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Monoclonal therapy against calcitonin gene-related peptide lowers hyperglycemia and adiposity in type 2 diabetes mouse models

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ABSTRACT

Objective: Calcitonin Gene-Related Peptide α (CGRPα) is a multifunctional neuropeptide found in the central and peripheral nervous system with cardiovascular, nociceptive, and gastrointestinal activities. CGRPα has been linked to obesity and insulin secretion but the role of this circulating peptide in energy metabolism remains unclear. Here, we thought to utilize a monoclonal antibody against circulating CGRPα to assess its ability to improve glucose homeostasis in mouse models of hyperglycemia and diabetes.

Methods: We examined the outcome of anti-CGRPα treatment in mouse models of diabetes and diet-induced obesity, using db/db mice, Streptozotocin (STZ) treatment to eliminate pancreatic islets, and high fat diet-fed mice. We also correlated these data with application of recombinant CGRPα peptide on cultured mature adipocytes to measure its impact on mitochondrial bioenergetics and fatty acid oxidation. Furthermore, we applied recombinant CGRPα to primary islets to measure glucose-stimulated insulin secretion (GSIS) and gene expression.

Results: BL6-db diabetic mice receiving anti-CGRPα treatment manifested weight loss, reduced adiposity, improved glucose tolerance, insulin sensitivity, GSIS and reduced pathology in adipose tissue and liver. Anti-CGRPα failed to modulate weight or glucose homeostasis in STZ-treated animals. High fat diet-fed mice showed reduced adiposity but no benefit on glucose homeostasis. Considering these findings, we postulated that CGRPα may have dual effects on adipocytes to promote lipid utilization while acting on pancreatic β-cells to modulate insulin secretion. Analysis of CGRPα in the pancreas showed that the peptide localized to insulin-positive cells and perivascular nerves surrounding islets. Ex-vivo analysis of pancreatic islets determined that CGRPα blocked GSIS and reduced insulin-2 gene expression. Mechanistical analysis revealed that recombinant CGRPα was able to reduce glycolytic capacity as well as fatty acid oxidation in primary white adipocytes.

Conclusions: These results establish a multifaceted role in energy metabolism for circulating CGRPα, with the ability to modulate thermogenic pathways in adipose tissue, as well as pancreatic β-cell dependent insulin secretion. Reducing circulating CGRPα levels with monoclonal therapy presents therapeutic potential for type 2 diabetes as shown in BL6-db/db mice but has reduced potential for models of hyperglycemia resulting from loss of β-cells (STZ treatment).

1. Introduction

Calcitonin Gene-Related Peptide (CGRP) CGRP is 37-amino acid peptide, distributed in discrete areas of the central and peripheral nervous systems [1]. The CGRPα peptide is found in motor neurons containing acetylcholine, Aδ and C fibers sensory nerves and its release within tissues is stimulated by activation of the transient

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receptor potential vanilloid 1 [2]. The two peptides CGRPα and CGRPβ differ by three amino acids in humans and one amino acid in rodents [3], giving them similar biological activities [4]. CGRPα and calcitonin are derived from tissue-specific alternative splicing of the primary transcript of the *Calca* gene [5] whereas CGRPβ is the sole product encoded by a distinct *Calcb* gene [6]. CGRPβ is mostly found in the enteric nervous system [7].

Remarkably, CGRPα has been shown to be involved in vasodilation [8], neurogenic inflammation [8], immune function [9] and hypertension [4]. CGRPα abundant localization to perivascular sensory nerves makes it also perfectly situated to act at a local level with "overspill" material being released into plasma [4,10]. Elevation of plasma CGRPα has been found in pathophysiological situations including pregnancy, thyroid carcinoma, obesity and aging [4,11–13].

CGRPα has been the object of multiple studies investigating its function in metabolic homeostasis, but the complexity of its transcriptional regulation and tissue localization confounds the interpretation of these results. A role in energy metabolism has been evidenced using CGRPα knockout mice, which are resistant to diet-induced obesity and manifest increased energy expenditure while having a higher food intake [14]. Blockage of downstream action on CGRP receptor using CGRP (8–37) produced increased oxygen consumption and energy expenditure in aged mice without affecting food intake [11]. The effects observed on feeding in genetic deletion point to the importance of defining the central versus peripheral role of CGRPα in energy metabolism. Recently, CGRPα-expressing neurons in the parabrachial nucleus have been associated with acute modulation of hunger, but the direct role for the peptide itself in this phenotype is unknown [15]. Finally, evidence for peripherally injected CGRPα and loss of TRPV1 sensory nerves suggests a role in antagonizing insulin release from pancreatic β-cells [13,16–24]. Taken together, these findings suggest that central and peripheral CGRPα may differ in their biological activities, with peripheral action showing promise to regulating glucose homeostasis. Recently, monoclonal antibodies against CGRPα have reached U.S Food and Drug Administration's approval for the treatment of migraine [25]. Due to their large size, these antibodies do not cross the blood brain barrier [25], and constitute a highly attractive route to tease apart the peripheral versus central role of CGRPα in glycemic control and energy metabolism.

2. Materials and methods

Experimental design. All procedures were approved by the Animal Care and Use Committee of Cedars Sinai Medical Center and University of California, Berkeley. BL6-db/db (Jax 000697), BL6-ob/ob (Jax 000632), BL6 (Jax 000664) mice were obtained from Jackson Laboratories. CGRPα-hDTR mice were donated by Marc Zylka (UNC Chapel Hill). For STZ treatment, 6-week-old BL6 mice received 6 × 50 mg/kg daily intraperitoneal injections (Enzo Life Sciences). Male mice were single housed and fed a normal chow (PicoLab Rodent 20 5053*, LabDiet), or high fat diet (HFD) chow (BioServ, 60% fat calories). Monoclonal CGRPα therapy (α-CGRP, MuMab 7E9) or control IgG (KLH-C3) were a gift from Teva Pharmaceuticals and prepared following published methods [26]. α-CGRP and control IgG were administered by weekly subcutaneous injections at 10, 25 and 40 mg/kg.

Indirect calorimetry, physical activity, and food intake. Indirect calorimetric studies were conducted in an automated home cage eight-chamber phenotyping system (CLAMS, Columbus Instruments). Body composition was assessed by EchoMRI. Mice were acclimatized in the chambers for at least 24 h. Locomotor activity and parameters of indirect calorimetry were measured for at least the following 48 h. Food intake was measured weekly in-home cages.

Metabolic studies. To perform the glucose tolerance test (GTT) and the glucose-stimulated insulin secretion (GSIS), mice were fasted for 16 h and tails were bled for the initial blood glucose concentration measured using a Bayer Contour glucometer (Bayer). Glucose (db/db: 0.5 g/kg weight and HFD: 1 g/kg weight) was intraperitoneally administered and blood glucose was measured at indicated times after injection. For the insulin tolerance test (ITT), 5-h-fasted mice were injected with 1–2 U/kg of human insulin (Humulin, Eli Lily) and glucose was measured as in the GTT.

Tissue and serum measurements. Protease inhibitor cocktail (Complete^{TM} Mini, Millipore Sigma) was added to freshly collected blood by cardiac puncture and the serum fraction was separated by coagulation at room temperature. CGRP was measured by ELISA (Cayman Chemical). Hepatic lipids were extracted with chloroform methanol (2:1) using previously described techniques [27]. Triglycerides (TG) were measured with a TG kit (TR0100, Sigma-Aldrich) and serum FFA were measured with FFA kit (MAK044; Sigma Aldrich).

