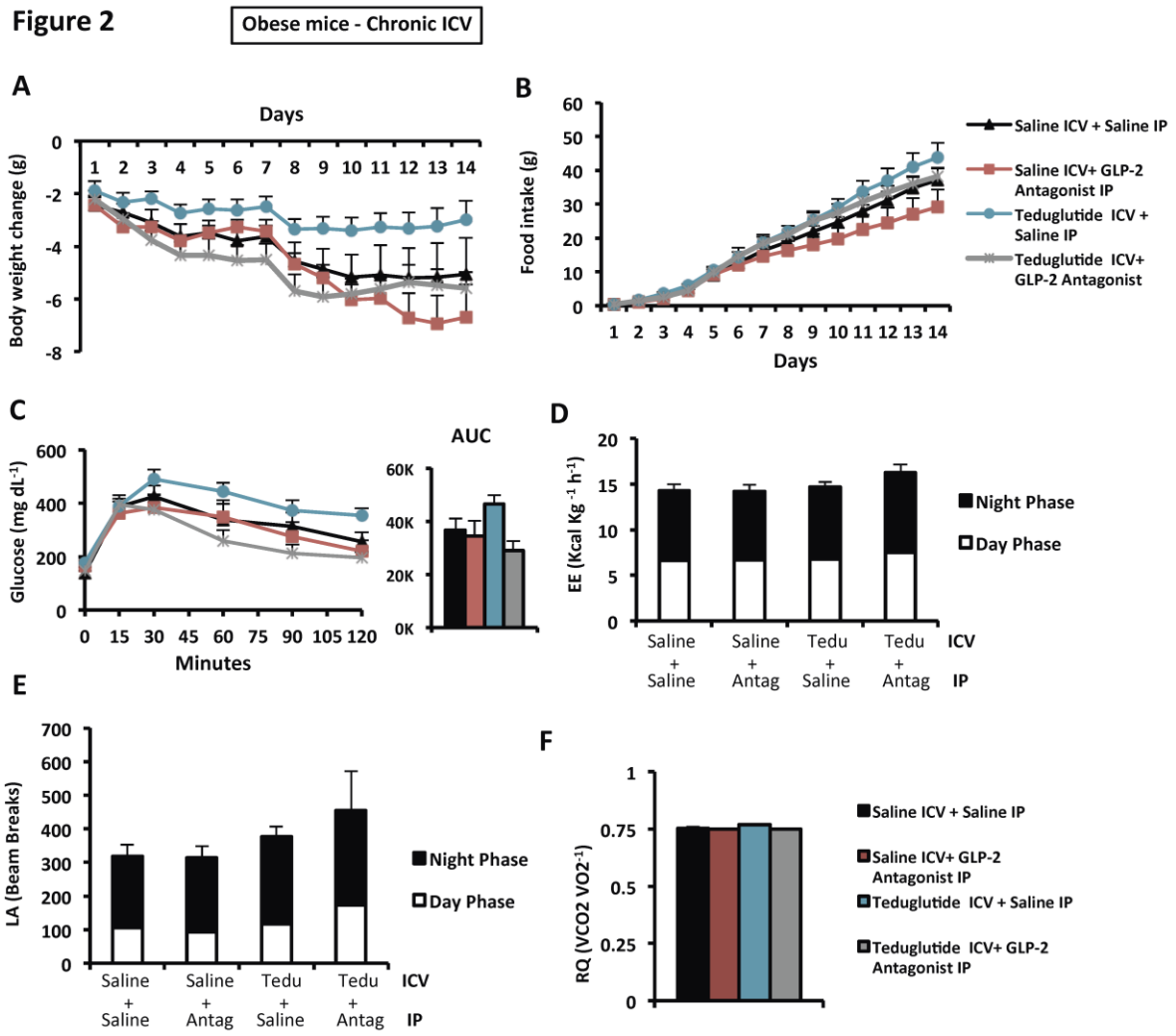

Figure 2. Chronic modulation of central GLP-2R affects energy metabolism during nutrient excess. Mice were placed on a high-fat diet for 12 weeks to establish diet-induced obesity. Effects of 14-day intracerebroventricular (ICV) infusion of the GLP-2R agonist teduglutide (10 µg/mouse) with or without the GLP-2 antagonist (GLP-2 3-33, 10 ng/mouse, intraperitoneal [IP] injection) in obese mice on **(A)** Body weight change, **(B)** Cumulative food intake, **(C)** Intraperitoneal glucose tolerance test and area under the curve (AUC) analysis, **(D)** Energy expenditure (EE), body mass-corrected, **(E)** Locomotor activity (LA), body mass-corrected, and **(F)** Respiratory quotient (RQ). n=7 saline ICV+saline IP, saline ICV+GLP2 antagonist IP, teduglutide ICV+GLP2 antagonist; n=9 teduglutide ICV+saline IP.

