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CagriSema drives weight loss in rats by reducing energy intake and preserving energy expenditure

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Julie Mie Jacobsen[1], Jens Frey Halling[1,2], Ida Blom[1,2], Jaime Moreno Martinez[3], Bjørn Hald[1], Kent Pedersen[1], Johannes Josef Fels[1], Søren Snitker[4], Anna Secher[1], Sofia Lundh[1], Carel W. le Roux[5], Kirsten Raun[1], Marc L. Reitman[6] & Rune Ehrenreich Kuhre[1,2]

CagriSema is a combination of amylin (cagrilintide) and glucagon-like peptide-1 (semaglutide) analogues being developed for weight management. Here, we show that CagriSema blunts metabolic adaptation in rats. Quantifying CagriSema's action on energy intake and expenditure in rats we observe 12% weight loss with a 39% reduction in food intake. By contrast, pair-feeding causes less-pronounced weight loss, while weight matching requires a 51% decrease in food intake. Therefore, approximately one-third of CagriSema's weight loss efficacy arises from an effect on energy expenditure, the blunting of metabolic adaptation, which contributes to the successful treatment of obesity.

Historically, centrally acting obesity medications are typically characterized as anorectic agents[1]. However, weight loss from calorie restriction is accompanied by metabolic adaptation, a decrease in metabolic rate below that predicted from the body weight (BW) and composition[2–5]. By preserving energy stores, this decrease contributes to the inability of most people to maintain their weight loss[6–8]. There are multiple drugs producing clinically relevant weight loss[9], including semaglutide (S)—a long-acting glucagon-like peptide-1 (GLP-1)[10] analogue and cagrilintide (C)—a long-acting amylin analogue[11]. When these are taken in combination (CagriSema), greater weight loss is achieved[12,13]. While C and S drive weight loss by reducing energy intake (EI)[11,14,15], it is less clear whether they also affect energy expenditure (EE). This study quantifies the relative contributions of EI versus EE to CagriSema-induced weight loss.

Diet-induced obese (DIO) male rats were treated with (1) vehicle with ad libitum feeding (V), (2) CagriSema with ad libitum feeding (CS), (3) vehicle with pair-feeding matched to the caloric intake of the CS rats (PF) or (4) vehicle with caloric restriction to match the weight of the CS rats (weight matched (WM)) (Fig. 1a,b and Extended Data Table 1). After 21 days, BW increased by 2.6% ± 0.71% in V rats and was reduced in the other cohorts by 8.7% ± 0.91% (CS), 5.6% ± 0.50% (PF) or 9.0% ± 0.42% (WM) (all $P < 0.0001$ compared with V; $P < 0.01$ between CS or WM and PF, one-way analysis of variation (ANOVA) with Tukey multiple comparison). Thus, the vehicle-adjusted weight loss was ~12% in the CS and WM rats, but only ~8% in the PF animals (Fig. 1c). The weight loss consisted primarily of fat (CS 15.2% ± 2.7%, PF 11.3% ± 2.9% and WM 19.7% ± 1.0% of baseline) with a smaller contribution of fat-free mass (FFM; CS 4.59% ± 1.67%, PF 2.25% ± 1.25% and WM 5.89% ± 1.81% of baseline, respectively) (Fig. 1d). CS treatment reduced cumulative EI by 39%, but the WM rats required a 51% reduction ($P = 0.03$ CS versus WM) to reach same weight loss (Fig. 1e,f). The cumulative total energy expenditure (TEE) during treatment was similar in the V and CS cohorts, but was reduced by 17% in the WM rats ($P = 0.008$) (Fig. 1g,h). Initially, CS treatment drastically reduced EI, which attenuated during treatment to a 25% reduction ($P = 0.001$) (Fig. 1i–l). For the WM group, the required EI restriction plateaued at 40% below baseline. Thus, the WM

[1]Global Drug Discovery, Novo Nordisk A/S, Måløv, Denmark. [2]Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark. [3]Digital Science and Innovation, Computational Biology, Novo Nordisk A/S, Måløv, Denmark. [4]Medical & Science, Clinical Drug Development, Novo Nordisk A/S, Søborg, Denmark. [5]Diabetes Complications Research Centre, University College Dublin, Dublin, Ireland. [6]Diabetes, Endocrinology, and Obesity Branch, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD, USA. e-mail: RUKU@novonordisk.com