Pancreatic islet isolation. Islets were isolated from a subset of wild-type C57BL6/J mice. Liberase TL (Millipore Sigma), dissolved in RPMI 1640 medium (Thermofisher), was injected into the common bile duct while the ampulla of Vater was clamped. The distended pancreas was then dissected out, digested for 13 min in a 37 °C water bath, and passed through a sieve to remove tissue debris. Islets were then separated from exocrine tissue by density gradient separation using Histopaque (Millipore Sigma) at 2400 rpm, 22 °C for 20 min. Islets were then carefully hand-picked from the interface using a plastic pipette, washed several times using RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco), and manually counted under a dissection microscope.

Ex-vivo glucose-stimulated insulin secretion. Isolated islets were transferred to a Petri dish containing RPMI 1640 medium with L-glutamine (20 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and FBS (10%). Following 48 h of incubation in 5% CO2 incubator at 37 °C, the islets were processed for glucose-stimulated insulin secretion (GSIS). The islets (n = 13/group in triplicate) were preincubated in 2.8 mM glucose-Krebs-Ringer bicarbonate HEPES buffer (KRBH) solution (in mM: 115 NaCl, 5 KCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 2% wt/vol bovine serum albumin (BSA), pH 7.4) for 30 min at 37 °C. The KRBH solution was then aspirated, and the islets incubated in KRBH solution containing different concentrations of glucose (2.8, 16.7 mM) and CGRPα peptide (Sigma-Aldrich) for 1 h at 37 °C. After incubation, the solution was collected and stored at −80 °C for insulin assay using an UltraSensitive Insulin ELISA kit (Crystal Chem).

Ex-vivo lipolysis. Gonadal fat pads were isolated from mice and weighed. Each pad was cut into 3 equal pieces and incubated in Krebs-Ringer Bicarbonate Buffer containing 1% fatty acid–free BSA (Sigma-Aldrich). The samples were treated with either PBS or isoproterenol (25 μM, Sigma-Aldrich) at 37 °C with mild shaking at 300 rpm. Glycerol release was measured for 2 h using the Free Glycerol Determination Kit (Sigma-Aldrich).

Ex-vivo islet gene expression assay. To study the effect of CGRPα on gene expression, islets isolated from six wild type (wt) mice were divided into two pools and incubated in RPMI 1640 medium (Gibco), supplemented with 10% FBS (Gibco) and penicillin/streptomycin (Gibco) for 24 h. After 24 h, islets were treated with either normal glucose (2.8 mM) or high glucose (16.7 mM) concentrations, dissolved in RPMI 1640 medium (Gibco) supplemented with 10% FBS and penicillin/streptomycin in the presence and absence of 500 nM CGRPα (Sigma-Aldrich) for 5 h mRNA was extracted using Qiagen RNeasy mini kit (Qiagen).

Quantitative real-time PCR. RNA was isolated from islets using

Qiagen RNeasy mini kit (Qiagen). One-step quantitative RT-PCR (qRT-PCR) was performed using Power SYBR™ Green RNA-to-CT™ 1-Step Kit (Applied Biosystems). A total of 5 ng of total mRNA was added per well and assayed on a LightCycler 480 II (Roche) using 18S rRNA as an internal control. Fold changes in target genes were determined by the $2^{-\Delta\Delta Ct}$ method.

Immunofluorescence and histological studies. Animals were perfused with PFA prior to sacrifice and tissues were cryoprotected in 30% sucrose in PBS overnight, then frozen in OCT (Tissue-Tek) and sectioned on a cryostat. 12-μm-thick sections were immunoblotted with primary antibodies in PBS containing 2% Donkey serum and 0.4% Triton X-100. Antibodies used were anti-CGRPα (BML-CA1134, Enzo Life Sciences) at 1:200, anti-CGRPα (ab36001, Abcam) at 1:200, anti-GFP (Thermo Fisher Scientific) at 1:200, and secondary antibodies (Alexa Fluor) at 1:600. For histological analysis, tissues were immersed in formalin overnight, dried in 70% Ethanol and embedded in paraffin. 8-μm-thick sections were stained with hematoxylin and eosin. Sections were imaged using a Keyence microscope BZ-X700 and a Leica LSM 780 Confocal.

OCR and ECAR measurements of CGRPα-treated adipocytes. For the mitochondrial and glycolysis stress tests, primary iWAT stromal vascular fraction cells were plated at 20,000 cells per well of a XF24 V7 cell culture microplate in growth medium (DMEM/F-12 with GlutaMAX (Gibco) supplemented with 10 % fetal bovine serum and penicillin-streptomycin (Gibco). Next day (day 0), differentiation was induced by changing medium to growth medium supplemented with 5 μg ml^{-1} insulin (Roche), 1 μM dexamethasone (Sigma-Aldrich), 1 μM rosiglitazone (ApexBio) and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich). Medium was then changed every other day with growth medium supplemented with 5 μg ml^{-1} insulin. For the mitochondrial stress test, on day 8 this medium was supplemented with either 100 nM CGRPα, 500 nM CGRPα, 500 nM CGRPα + 1 μM CGRP (8–37), or vehicle (PBS), and cells were incubated overnight. On the day of analysis, cells were washed twice with assay medium (Seahorse XF Base Medium (Agilent Technologies) supplemented with 20 mM glucose, 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), pH 7.4), and incubated at 37 °C in a CO_2-free incubator for 1 h in assay medium containing either 100 nM CGRPα, 500 nM CGRPα, 500 nM CGRPα + 1 μM CGRP (8–37), or vehicle. OCR and ECAR were measured in a Seahorse XFe24 Analyzer, using the following injections and final concentrations: oligomycin (1.5 μM), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 2 μM), rotenone/antimycin A (Rot/AA, 0.5 μM). For the glycolysis stress test, on day 9, insulin-containing growth medium was supplemented with either 500 nM CGRPα or vehicle and cells were incubated overnight. On the day of analysis, cells were washed twice with assay medium (Seahorse XF Base Medium supplemented with 2 mM L-glutamine (Gibco), pH 7.4), and incubated at 37 °C in a CO_2-free incubator for 1 h in assay medium containing either 500 nM CGRPα or vehicle. OCR and ECAR were measured in a Seahorse XFe24 Analyzer, using the following injections and final concentrations: glucose (10 mM), oligomycin (1.5 μM) and 2-deoxy-D-glucose (2-DG, 50 mM). For the FAO assay, 8000 cells per well were plated in a XF96 V3 PS cell culture microplate and differentiated as described previously. On day 9, cells were incubated overnight with substrate-limited medium (Seahorse XF Base Medium containing 0.5 mM glucose, 1 mM GlutaMAX, 0.5 mM carnitine, 1% FBS, and either 500 nM CGRPα or vehicle, pH 7.4). Cells were then washed twice with FAO assay medium (111 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 2 mM $MgSO_4$, 1.2 NaH_2PO_4, 2.5 mM glucose, 0.5 mM carnitine, 5 mM HEPES, pH 7.4), and incubated for 45 min at 37 °C in a non-CO_2 incubator with FAO assay medium supplemented with either 500 nM CGRPα or vehicle. Etomoxir (40 μM) or vehicle (FAO assay medium) were then added and cells were incubated for 15 min at 37 °C in a non-CO_2 incubator. Palmitate-BSA conjugate or BSA control were added immediately before measuring OCR and ECAR in a Seahorse XFe96 Analyzer, using the following injections and final concentrations: oligomycin (1.5 μM), FCCP (2 μM), Rot/AA (0.5 μM). The following parameters were used for the tests: mix 3 min, wait 2 min, measure 3 min.