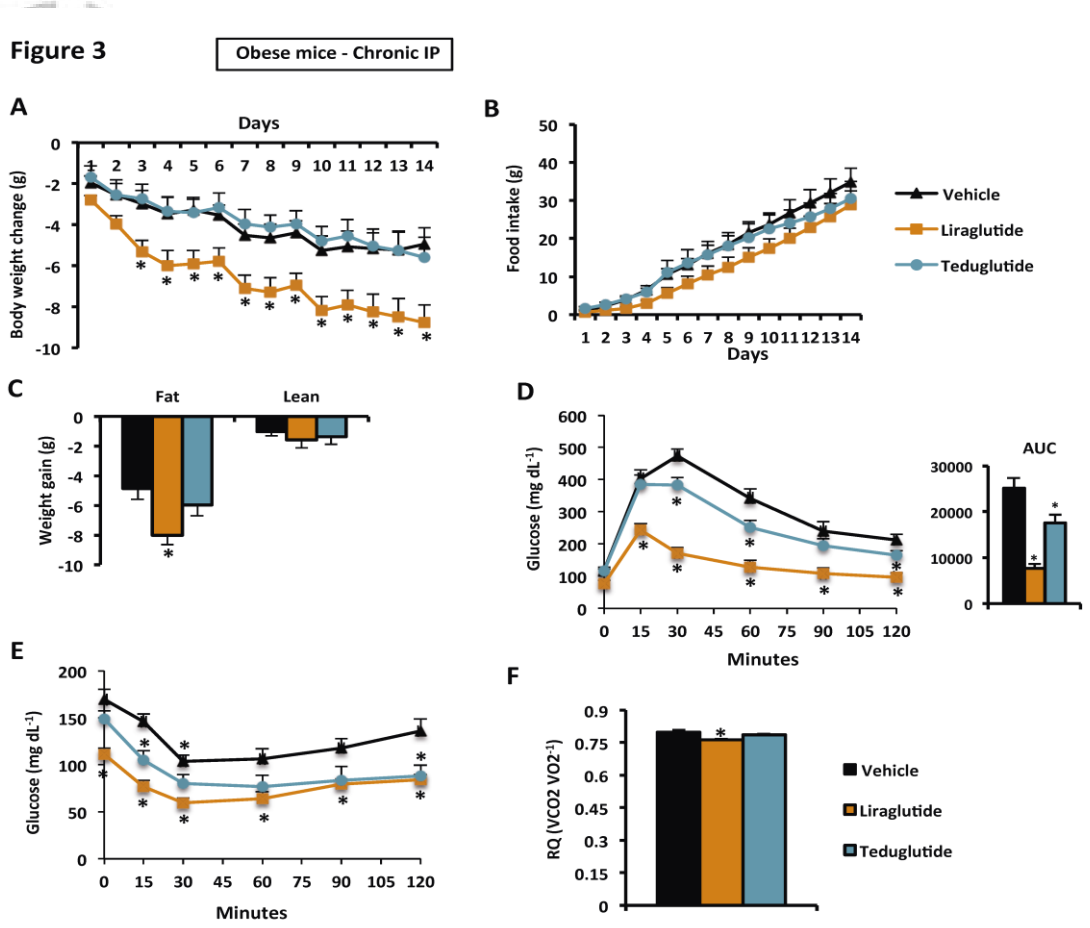

Figure 3. Chronic activation of peripheral GLP-2R provides body weight-independent glucose tolerance in diet-induced obese mice. Mice were placed on a high-fat diet for 12 weeks to establish diet-induced obesity (DIO). Mice were treated with once-daily intraperitoneal injection of saline, liraglutide (50 μg kg^{-1}), or teduglutide (4 μg) for 14 days. **(A)** Body weight change, **(B)** Cumulative food intake, **(C)** Body composition, **(D)** Glucose tolerance and are under the curve (AUC) analysis, **(E)** Insulin sensitivity, and **(F)** Respiratory quotient (RQ). * $p<0.05$ vehicle *versus* treatment (t-test in bar graphs and two-way ANOVA in body-weight, food intake, GTT and ITT curves). Panels A–C and F, vehicle and teduglutide n=8; liraglutide n=7. Panel D and E, vehicle and teduglutide n=14, liraglutide n=7. (First series of experiments: control, vehicle and teduglutide group (n=8), liraglutide group (n=7); second series of experiments: vehicle and teduglutide (n=6)).