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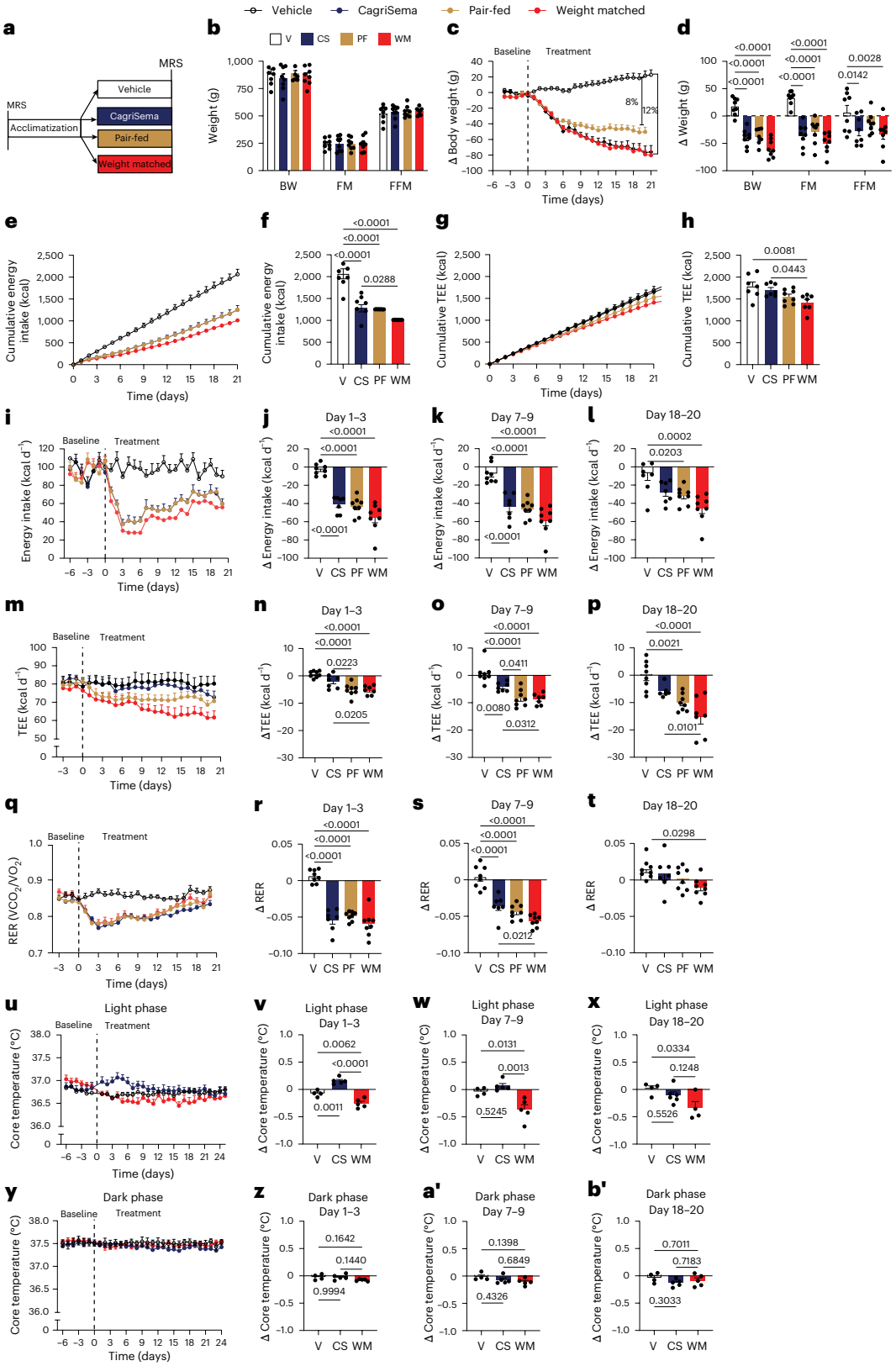

Fig. 1 | CagriSema drives weight loss by acting on both EI and EE. a, Study design. **b**, Baseline body weight (BW), fat mass (FM) and fat free mass (FFM). **c**, Body weight time course during treatment. **d**, Change in body weight, fat mass and fat free mass between day −5 and day 21. **e**,**f**, Cumulative energy intake (EI). **g**,**h**, Cumulative total energy expenditure (TEE). **i**–**l**, Daily EI. **m**–**p**, Daily TEE. **q**–**t**, Respiratory exchange ratio (RER). **u**–**x**, Light phase body temperature. **y**–**b′**, Dark phase body temperature. In **i**–**b′**, each row shows the time course followed by the change from baseline (mean of days −3 to −1) to the mean of days 1–3, 7–9 or 18–20, as indicated. A dashed line indicates the first day of treatment. Body temperature is from a cohort detailed in Extended Data Fig. 3. Data are mean ± s.e.m., $n = 10$ or 11 per group (**b**–**t**) and $n = 4$ or 5 per group (**u**–**b′**). *P* values from one-way ANOVA with Tukey post hoc testing. VCO_2, rate of CO_2 production; VO_2, rate of O_2 consumption; MRS, magnetic resonance spectroscopy.

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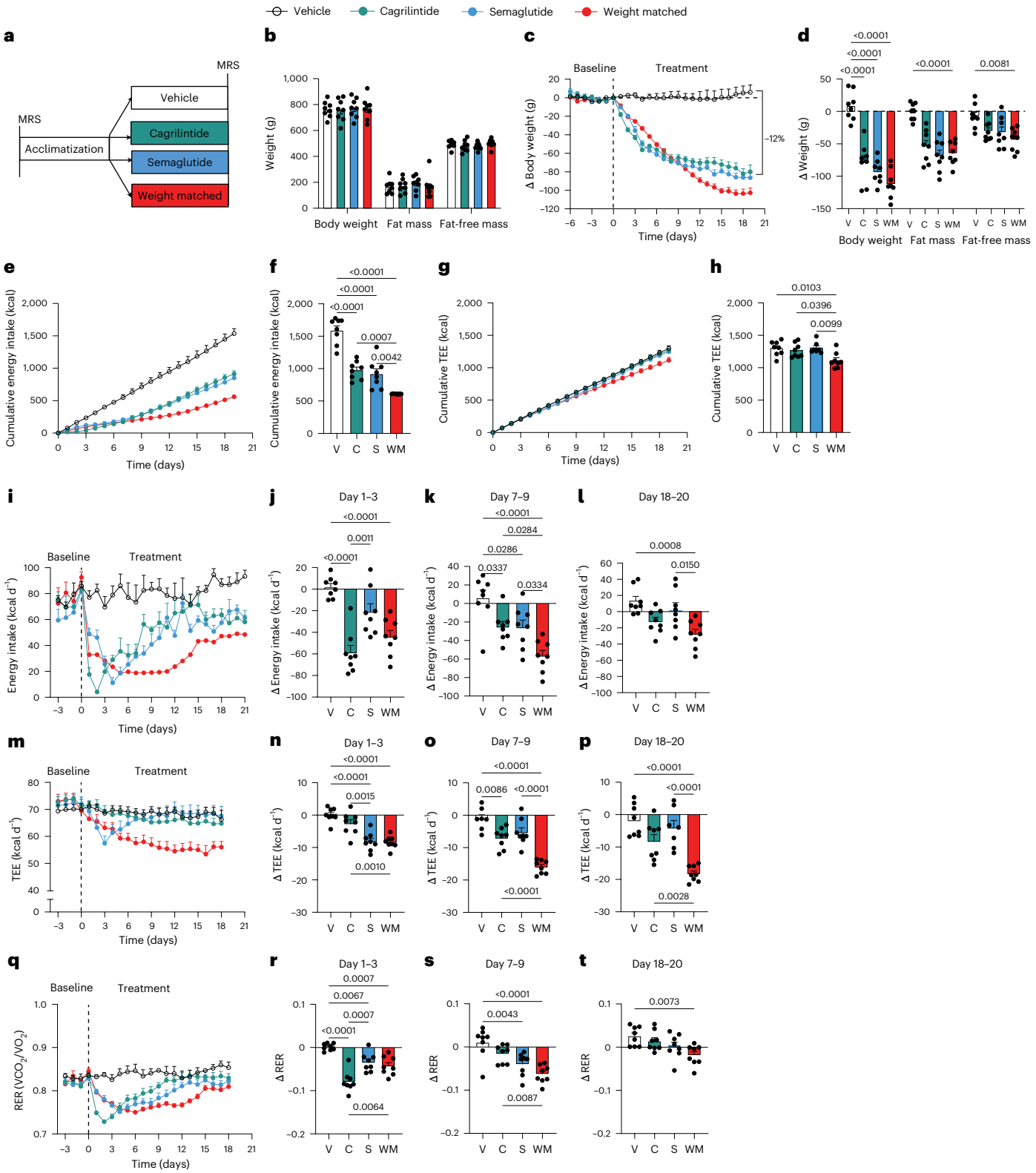