Statistical methods. GraphPad Prism 8 was used for statistical analysis. Data are expressed as mean ± SEM. P-values were calculated using unpaired Student's t test or two-way ANOVA with Holm–Sidak post hoc analysis as appropriate. P-values less than 0.05 were considered significant.

3. Results

3.1. The CGRP pathway is upregulated in gonadal fat and pancreatic islets from diabetic mice

As levels of circulating CGRPα were found increased in obesity, aging and other models of disease [4,11–13], we asked if plasma levels of CGRPα in diabetic mice (db/db or $Lepr^{db}$ mice) were altered. We observed significantly higher levels of circulating CGRPα in BL6-db/db mice compared to controls (Fig. 1a), while these mice presented marked hyperglycemia at 10 weeks old (not shown).

Once released, CGRPα plays multifunctional roles at different sites by binding to its receptor calcitonin receptor-like receptor (CALCRL) and its receptor activity-modifying protein 1 (RAMP1). Interestingly, when comparing transcript levels for ramp1 between db/db and wt in BL6 background (Fig. 1b–f), a large upregulation of approximately 20 fold was observed in gonadal white adipose tissue (gWAT), while a 6 fold upregulation was visible in pancreatic islets and a 2 fold increase in liver, while higher calcrl expression was observed exclusively in gWAT (8 fold). Other tissues surveyed were spleen and skeletal muscle due to their relevance to diabetes and energy metabolism, where we did not monitor any differential expression (Fig. 1e and f). These data are consistent with an abnormal upregulation of CGRPα signaling in diabetes.

3.2. Chronic monoclonal therapy against CGRP improves db/db and ob/ob diabetes

To assess the relevance of CGRPα in metabolic disease, we tested if prolonged reduction of plasma CGRPα could revert hyperglycemia in mouse models of diabetes. To do so, we used a monoclonal antibody therapy (muMab 7E9, Teva Pharmaceuticals Ltd) which kinetics have been described previously [26]. Anti-CGRPα antibody is stable in circulation for a week, and inhibits CGRPα and β peptides through select C-terminal epitope binding [26]. We treated BL6-db/db male mice with weekly subcutaneous injections of this antibody (α-CGRP) or a control IgG starting at 8 weeks old, when these mice already harbor symptoms of diabetes. The MuMab dosage used in our study was previously shown to inhibit neurogenic vasodilation of meningeal blood vessels in a CGRPα-dependent process (10–25 mg/kg), without affecting blood pressure or heart rate [26]. MuMab 7E9 presents strong affinity for CGRPα, binding the C-terminus of the peptide in a dose-dependent manner (IC_{50} = 10 nM to inhibit 10pM of CGRPα), therefore the injected dose neutralizes free CGRPα peptide.

Within a week after injection, delayed weight gain was visible with two different dosages (Fig. 2a and b). Because the lower dose (10 mg/kg) did not confer benefits on fasting blood glucose (not shown), we focused our analysis on the higher dosage. No difference in food intake was observed, as all db/db mice were similarly hyperphagic throughout the course of the study (Fig. 2c). Remarkably, after 6 weeks of treatment, reducing plasma CGRPα dramatically

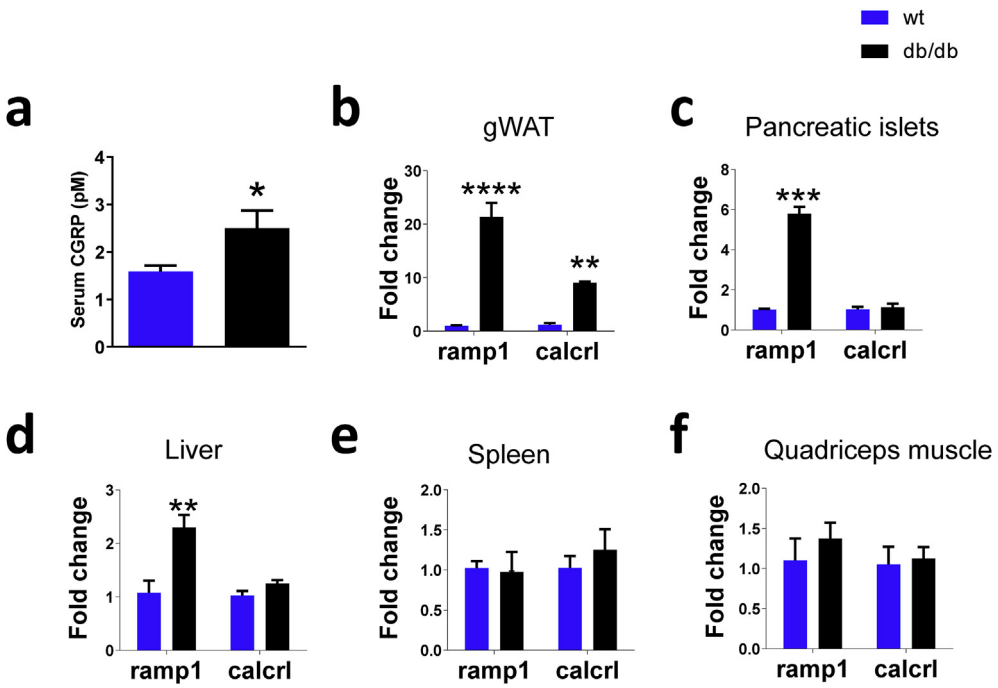

Fig. 1. CGRP signaling increases upon diabetes. (a) Plasma levels of CGRP from db/db and wt male mice in BL6 background. n = 8, means ± S.E.M. (b–f) RTqPCR analysis of gonadal fat, isolated islets from pancreas, liver, spleen, and quadriceps muscle tissues. n = 4, means ± S.E.M., *p < 0.05, Student's t test.

improved fasting blood glucose by 24% (324.7 versus 427.8 mg/dl) and overall ameliorated glucose tolerance (Fig. 2d). These results were observed in two independent cohorts of db/db mice (Supp Fig. 1a and b). Because hyperglycemia in diabetes is associated with insulin resistance [28], we performed an insulin sensitivity test after seven weeks of treatment (Fig. 2e). In these severely diabetic mice, insulin injection had diminished ability to lower blood glucose levels, with α-CGRP treatment resulted in improved management of blood glucose in response to insulin (Fig. 2e). We then asked whether the beneficial effects on glucose tolerance and insulin sensitivity were associated with ameliorated glucose-stimulated insulin secretion (GSIS). Diabetic db/db mice manifest hyperinsulinemia to compensate for reduced insulin action in peripheral tissues [29]. Remarkably, α-CGRP treatment improved GSIS in db/db animals after 7 weeks of treatment (Fig. 2d).