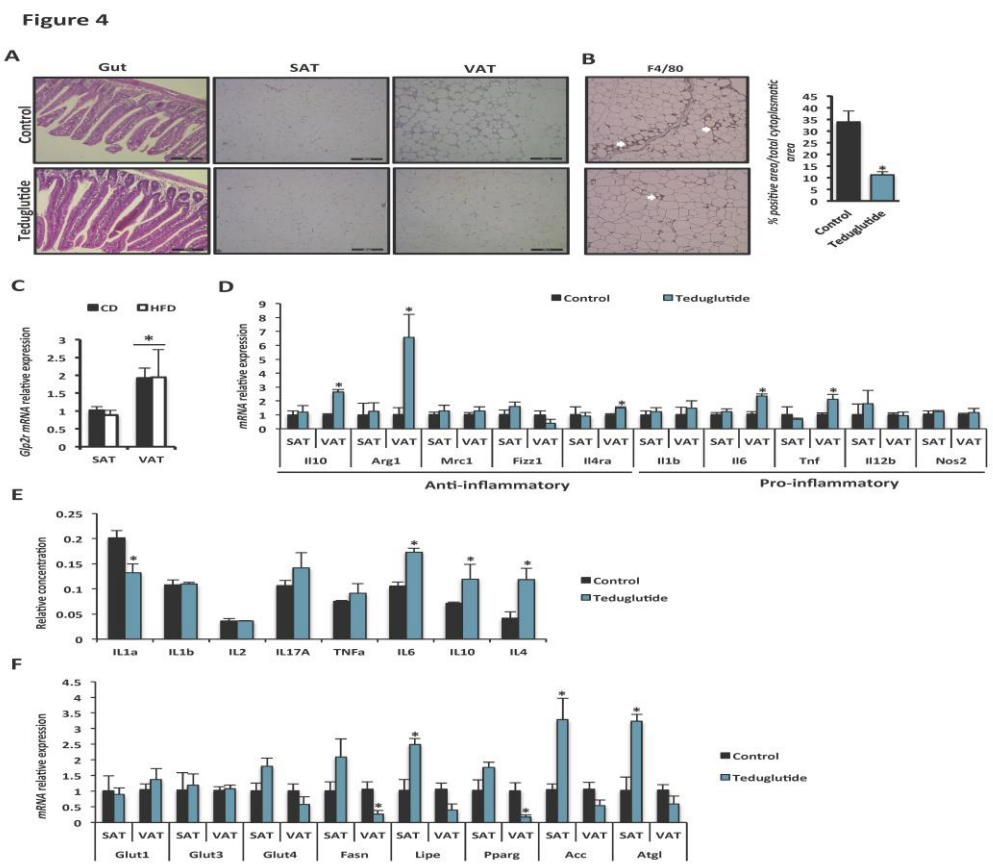

Figure 4. Effects of chronic modulation of peripheral GLP-2R in adipose tissue of diet-induced obese mice. Mice were placed on a high-fat diet for 12 weeks to establish diet-induced obesity. Mice were treated with once-daily intraperitoneal injection of saline or teduglutide (4 µg) for 14 days. **(A)** Hemtaoxylin & eosin staining of tissue sections. **(B)** F4/80 staining of VAT sections and quantification. Arrows point to crown-like structures) (n=5). **(C)** GLP-2R mRNA relative expression in subcutaneous (SAT) and visceral (VAT) adipose tissue. Normalization set at 1 *versus* respective control SAT (n=8). **(D)** mRNA expression of pro- and anti-inflammatory markers. Normalization set at 1 *versus* respective control (n=8). **(E)** Systemic effects of teduglutide treatment by quantikine ELISA (n=5). **(F)** Relative expression of metabolism markers (n=8). CD = chow diet; HFD = high-fat diet. * $p<0.05$ control *versus* treatment (t-test).