Fig. 2 | Cagrilintide and semaglutide act individually on both EI and EE. **a**, Study design. **b**, Baseline body weight (BW), fat mass (FM) and fat free mass (FFM). **c**, Body weight time course during treatment. **d**, Change in body weight, fat mass and fat free mass between day −6 and day 22. **e**,**f**, Cumulative energy intake (EI). **g**,**h**, Cumulative total energy expenditure (TEE). **i**–**l**, Daily EI. **m**–**p**, Daily TEE. **q**–**t**, RER. In **i**-**t**, each row shows the time course followed by the change from baseline (mean of days −3 to −1) to the mean of days 1–3, 7–9 or 18–20, as indicated. A dashed line indicates the first day of treatment. Data are mean ± s.e.m., $n = 8$ per group. P values from one-way ANOVA with Tukey post hoc testing. Rate of CO_2 production; VO_2, rate of O_2 consumption. MRS, magnetic resonance spectroscopy.

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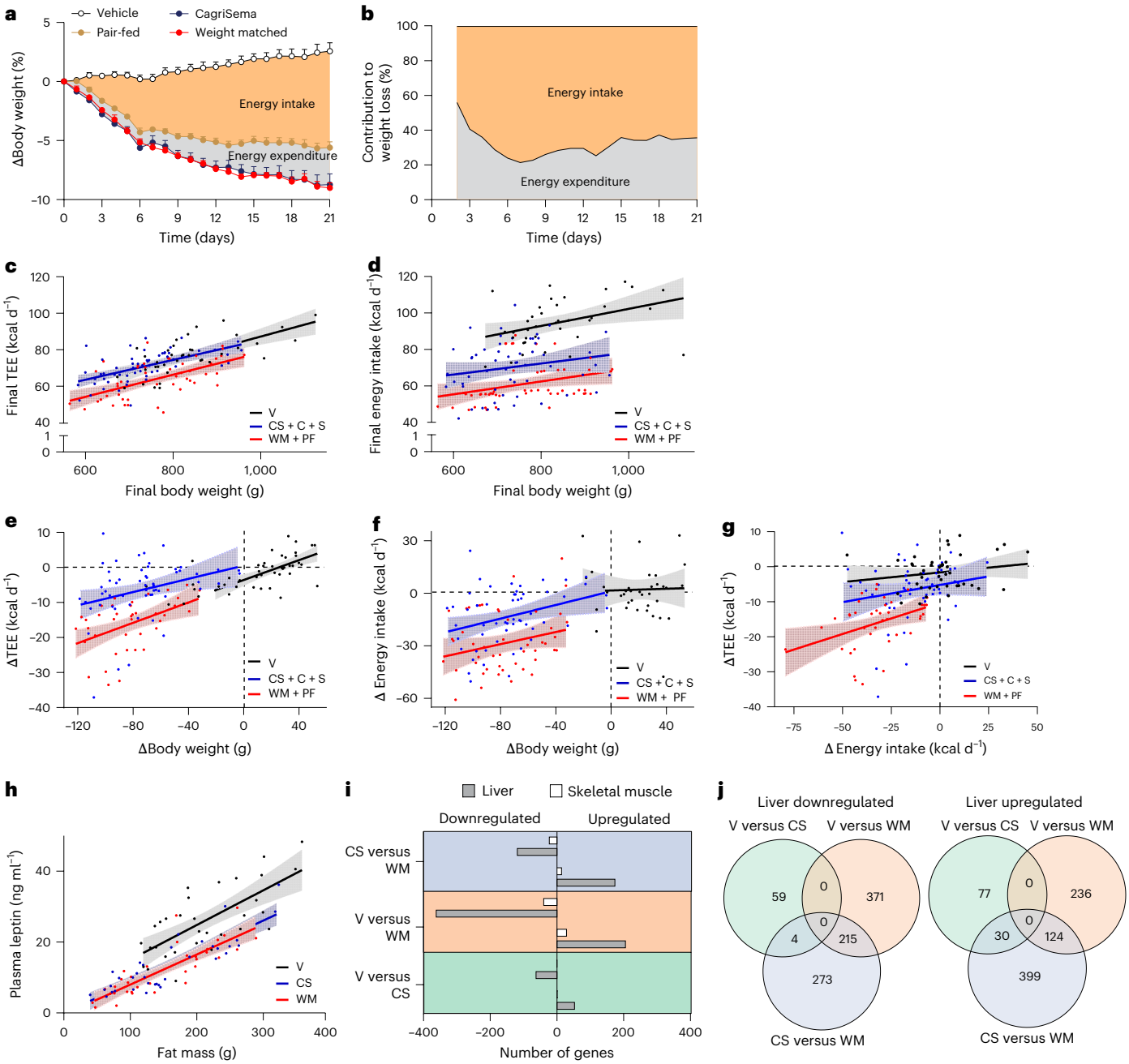