After 8 weeks of treatment, we assessed tissue pathology in various organs (Fig. 2g). Brown adipose tissue (BAT) was necrosed in control BL6-db/db, with a high level of visible lipid vesicles, whereas α-CGRPα treatment preserved the cellular morphology of the adipocytes without reducing lipid vesicles content. Large adipocytes and infiltrated macrophages were visible in gWAT of control IgG, but these characteristics were less pronounced in α-CGRP treated mice. Hepatic steatosis, or fatty liver, is a typical feature of type 2 diabetes genetic mouse models [30] and was reduced in antibody-treated mice (Fig. 2g). We further measured liver TG content and found a reduction in antibody-treated mice, concomitant with lower serum FFA levels (Fig. 2h). These results indicate that α-CGRP (25 mg/kg) was beneficial to improve multiple metabolic parameters in severely diabetic db/db mice.

To further evaluate whether this antibody could improve diabetes in a different model, we tested weekly injections in another commonly used monogenetic diabetes mouse, the leptin deficient (ob/ob) mouse. These mice exhibit faster onset obesity but milder glucose intolerance than db/db animals [31]. Here, we found that weekly administration of the antibody modestly diminished weight gain over time, with improved weight loss appearing after 5 weeks of injections (Supp Fig 1c). Remarkably, glucose tolerance after 6 weeks of treatment was significantly ameliorated (Supp Fig 1d). These data demonstrate that neutralization circulating CGRPα improves hyperglycemia and weight loss in severely diabetic animals.

3.3. *The CGRPα neuropeptide acts on pancreatic β-cells to reduce insulin secretion*

Our results thus far indicate that α-CGRP reduces hyperglycemia and improved GSIS in two monogenic diabetes mouse models in the BL6 background (Fig. 2 and Supp Fig. 1). The ability of α-CGRP to ameliorate insulin secretion in these mice prompted us to examine the action of CGRPα on pancreatic β-cells.

We first asked if CGRPα was found within pancreatic islets using a reporter model and antibody staining against CGRPα. In CGRPα-GFP-hDTR knockin mice, a GFP tracer has been knocked in to the Calca locus, therefore allowing to visualize CGRPα expressing cells [32,33]. GFP is visible in discrete cells within the islets (5%), which colocalize with CGRPα immunoreactive cells (Fig. 3a). A higher fraction of pancreatic islet cells was immunoreactive for CGRPα, suggesting that the peptide may be endocytosed by these cells which do not express GFP, and therefore do not produce CGRPα. The presence of CGRPα-positive islet cells was confirmed using two different polyclonal antibodies against CGRPα (Fig. 3a and b). Interestingly, CGRPα-positive neural fibers were also observed around the islets (Fig. 3a and b) and these nerves were associated with blood vessels, exocrine and endocrine compartments, consistently with previous observations made in various mammalian species [34]. CGRPα-positive cells within islets contained insulin (Fig. 3c), therefore demonstrating that β-cells were primarily uptaking the secreted peptide.

We then asked if recombinant CGRPα could alter GSIS from primary islets, as our previous study showed that CGRPα reduced GSIS from MIN6 cells [11] and higher GSIS was observed in db/db mice receiving α-CGRPα. Here, ex-vivo stimulation of primary islets incubated for 5 h with 500 nM recombinant CGRPα reduced GSIS

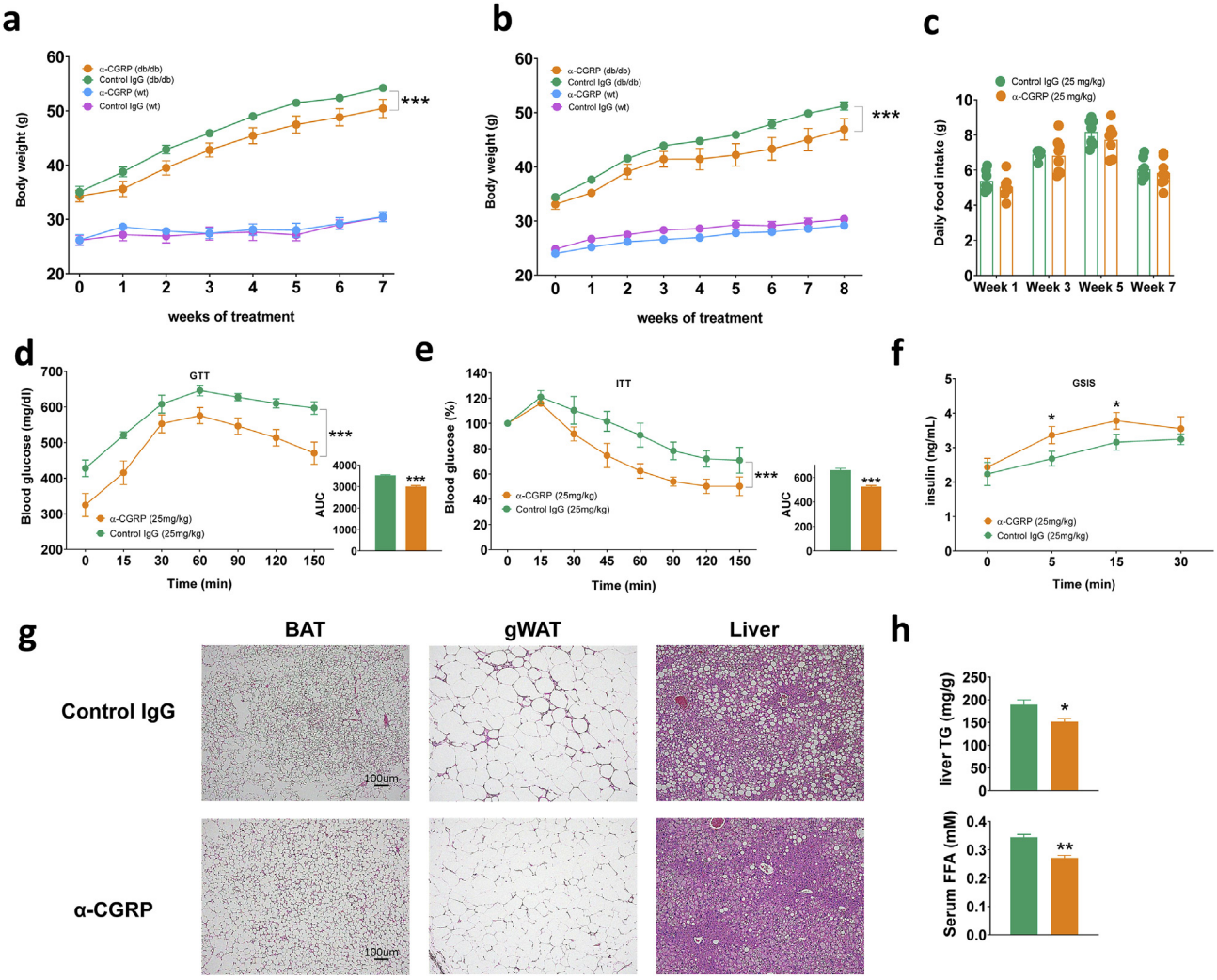

Fig. 2. Monoclonal therapy against CGRP improves diabetes in db/db mice. (a) Body weights of 8 weeks old diabetic BL6 db/db or control wild-type males treated with weekly injection of 10 mg/kg α-CGRP antibody or control IgG and (b) 25 mg/kg α-CGRP antibody or control IgG (n = 6). (c) Daily food intake from 25 mg/kg α-CGRP or control IgG recorded at 1, 3, 5 and 7 weeks (n = 6). (d) Glucose tolerance test at week 5 and area under the curve (n = 6). (e) Insulin sensitivity test at week 6 and area under the curve (n = 6). (f) GSIS at week 7 (n = 4). (g) Representative H&E stainings of brown fat, gonadal adipose tissue (gWAT) and liver from 25 mg/kg treated animals. (h) Analysis of liver triglyceride (TG) levels and quantification of serum free fatty acids (FFA), (n = 6). ***p < 0.0001, **p < 0.001, *p < 0.05, all values denote means ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

compared to vehicle (Fig. 3d). Additionally, using RTqPCR, we found that this treatment led to transcriptional downregulation of the ins2 gene, without affecting glucagon or caspase-3 gene expression.