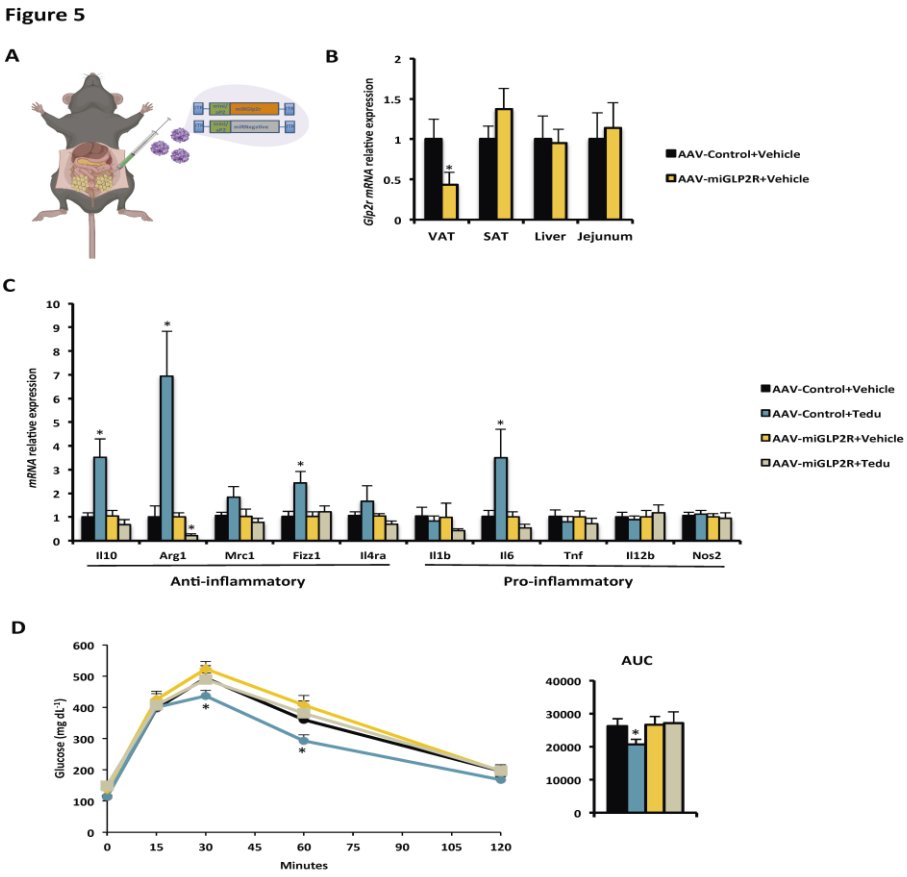

Figure 5. GLP-2R silencing in visceral adipose tissue abolishes the beneficial metabolic effects of teduglutide. Mice were placed on a high-fat diet for 12 weeks to establish diet-induced obesity. **(A)** Mice were injected with 1×10^{11} vg (viral genomes) of AAV-mini/aP2-miRGLP2R or AAV-mini/aP2-miRNA negative control (control). Two weeks later, mice were treated daily with saline or teduglutide (4 μg) by intraperitoneal injection over 14 days. **(B)** RNA from visceral (VAT) and subcutaneous (SAT) adipose tissue, liver and jejunum was extracted and analyzed for *Glpr-2r* expression. Normalization set at 1 *versus* respective control. **(C)** Expression of inflammatory and anti-inflammatory markers in VAT of control and *Glp-2r* knock-down mice. Normalization set at 1 *versus* respective vehicle. **(D)** Intraperitoneal glucose tolerance test. (A-D) n=10. * $p<0.05$ *versus* AAV-control+vehicle (t-test in bar graphs and two-way ANOVA in GTT curve).