Fig. 3 | Prevention of metabolic adaptation is responsible for close to one-third of CagriSema-induced weight loss. a,**b**, Contributions of energy intake (EI) and energy expenditure (EE) to body weight (BW) loss. Data are taken from Fig. 1c. **c**–**g**, Effect of vehicle (V), drug (CS + C + S) or caloric restriction (WM + PF) treatments on final BW, EE and EI, and on the changes in these parameters. **e**–**g**, Dotted lines are at the zero of each axis (represent no changes). **h**, Plasma leptin levels after the indicated treatments (taken data from Fig. 1 and Extended Data Figs. 1, 2 and 3). **i**,**j**, Upregulated and downregulated genes by treatment. Pathway enrichment analysis from liver can be found in Extended Data Fig. 7. Detailed statistical analyses are given in Extended Data Table 2 and Supplementary Table 1. Group size: **a**,**b**, $n = 8$; **c**–**g**, $n = 38$ (V), $n = 57$ (CS + C + S), $n = 50$ (WM + PF); **h**, $n = 40$ (V), $n = 35$ (CS), $n = 34$ (WM); **i**,**j**, $n = 4$ (V, CS and WM). Data are presented as mean ± s.e.m. (**a**), absolute numbers (**b**, **i** and **j**) and as regression lines with 95% confidence bands (**c**–**h**).

group tended to require a greater reduction in EI than the CS cohort ($P = 0.0288$), suggesting differences in TEE. Indeed, TEE in CS-treated rats was higher than in WM rats at all points and was statistically different from V at days 7–9, but not at days 1–3 or 18–20 (Fig. 1m–p). The TEE of the WM rats was reduced by 22% on day 18–20 ($P < 0.0001$ versus V; $P = 0.01$ versus CS). The respiratory exchange ratio (RER) was only transiently reduced (Fig. 1q–t). In additional experiments with two other cohorts (Extended Data Figs. 1–3), TEE was significantly reduced in CS rats compared with V rats during days 1–3 and 7–9 in one experiment (Extended Data Fig. 3n–p), but not in the other (Extended Data Fig. 1n–p). The CS TEE reductions were less than the reduction seen in WM rats. Both studies replicated the differences in EI and EE between CS and WM rats during treatment and at the reduced BW state (days 18–20) (Extended Data Fig. 1 and Extended Data Fig. 3e–p). Additional experiments generally replicated the differences in EI and EE between CS and WM rats and found no effect of CS or WM on light or dark phase physical activity, circadian rhythm or body temperature. Analysing EE separately for the dark and light phases showed that the difference in TEE between CS and WM was driven by differences in both phases (Fig. 1u–b′ and Extended Data Figs. 1–3). In summary, the

WM group showed metabolic adaptation, which was diminished in the CS cohort.

Individually, C and S at 2 nmol kg^{-1} per day each produced about half the weight loss of the CS combination (2 + 2 nmol kg^{-1} per day), with similarly smaller effects on body composition, EI, TEE and RER (Extended Data Fig. 4). At higher doses (C, 10 nmol kg^{-1} per day; S, 30 nmol kg^{-1} per day), the C, S and WM rats lost similar amounts of BW, fat mass (FM) and FFM as the CS rats (Fig. 2a–d). Cumulative EI was reduced 39% and 42% in the C and S groups, less than the 62% seen in the WM rats ($P = 0.0007$, C versus WM; $P = 0.004$, S versus WM) (Fig. 2e,f). Cumulative TEE was again reduced in the WM cohort, but not the C or S rats (Fig. 2g,h). Initially, both C and S profoundly reduced daily EI, which attenuated with continued treatment (C was started at the full dose and S was dose-escalated over four days) (Fig. 2i–l). TEE was lower in C and S compared with V rats during the phase of profound EI inhibition and weight loss (days 1–3 and 7–9), but did not differ significantly at steady state (days 18–20), although TEE in C rats tended to be lower ($P = 0.08$). As with CS treatment, a reduced thermic effect of food and reduced BW contribute to these reductions and the TEE reductions were less than in WM rats. RER in the C and S cohorts was not different from RER in V rats (Fig. 2m–t). These results demonstrate that individually, C and S each counteract metabolic adaptation.