Based on these observations, the results presented so far indicate that CGRPα reduces pancreatic islet-dependent insulin secretion upon glucose challenge. Our observations are in accordance with rat data on isolated perfused pancreas [20], as well as in-vivo mouse studies [22].

To further assess if α-CGRP could improve hyperglycemia in type-1 diabetes, we examined weight and glucose homeostasis in STZ-treated mice. STZ, an alkylating agent, leads to β-cell death and is used as a model to mimic type-1 diabetes [35]. STZ was given through daily injections to wild-type B6 male mice for two weeks until fasting blood glucose reached a diabetic hyperglycemic state (400 mg/dl). STZ-treated mice harbored lower body weights than saline control animals on NC, with α-CGRP therapy failing to produce durable weight loss in these hyperglycemic mice (Fig. 4a). In saline-treated controls, α-CGRP therapy had no effect on NC body weights, but reduced HFD-dependent weight gain (Fig. 4b). Furthermore, α-CGRP treatment did not ameliorate glucose tolerance in STZ-treated mice at 12 weeks old (Fig. 4c). Interestingly, insulin sensitivity was not significantly improved in the STZ group (Fig. 4d). Because α-CGRP treatment could improve hyperglycemia in db/db mice, these findings suggest that circulating CGRPα may alter glycemia through pancreatic islet-dependent action in diabetic mouse models.

3.4. Anti-CGRP monoclonal therapy promotes lipolysis in high fat diet-fed mice

Because we observed weight loss in HFD-fed mice, we further tested the effects of this antibody in a diet-induced obesity (DIO) model. To do so, we treated wild-type (BL6) male mice with weekly subcutaneous injections of α-CGRP (25 mg/kg) or control IgG starting at 6 weeks of age and subjected these mice to high fat diet (HFD) or normal chow (NC). Within a week after injection, we observed a modest but significant delay in weight gain during HFD of the α-CGRP treated animals (Fig. 5a), with no notable difference in NC-fed animals. The changes in weight gain on HFD were due to reduced fat mass in antibody-treated mice (Fig. 5b).

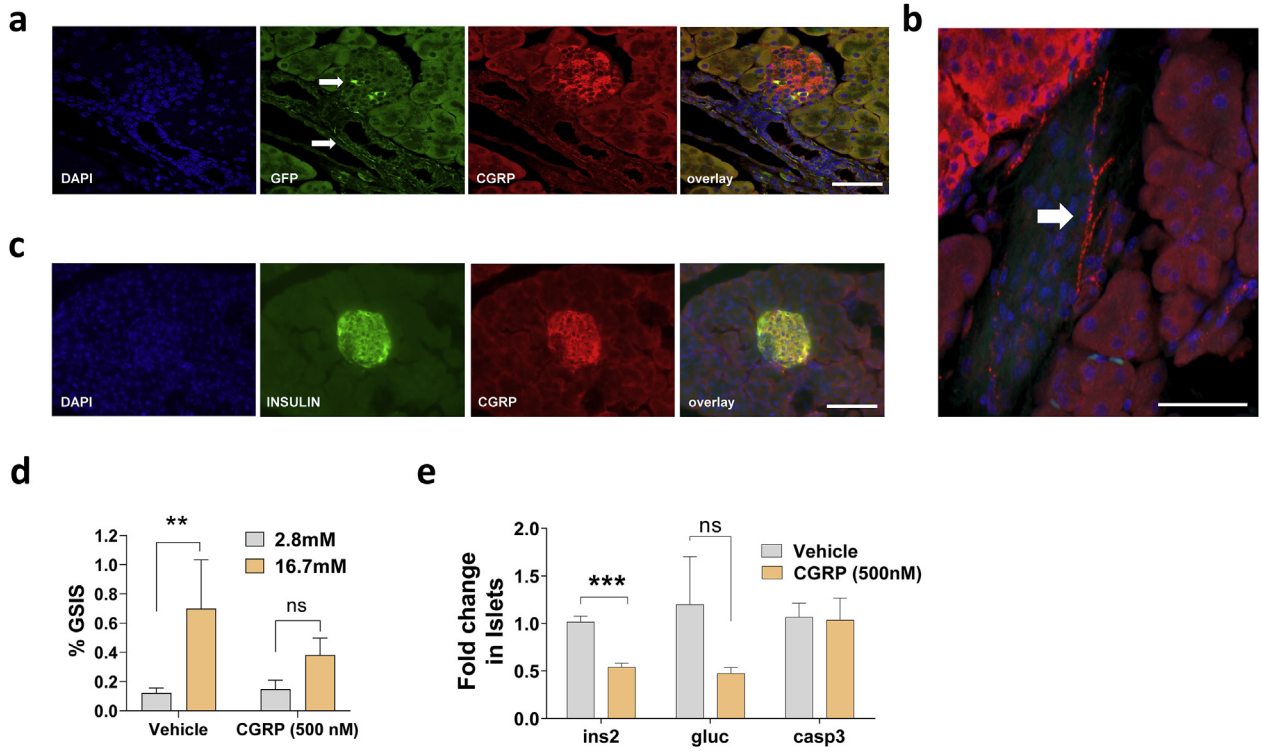

Fig. 3. CGRP innervation modulates pancreatic β-cells response to glucose. (a) Immunohistochemistry of pancreatic section from CGRPα-GFP mice, blue (dapi), green (GFP) and red (anti-CGRP), scale bar 100 μm. (b) Immunohistochemistry of pancreatic section, blue (dapi), red (anti-CGRP), scale bar 10 μm. (c) Immunohistochemistry of pancreatic section from CGRPα-GFP mice, blue (dapi), green (insulin) and red (anti-CGRP), scale bar 100 μm. (d) Glucose stimulated insulin secretion assay from isolated pancreatic islets (wild-type males) treated with vehicle or 500 nM CGRP (n = 6). (e) Transcript expression level from isolated pancreatic islets pretreated for 5 h with vehicle or 500 nM CGRP (n = 6). (f) Body weights from STZ-treated and (g) wild-type BL6 saline controls receiving weekly subcutaneous injections of 40 mg/kg α-CGRP or Control IgG at 8 weeks old and subjected to HFD at 12 weeks (n = 8). (e) Glucose tolerance test of 12 weeks old STZ-mice on normal chow (n = 8). (f) Insulin sensitivity test of 14 weeks old STZ-mice on HFD (n = 8). ***p < 0.0001, **p < 0.001, *p < 0.05, two-way ANOVA, all values denote means ± SEM. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Surprisingly, there was no improvement in glucose tolerance or insulin sensitivity in these mice (Fig. 5c and d) after 18 and 21 weeks of DIO (Fig. 5d and e). In HFD-fed animals, CGRPα blockade promoted increased energy expenditure as demonstrated by higher oxygen consumption after 7 weeks of HFD (Fig. 5e). There was a minor difference in substrate utilization as indicated by analysis of the respiratory exchange ratio (RER), characterized by amelioration of glucose utilization during the night cycle (Fig. 5f). The improved oxygen consumption occurred without affecting total activity (Fig. 5f) or food intake (not shown). Intrigued by the effects observed on energy expenditure, we asked if fatty acid oxidation pathways were upregulated in the gonadal fat of these mice. Increased expression of genes involved in fatty acid oxidation (FAO) metabolism, such as mcad, acaa2, acc-β and cpt1b were measured (Fig. 5h). These results are suggestive of higher lipolysis in these animals.