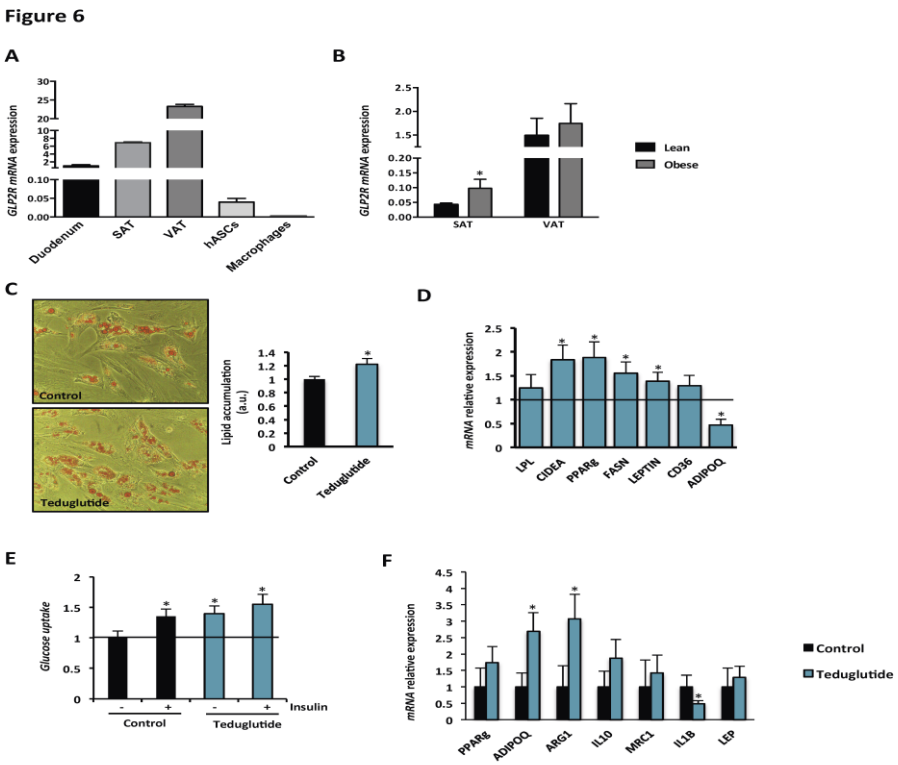

Figure 6. GLP-2 acts as a lipogenic and anti-inflammatory signal in human adipose tissue. **(A)** mRNA expression of *GLP2R* in various tissues and cells: duodenum, subcutaneous (SAT) and visceral (VAT) adipose tissue, human adipose stem cells (hASCs) and macrophages (n=5). **(B)** *GLP2R* mRNA expression in SAT and VAT depots from lean and obese subjects (n=7). Adipocytes differentiated from hASCs derived from lean SAT were treated for 24 h with teduglutide (10 nM), **(C)** stained with Oil Red O and, **(D)** assayed for the expression of adipogenic markers. Normalization set at 1 *versus* untreated cells. **(E)** Basal (set at 1) and insulin-induced (100 nM) glucose uptake in control and teduglutide-treated (10 nM, 24 h) adipocytes (n=5). **(F)** Obese human VAT explants were treated with teduglutide (10 nM) for 24 h, and RNA was extracted and analyzed for gene expression (n=6). * $p<0.05$ control *versus* treatment (t-test).