To quantify the contribution of metabolic adaptation, we examined the weight difference between the CS and PF rats. Based on the areas between the curves, 28% of the weight loss was due to EE and 72% to EI (Fig. 3a). The EE contribution increased during treatment, reaching 34% at 3 weeks (Fig. 3b). Next, all experiments (145 rats) were pooled into vehicle (V), drug-treated (CS + C + S) and caloric restriction (WM + PF) groups to incorporate BW into the analysis of TEE (Extended Data Table 1). We focused on the physiology at 3 weeks of treatment, when the drug dose is stable and the pharmacodynamic effects are near steady state, avoiding the earlier transient changes such as the massive reduction in food intake, which will lower TEE from a smaller thermic effect of food. Using analysis of covariance (ANCOVA), the final TEE did not differ between the V and drug groups, but was greater than in the calorically restricted rats (V versus WM + PF, $P < 0.0001$; CS + C + S versus WM + PF, $P < 0.0001$; CS + C + S versus V, $P = 0.97$) (Fig. 3c). The overlap of the vehicle and drug-treated groups suggests near complete prevention of metabolic adaptation at this time point. ANCOVA of final EI versus final BW demonstrated the highest EI in the vehicle group, intermediate levels in the drug-treated group and lowest intake with caloric restriction (V versus CS + C + S, $P < 0.0001$; V versus WM + PF, $P < 0.0001$; CS + C + S versus WM + PF, $P = 0.0002$) (Fig. 3d). When analysed as the change in BW from baseline (ΔBW), at matched ΔBW there was less TEE reduction in the drug-treated group than in the caloric restriction group ($P < 0.0001$) (Fig. 3e). Similarly, at matched ΔBW, the change in EI from baseline, ΔEI, was less in the drug-treated group than in the caloric restriction group ($P < 0.0001$) (Fig. 3f). Comparing the change in TEE from baseline, ΔTEE, versus ΔEI indicates that the three cohorts have a different ΔTEE for a given ΔEI (V versus WM + PF, $P < 0.0001$; CS + C + S versus WM + PF, $P = 0.0003$; CS + C + S versus V, $P = 0.081$) (Fig. 3g). Regression parameters for Fig. 3c–g are given in Extended Data Table 2. Thus, the ΔTEE–ΔEI relationship is fundamentally different among the ad libitum fed, calorically restricted and drug-treated weight-reduced states. Taken together, these results demonstrate that C, S and CagriSema act by reducing food intake and by blunting the metabolic adaptation that accompanies weight loss.

We examined possible mechanisms for this effect. Plasma leptin levels were similarly reduced in the CS and WM cohorts (Fig. 3h and Extended Data Fig. 5a). Triiodothyronine (T_3) and thyroxine (T_4) levels did not change (Extended Data Figs. 6a–d and 7a–d). Informative changes were also not detected for plasma glucagon, glucose, free fatty acids (FFA), glycerol, triglyceride, cholesterol, high-density lipoprotein (HDL), alanine aminotransferase or aspartate aminotransferase concentrations. As expected, insulin levels were reduced in the CS and WM cohorts, and β-hydroxybutyrate levels were elevated in the WM cohort (Extended Data Fig. 5b–l). Transcriptome analysis showed many changes in the WM rats, more in liver than muscle. By contrast, the CS rats had far fewer changes, closely matching the V profile (Fig. 3i–k and Extended Data Fig. 7).

Although analysis of the circulating factors and gene expression failed to identify mechanism(s) behind the abolished metabolic adaptation, it highlighted that physiology and gene expression are strikingly unperturbed in CS-treated rats compared with V rats, supporting the idea that after ~3 weeks of treatment the rats have achieved a metabolic steady state similar to their baseline despite a significantly reduced BW.

We demonstrate that attenuation of metabolic adaptation is an important mechanism of action for the weight-reducing efficacy of CagriSema in rats. The typical characterization of obesity medications as anorectics[1] is probably because food intake effect is visible, larger and easier to measure, especially in humans. We hypothesize that reducing metabolic adaptation is a general property of a successful obesity pharmaceutical. Further testing of this idea requires studies of longer duration, examination of other drug mechanisms, females, diets and species, including those that do not have considerable thermogenesis from brown adipose tissue. The influence of other weight-reducing drugs on EE has been examined in humans[16,17]. Although hampered by lack of weight matching in the control group, a clinical study of the dual GLP-1–glucagon receptor agonist SAR435899 (bamadutide) supports that pharmacological attenuation of metabolic adaptation is indeed a biological possibility in humans[16]. Because CagriSema changes both factors contributing to energy balance, a unifying concept of CagriSema's action is that it lowers the body's target weight. Presumably multiple brain regions contribute, including at least the hypothalamus and hindbrain[18]. Further mechanistic insights would be gained by identifying the specific neuronal circuits that coordinate this. It is expected that the neurologic effects of CagriSema are dependent on chronic drug treatment, because discontinuation of other GLP-1-based drugs causes weight regain[19,20]. The relatively normal physiology associated with CagriSema-induced weight loss compared with WM and PF animals can be understood as if CagriSema suppresses not only the subjective feeling of hunger, but also the starvation response in general. Future studies in humans could investigate whether there are associated clinical advantages such as a reduction in tiredness and stress levels compared with an equal weight loss by dieting alone.

In summary, CagriSema causes weight loss in rats by both reducing EI and preventing metabolic adaptation, with approximately one-third of the weight loss due to counteraction of suppression of EE.

Methods

Experimental models

Ethical considerations. Animal studies were conducted with permission from the Danish Animal Experiments Inspectorate (2020-15-0201-00683) in accordance the National Institutes of Health (publication number 85-23) and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1985).

Animals, housing and pre-experimental procedures. DIO male Sprague Dawley rats were obtained from Taconic and housed three per cage at ~22 °C with ad libitum access to 45% high-fat diet throughout all studies (Research Diets, catalogue no. D12451) and water, following a 12:12 h light and dark cycle (lights on at 0600 hours). At least two weeks before transfer to study, the light and dark cycle was reversed with lights on from 2300 to 1100 hours.

Measurement of energy expenditure. Five independent EE studies were performed (Extended Data Table 1). In each case, rats of similar BW and body composition (EchoMRI) were transferred to a Promethion Core Metabolic indirect calorimetry system (Sable Systems