To directly assess endogenous lipolysis, we measured isoproterenol-induced lipolysis in biopsies from gWAT of HFD-fed mice using established procedures [36]. Isoproterenol is an agonist of β-adrenergic receptors, which activation leads to lipase-dependent breakdown of cellular TG. Consistent with our FAO gene expression, we found that lipolysis rate was increased in α-CGRP treated mice (Fig. 5i).

3.5. *CGRPα neuropeptide impairs glycolysis and fatty acid oxidation in white adipocytes*

To assess if CGRPα peptide directly impairs adipocytes' ability to dissipate energy, we investigated oxygen consumption and glycolysis response to recombinant CGRPα peptide. Using cultured primary adipocytes subjected to mitochondrial stress test using XF Seahorse technology, we measured oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) profiles upon treatment with recombinant CGRPα, at physiological circulatory concentration (100 and 500 nM). CGRP (8–37), a selective CGRP receptor antagonist was also applied to block downstream CGRP signaling [37]. The addition of oligomycin blocks complex V of the ETC to measure ATP-linked respiration, the uncoupling agent carbonylcyanide p-triflouromethoxyphenylhydrazone (FCCP) forces the maximal respiration capacity of adipocytes, whereas a mix of rotenone and antimycin A, which are inhibitors of complexes I and III of the mitochondrial ETC are used to fully deplete mitochondrial O_2 consumption. These various treatments did not alter adipocytes OCR responses to a mitochondrial stress test (oligomycin, FCCP and Rot/AA) compared to vehicle treatment (Fig. 6a). When we analyzed the glycolytic profile of these adipocytes, we observed that CGRPα reduced ECAR responses in a dose dependent manner (Fig. 6b). Importantly, CGRP (8–37) restored ECAR responses to vehicle-treated adipocytes levels (Fig. 6b). We further investigated the effects of recombinant CGRPα in a glycolytic stress test using Seahorse XF analyzer (Fig. 6c). CGRPα had no effect on glucose-dependent activation of glycolysis but lowered oligomycin-dependent increase in ECAR.

As α-CGRP promoted reduced fat mass in DIO and diabetic mice, we also asked if the CGRPα neuropeptide had an impact on FAO in primary adipocytes. Using XF Seahorse, we probed free fatty acid (FFA) oxidation using an established protocol [38]. CGRPα did not

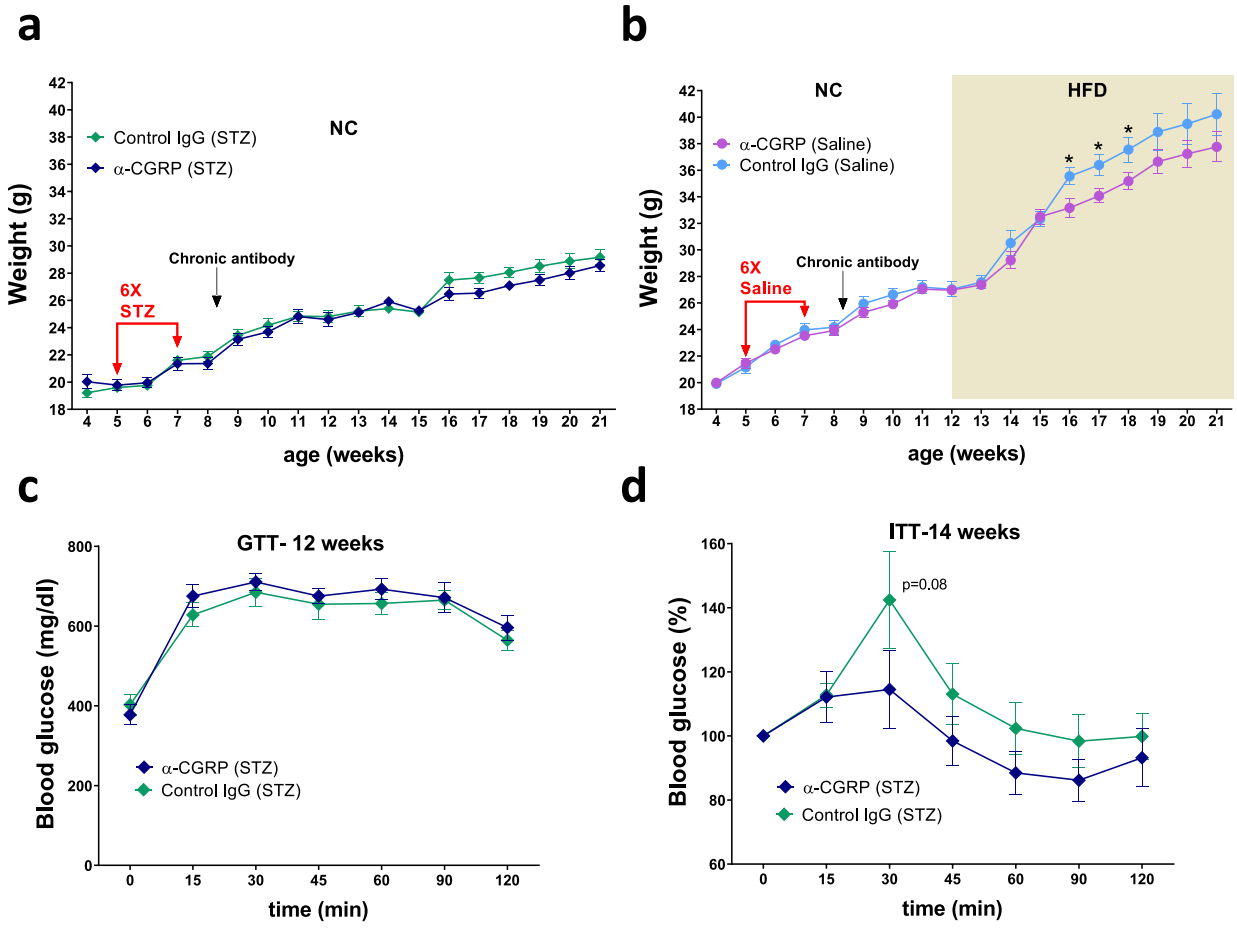

Fig. 4. Hyperglycemia in mice lacking pancreatic β-cells is not improved by CGRP antibody treatment. (a) Body weights from STZ-treated and (g) wild-type BL6 saline controls receiving weekly subcutaneous injections of 40 mg/kg α-CGRP or Control IgG at 8 weeks old and subjected to HFD at 12 weeks (n = 8). (e) Glucose tolerance test of 12 weeks old STZ-mice on normal chow (n = 8). (f) Insulin sensitivity test of 14 weeks old STZ-mice on HFD (n = 8). ***p < 0.0001, **p < 0.001, *p < 0.05, two-way ANOVA, all values denote means ± SEM.

impact basal OCR but reduced FCCP-maximal respiration upon palmitate oxidation by mitochondria (Fig. 6d). Taken together, these findings suggest that circulating CGRPα levels may communicate with adipocytes to negatively regulate energy utilization.