International) and acclimated for 5–7 days to single housing with lights on from 2300 to 1100 hours at 22 °C with bedding material, a transparent plastic house or hide, nesting material (~15 g) and a wooden stick chew toy. BW, food intake and EE were measured daily for 3–5 days and used for distributing rats into groups matched all three parameters. Next, baseline data were collected for 3–5 days in conjunction with daily handling and restraint (at 0800 to 1000) to accustom the rats to the treatment phase. For treatments, rats were given a subcutaneous injection at weighing (0.5 ml per kg BW, 0800–1000) of vehicle (V, PF and WM groups), C, S or CagriSema. In all experiments, the vehicle was 0.007% polysorbate 20, 50 mM phosphate and 70 mM sodium chloride pH 7.4. CagriSema was dosed as follows: day 1, 0.5 + 0.5 nmol kg^{-1}; day 2, 1 + 1 nmol kg^{-1}; and day 3 to end, 2 + 2 nmol kg^{-1} d^{-1}. High-dose C was given at 10 nmol kg^{-1} d^{-1} from day 1 to the end. High-dose S was given as follows: day 1, 1 nmol kg^{-1}; day 2, 3 nmol kg^{-1}; day 3, 10 nmol kg^{-1}; and day 4 to end, 30 nmol kg^{-1} d^{-1}. The 10 and 30 nmol kg^{-1} doses of C and S were chosen based on previous preclinical toxicology studies during development demonstrating that these doses were well tolerated and matched the efficacy of CagriSema 2 + 2 nmol kg^{-1}. Pharmacologically relevant plasma concentrations were achieved (Extended Data Table 1). Food was replaced daily and drinking water was replaced two or three times per week. Two days before the end of treatment, body composition was determined by magnetic resonance spectroscopy (0900 to 1100 hours). WM rats ate their restricted food faster than CS rats (which eat continuously throughout the dark phase), but this is unlikely to have been a driving factor in the detected differences during weight stability at the lower BW because fasting time itself did not influence EE in WM rats (Supplementary Data Fig. 1). At the end of the experiment, blood was collected from the abdominal aorta under isoflurane anaesthesia and the rats were euthanized by decapitation. Blood was collected into prechilled EDTA-coated tubes, placed on ice and centrifuged (6,000 *g*, 4 °C, 5 min) within 15 min. Plasma was transferred into fresh Eppendorf tubes or tubes with added sodium fluoride (final concentration in whole blood was 5 mg ml^{-1}) for measurements of FFA and glycerol. Plasma was stored at −20 °C until analysis.

Quantification of core body temperature

Before transfer to the indirect calorimetry system to generate the data presented in Fig. 1u–b′, male DIO rats had a temperature-recording capsule implanted in the middle of the abdominal cavity to allow measurement for core body temperature[21]. To implant the capsule, rats were put in surgical anaesthesia by isoflurane inhalation and injected subcutaneously with antibiotics and analgesics (Rimadyl, 5 mg kg^{-1}; Baytril, 10 mg kg^{-1}; Anorfin, 0.05 mg kg^{-1}) approximately 30 min before anaesthesia. The temperature-recording capsule (Anipill; Animals Monitoring) was 17.7 × 8.9 mm and weighed 1.7 g. Implantation was done by a midline incision (~2 cm) with a scalpel. After insertion of the capsule, the muscle layer was closed with single sutures and the skin was closed with running hidden sutures. Surgery was performed under sterile conditions. For the next 3 days, rats were treated daily with Rimadyl (subcutaneously, 5 mg kg^{-1}). The rats were given 7 days to recover because thermoregulation is perturbed for approximately 5–7 days following surgery[22]. Rats were transferred to the indirect calorimetry system and the protocol described above was followed. During recovery, rats were monitored daily for clinical observations.

Measurement of activity level. Although the Sable system includes activity frames for measurements of activity level, such measurements were not possible in our experimental set-up because the DIO rat cages were too large to fit into the activity frames. To obtain information about potential differences in physical activity between the CS and WM cohorts, quantification of physical activity was based on implanted telemetry devices. Normal weight male Sprague Dawley rats were purchased from Charles River. On arrival at Novo Nordisk's animal facility, rats were housed under a 12:12 h light and dark cycle with lights on at 0600 hours. Rats were shifted from standard chow diet to ad libitum access to 60% high-fat diet (Research Diet, catalogue no. D12492) to induce obesity. After 9 months on the high-fat diet, rats weighted ~920 g at which point they were entered into the study. Rats were allocated to one of three groups (matched, CS and WM) matched by BW and body composition (FM and FFM) (Extended Data Fig. 2). Body compositions were obtained by magnetic resonance imaging, as described above. The surgical procedure and anaesthesia and analgesia were induced as described for the Anipill insertion, and a telemetry transducer (Data Sciences International, catalogue no. TA-F40) was inserted into the abdomen. After the operation, rats were returned to their home cages and the procedure used for recovery in the core body temperature study was followed. After recovery, baseline activity was obtained by moving rats in their home cages to telemetry receivers, to allow tracking of physical activity. Rats were single-housed during activity measurements. No changes were made to the light and dark rhythm, food supply or food type. Baseline physical activity was quantified over four consecutive days. After measurements, the treatment phase was initiated. During the treatment phase, a weight loss matching the trajectory and maximal weight loss in the indirect calorimetry studies was induced by either daily treatment with CagriSema (following the same dosing regimen as for the EE studies) or by calorie restriction to match weight loss in the CS group (WM group) using same dosing regimen as in the other CS experiments. Untreated rats were included for comparison (V). At the end of treatment period, recording of physical activity was repeated as outlined above. Activity data were processed using Ponemah software (Data Sciences International) and are presented as traces (counts per 15 min) as well as average counts, covering the respective four days of measurements. By end of activity measurements, body composition data were obtained by magnetic resonance image scanning.

Recordings and data processing

The rate of O_2 consumption, the rate of CO_2 production and water vapour pressure were recorded at 1 Hz with a 2.5 min cage time constant and flow rate of 3,500 ml min^{-1}. Food intake and water intake were collected by continuous recording (1 Hz) of food and water hopper weights. Food intake was also recorded by manual weighing. Data were processed using Sable Systems Macro Interpreter v.2.45, calculating EE and RER and filtering out abnormal values (for example, erroneous food intake events). The Macro Interpreter was set to output data at 5 min intervals for all parameters. We found no evidence for torpor events (core body temperature drops >3 °C). The procedures are described in detail in ref. 23.