4. Discussion

Given the existing precedents linking CGRPα to energy metabolism [11,14], we characterized the role of circulating CGRPα in mouse models of diabetes and obesity. Using a selective method to sequester this peptide from the circulatory system, we found that loss of CGRPα reduced weight gain and mitigated hyperglycemia in diabetic mice lacking leptin receptor and leptin hormone. As these results pointed towards a potential communication between CGRPα and specific cell types, we performed ex-vivo analysis of pancreatic islets and adipocytes to confirm the existence of such interactions.

How is peripheral CGRPα regulating glucose levels in diabetic mice? Previously, we found that mutation of the sensory receptor TRPV1 increased energy metabolism and GSIS upon aging [11]. Upon TRPV1 deletion, the circulating levels of CGRPα were reduced. Such results are in accordance with the observation that α-CGRP enhanced GSIS in db/db animals (Fig. 2). Because glucose management was achieved in diabetic mice receiving the antibody but not in HFD-fed animals, we postulated that this effect could be the consequence of divergent pancreatic β-cells phenotypes in the mouse models used. β-cell plasticity is highly dependent on the strain and intervention performed. It is well established that DIO causes both rapid increase in β-cell insulin secretion and β-cell mass to ramp up insulin production [39,40]. β-cell proliferation is the primary method by which β-cell mass increases during diet-induced obesity, stimulated by increased blood insulin and glucose concentrations. As α-CGRP failed to ameliorate glucose tolerance in HFD-fed mice, it is plausible that CGRPα inhibition on β-cell GSIS is blunted by the larger β-cell to combat insulin resistance. In contrast, db/db mice develop transient hyperglycemia and slower β-cell hypertrophy [41]. Remarkably, antibody treatment efficiently lowered blood glucose in db/db animals and improved β-cell GSIS.

We confirmed our in-vivo results with ex-vivo analysis of pancreatic islets where exogenous CGRPα application lowered GSIS and reduced ins2 gene expression (Fig. 3). These findings complement previous observations from peripherally injected CGRPα and loss of TRPV1 sensory nerves on insulin release [13,16–24]. A rather novel finding in our study resulted from the dual utilization of CGRPα-GFP expressing mice combined with immunostaining against CGRPα (Fig. 3a). These stainings demonstrated that a very discrete number of pancreatic islet cells express Calca, the gene encoding for CGRPα, while most pancreatic β-cells contain CGRPα, consistent with uptake of the peptide secreted by perivascular

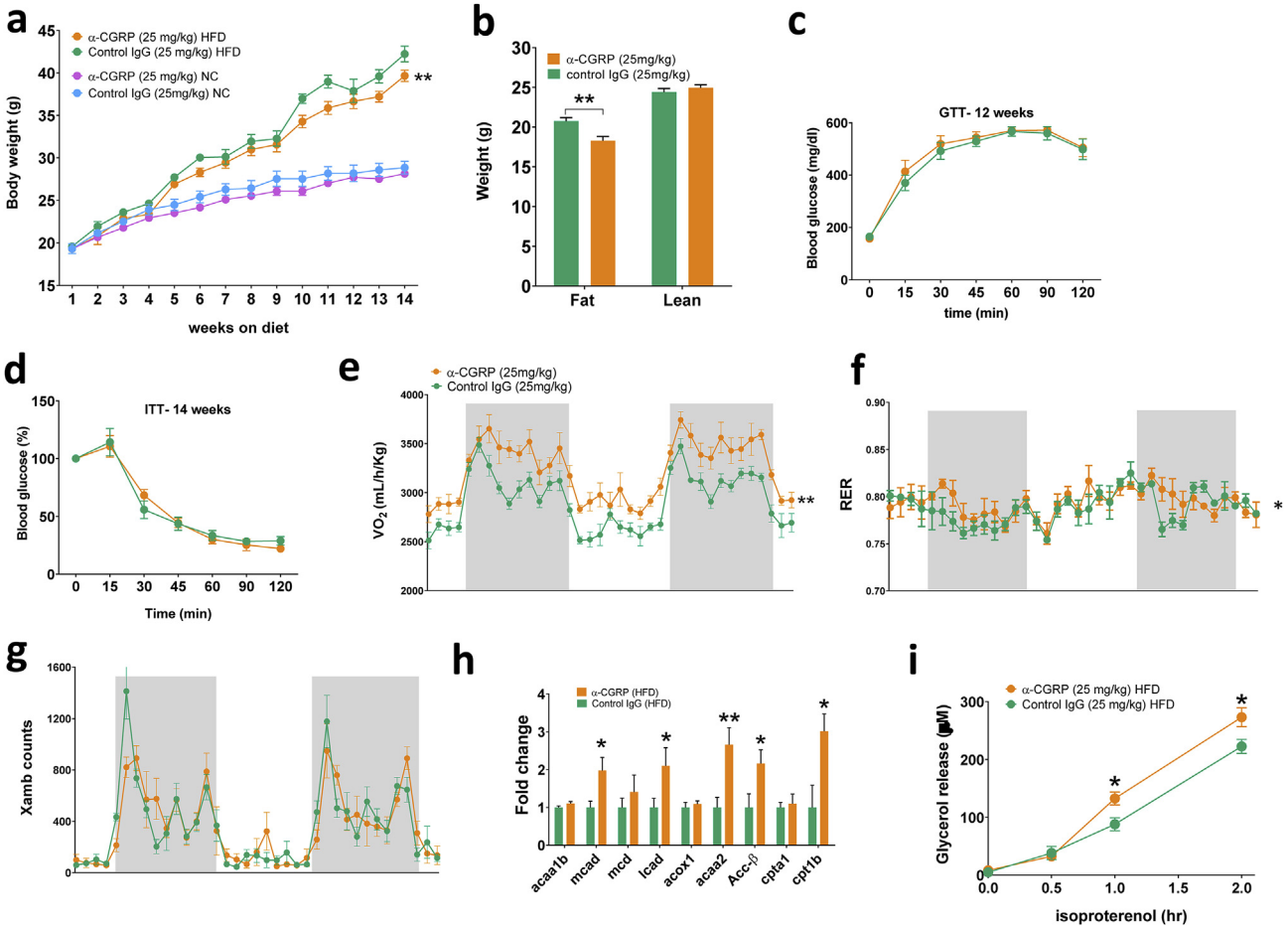

Fig. 5. Monoclonal therapy against CGRPα increases energy expenditure in high fat diet-fed mice. (a) Body weights of 6-week-old HFD and NC-fed mice upon weekly subcutaneous injections of 25 mg/kg α-CGRP or Control IgG (n = 8–14) (b) Fat mass and lean mass of α-CGRP and control IgG measured after 6 weeks of HFD (n = 8). (c) Glucose tolerance test of HFD mice at week 12 (n = 8). (d) Insulin sensitivity test of HFD mice at week 14 (n = 8). (e) Oxygen consumption of animals on HFD, (f) RER and (g) ambulatory counts measured by indirect calorimetry (n = 6). (h) Gene expression analysis of FAO-related transcripts (n = 5). (i) Isoproterenol-induced lipolysis rate of gWAT biopsies (n = 3). ***p < 0.0001, **p < 0.001, *p < 0.05, two-way ANOVA, all values denote means ± SEM.

nerves (Fig. 3). CGRPα-GFP was found in perivascular nerves located near blood vessels. The results of the present study raise the interesting possibility that sensory nerve endings release CGRPα, which may act as a modulator of pancreatic β-cell endocrine function. As CGRP receptor transcript levels were markedly increased in diabetic db/db mice, we postulate that increased CGRP signaling may contribute to negative regulation of insulin release. Interestingly, CGRPα shares 46% identity with amylin and both peptides strongly antagonize the ability of insulin to stimulate peripheral glucose disposal and inhibit hepatic glucose output in hyperinsulinemic glucose-clamp studies [42]. CGRPα and amylin show affinity for the different calcitonin receptors, suggesting that they may work as co-ligands for amylin and CGRP receptors on pancreatic β-cells [43].