Biochemical analysis

Plasma concentrations of FFA, glycerol, HDL, triglycerides, cholesterol, alanine aminotransferase and aspartate aminotransferase were quantified using a Cobas 6000 analyser (F. Hoffmann-La Roche AG), following instructions provided by the manufacturer. FFA and glycerol were quantified on the Cobas 6000 analyser in samples that were supplemented with sodium fluoride for stabilization. Plasma concentrations of insulin and leptin were measured using a custom developed Mesoscale (MSD) multiplex assay, which is a multi-array assay with electrochemiluminescence readout. The lower limit of quantification was 3.6 pM (insulin) and 1.8 pM (leptin). Plasma concentrations of T_3 and T_4 were quantified using commercial enzyme-linked immunosorbent assay kits (Antibodies Online, catalogue nos. ABIN6970859 (T_3) and ABIN6970688 (T_4)). Plasma concentrations of C and S (from samples taken 24 h after last dosing) were quantified by liquid chromatography–mass spectrometry (Supplementary Methods).

Gene expression analysis

RNA sequencing. Liver tissue and skeletal muscle tissue (quadriceps) (~0.5 cm^3 in each case) were extracted immediately after rats

were euthanized, placed into Eppendorf tubes with RNA (Invitrogen, catalogue no. AM7021) and kept on wet ice until storage at −20 °C. RNA was extracted using an RNeasy Mini Kit (Qiagen, catalogue no. 74106) and complementary DNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, catalogue no. 1708840) following the manufacturer's instructions. All samples had an RNA integrity number of >7, as determined using RNA ScreenTape Analysis (TapeStation, Agilent Technologies).

Bulk RNA transcriptomics sequencing. Bulk RNA sequencing was performed by Genewiz. Library preparation and sequencing were performed, followed by the trimming of sequence reads using fastp v.0.23.13 to remove potential adaptor sequences and low-quality nucleotides. Simultaneously, unique molecular identifier-based de-duplication was carried out using fastp v.0.23.1[24]. The resulting trimmed and de-duplicated reads were then aligned to the *Rattus norvegicus* Rnor6.0 reference genome, available on ENSEMBL, using STAR aligner v.2.5.2b4[25], producing BAM files. Subsequently, unique gene hit counts were calculated using featureCounts from the Subread package v.1.5.2 by summarizing and reporting the hit counts based on the gene id feature in the annotation file[26]. Only unique reads falling in exon regions were considered, and in cases in which a strand-specific library preparation was used, the reads were counted accordingly.

Principal component and differential expression analysis. Following the normalization of gene hit counts, the results underwent principal component analysis using the top 2,000 genes with the highest variance. Two samples were identified as markedly different from the remaining samples. Because of the potential of these outliers to distort the overall sample representation in the principal component analysis plot (Extended Data Fig. 7), they were excluded from the analysis solely for visualization purposes, without affecting subsequent analyses. In addition, DESeq26 (ref. 27) was utilized to compare gene expression across three distinct pair-wise tissue contrasts. The Wald test was used to derive P values and $\log_2$(fold change). Genes with an adjusted P value <0.05 were considered statistically significant as differentially expressed genes for each comparison.

Pathway enrichment analysis. A functional analysis was conducted on the statistically significant genes exhibiting an absolute $\log_2$(fold change) > 0.5. This analysis utilized clusterProfiler v.4.10.17[28] and encompassed three databases: Kyoto Encyclopedia of Genes and Genomes, Reactome and Gene Ontology terms. In addition, the Molecular Signature Database[29] was used to investigate the hallmark gene sets.

Data presentation and statistical analysis. Data are presented as mean ± s.e.m. Graphs were created in GraphPad Prism 9 and figures were edited in Adobe Illustrator (Adobe Systems). Statistical significance was assessed in GraphPad Prism and tested by one- or two-way ANOVA for repeated measurements followed by Tukey's multiple comparison test, or unpaired one-way ANOVA followed by Tukey's multiple comparison test. Gaussian distribution of data was confirmed before testing using the D'Agostino–Pearson normality test. Sample sizes are indicated in the 'Results' and figure legends. Consistent with current guidelines, TEE data are presented per animal, not adjusted to BW or FFM, and are analysed by ANCOVA when BW was included[30].

Analysis of pooled data. To increase the sample size and BW range, five independent experiments totalling 145 rats were pooled into vehicle (V, n = 38), drug (CS + C + S, n = 57) and calorie restriction (WM + PF, n = 50) groups (Extended Data Table 1). To reduce error, pretreatment values were the mean of measurements on days −2, −1 and 0, and final values were the mean of days 18, 19 and 20. Analyses were performed using JMP v.17.0.0. In a mixed model ANCOVA, the pretreatment TEE depended on BW, but also on experiment (adjusted (coefficient of determination) R^2 = 0.471, 68% of attributable variation due to BW and 32% to experiment). Pretreatment EI depended more on experiment than BW (adjusted R^2 = 0.437, 36% of attributable variation due to BW and 64% to experiment). These pretreatment differences reduce the power of the pooled analysis (more for EI than TEE). We did not perform prospective power calculations—each experiment used all available calorimetry chambers, and the pooled analysis included all experiments. Tissues contribute differently to metabolic rate, so we compared the model fit using different combinations of BW, FFM and FM. For both pretreatment TEE and pretreatment EI, BW gave similarly robust fits alone compared with adding FFM, FM or both, with or without 'experiment' as a covariate. However, removing BW, with or without FFM, FM or both, significantly worsened the fit. Thus, to avoid over-parameterization, we used BW without FFM and FM. These three groups did not differ by initial BW, TEE or EI. Because the treatments (V, CS, C, S, WM, PF) were not distributed evenly among the experiments, 'experiment' was not used as a covariate in the pooled analysis. For Fig. 3c–g, preliminary analyses demonstrated in each case that the interaction between 'treatment' and the dependent variable was not significant, so the interaction term was omitted. The plots in Fig. 3c–g are the simple linear regression lines (Extended Data Table 2). Statistical comparison between groups used mixed model ANCOVA (Supplementary Table 1). When native TEE, EI and BW units in Fig. 3e–g were changed to per cent changes, the results were quantitatively similar. Substituting FFM for BW in Fig. 3c–f also produced similar results (Supplementary Table 2).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

RNA sequencing data have been deposited under the accession code GSE295272. Data in Extended Data Figs. 1–7 and Exteneded Data Tables 1 and 2 are available from the corresponding author upon request. Source data are provided with this paper.