What is the consequence of CGRPα stimulation on adipocytes and insulin-secreting cells? CALCRL-dependent regulation of intracellular cyclic adenosine monophosphate (cAMP) is potential candidate. In a thorough cell line characterization, Zeller et al. demonstrated that muMab 7E9 powerfully prevented CGRPα-dependent cAMP intracellular increase in a dose dependent manner [26]. cAMP signaling is particularly important for β-cells ability to secrete insulin, by modulating granule exocytosis and is therefore considered an amplifier of insulin secretion triggered by Ca^{2+} elevation [44]. In addition to glucose and other nutrients, β-cells are also stimulated by specific hormones such as the incretins, which are secreted from the gut in response to a meal and act on β-cell receptors that increase the production of cAMP [45]. CGRPα may be a negative regulator of incretin's action under normal physiological conditions but may become maladaptive in disease state.

We also provide evidence that CGRPα modulates adipose tissue energy utilization in our in-vivo and ex-vivo data sets (Figs. 5 and 6). These results are in accordance with a previous study where exogenous CGRPα induced lipolysis and β-oxidation in soleus muscle [46]. We also observed increased gene expression in a select panel of fatty-acid oxidation related transcripts (Fig. 5). Therefore, the improved energy expenditure phenotype manifested by HFD-fed mice receiving the antibody is likely to be derived from improved adipose lipolysis and energy utilization. These phenotypes are reminiscent of the leaner profile of CGRP knockout mice on DIO [14].

5. Conclusions

Taken together, these results illustrate that CGRPα influences energy homeostasis through a dual action on adipocytes lipid utilization and pancreatic β-cells insulin secretion. Monoclonal antibody against CGRPα successfully improves glycemic control in a

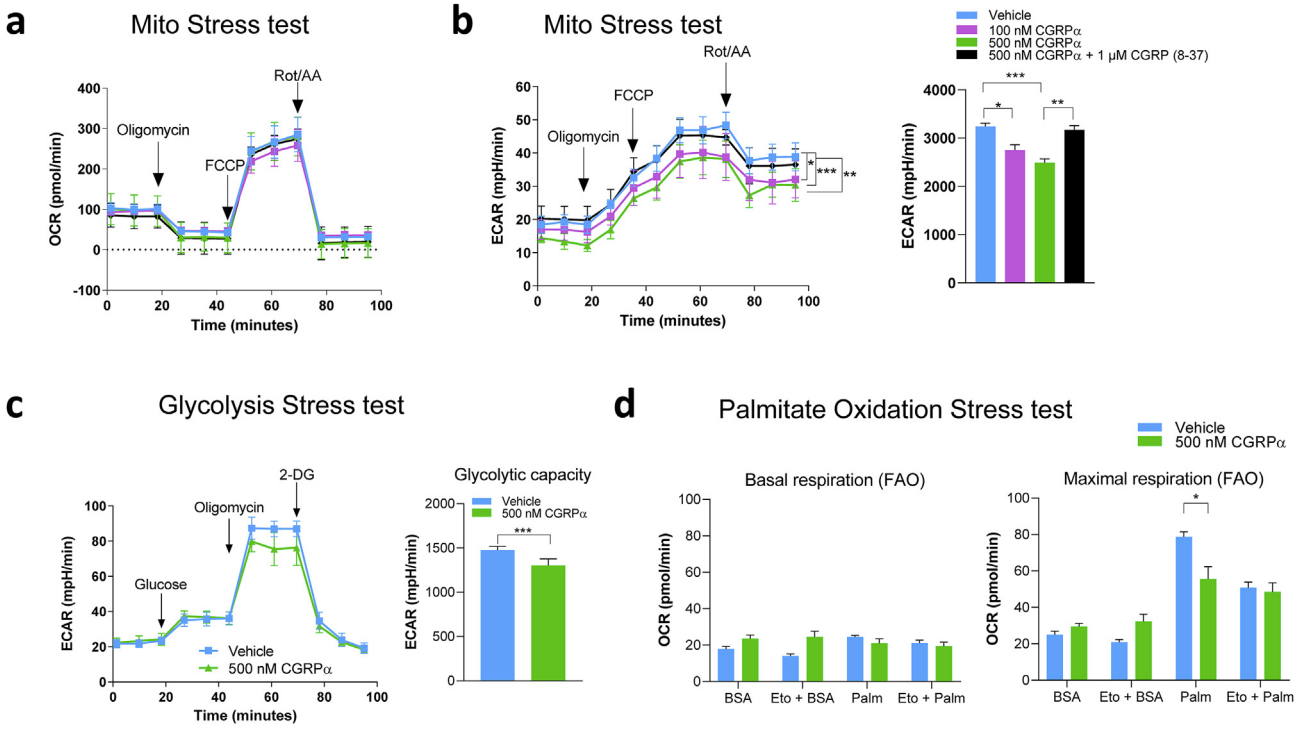

Fig. 6. Mitochondrial respiration analysis of primary adipocytes upon CGRPα treatment. (a) OCR data obtained from Seahorse XF analysis (mito stress test) from primary SVF inguinal adipocytes pre-treated with CGRPα and CGRP receptor antagonist CGRP (8–37), following the injection of oligomycin (ATP-linked respiration), FCCP (maximal respiratory capacity) and Rotenone/Antimycin A (reserve capacity). (b) ECAR data and cumulative ECAR responses to treatments in a (n = 5). (c) ECAR data from Seahorse glycolysis stress test of primary SVF inguinal adipocytes pre-treated with CGRPα following glucose, oligomycin and 2-deoxyglucose (2-DG) (n = 5). (d) OCR measured using Seahorse XF during a palmitate oxidation stress test under basal (left panel) and maximal respiration after the addition of FCCP (right panel). Adipocytes were pre-treated with CGRPα or vehicle (PBS) for 45 min. BSA was used as a control to Palmitate-BSA substrate, Etomoxir was used to inhibit carnitine palmitoyl transferase 1a import of long chain fatty acid for respiration (n = 6). ***p < 0.0001, **p < 0.001, *p < 0.05, two-way ANOVA, all values denote means ± SEM.

diabetic model of progressive islet hypertrophy. These data suggest that monoclonal therapy against CGRPα may be beneficial in lowering hyperglycemia in human type 2 diabetes.

Author contributions

JH, AL, MZ, HC and CER performed the experiments. CER designed the study and wrote the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metop.2020.100060.

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