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Author contributions

R.E.K. conceptualized the study. J.M.J., J.F.H., I.B., J.M.M., B.H., K.P., J.J.F., M.L.R. and R.E.K. designed and performed the experiments. J.M.J., J.F.H., I.B., J.M.M., B.H., K.P., J.J.F., S.S., A.S., S.L., C.W.l.R., K.R. and M.L.R. analysed and interpretated data. R.E.K. and M.L.R. drafted the manuscript. J.M.J., J.F.H., I.B., J.M.M., B.H., K.P., J.J.F., S.S., A.S., S.L., C.W.l.R., K.R. and M.L.R. edited the manuscript and provided important intellectual content. All authors approved the final version of the paper.

Competing interests

J.M.J., J.F.H., I.B., J.M.M., B.H., K.P., J.J.F., S.S., A.S., S.L., K.R. and R.E.K. are employed by Novo Nordisk A/S, and some are minor shareholders in Novo Nordisk A/S. The other authors declare no competing interests. All authors declare that this work was not influenced by economic or other types of conflicting interest.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s42255-025-01324-8.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s42255-025-01324-8.

Correspondence and requests for materials should be addressed to Rune Ehrenreich Kuhre.

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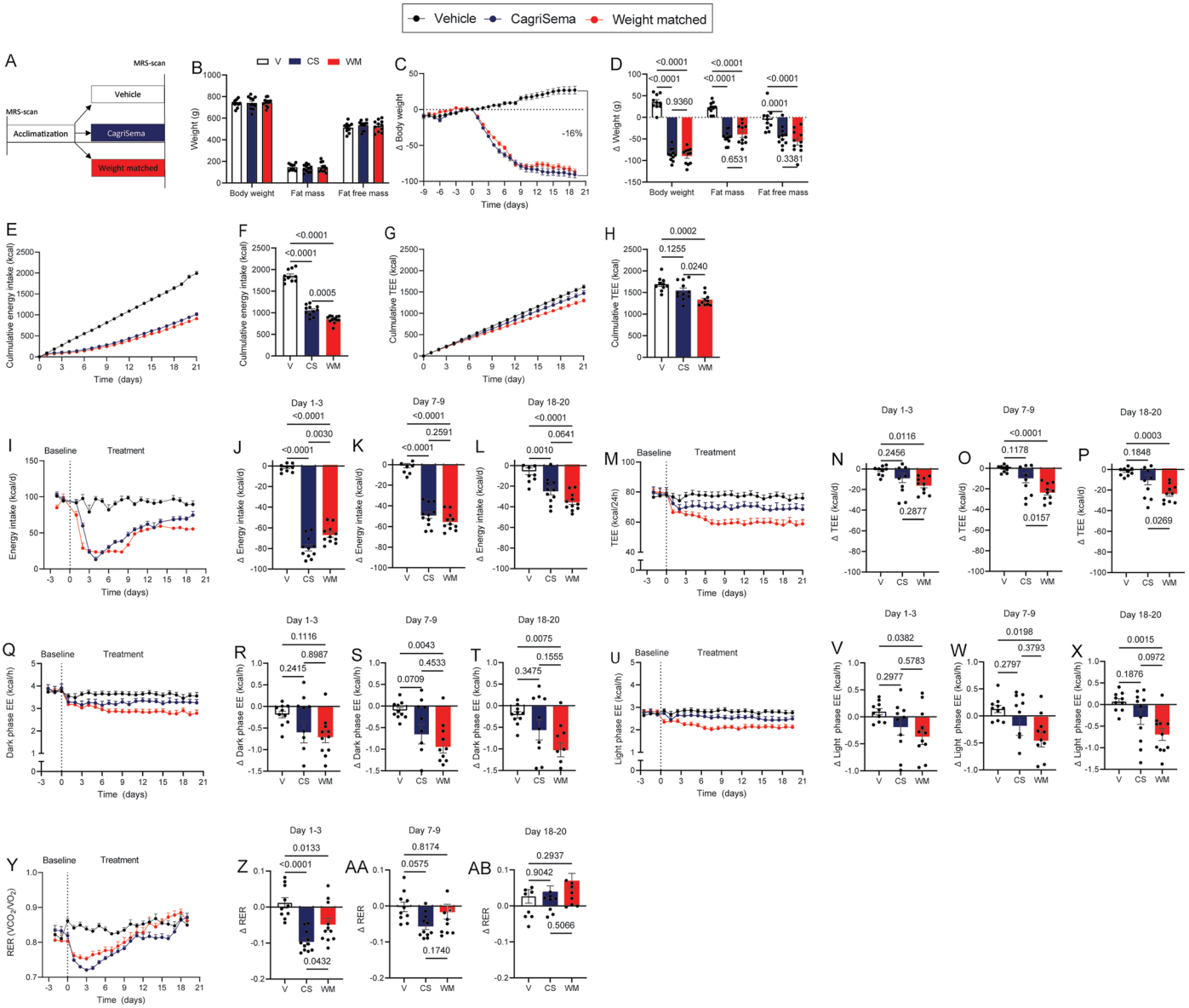

Extended Data Fig. 1 | Reproduction of main Fig. 1 results in an independent cohort. (**a**) Study design. Treatments were vehicle (V), CagriSema (CS, 2 + 2 nmol/kg), and calorie-restricted to match the weight of the CS rats (weight matched, WM). (**b**) Body weight, fat mass and fat free mass at day −5. (**c**) Body weight time course, (**d**) change in body weight, fat mass (FM), and fat free mass (FFM) from day −5 to day 21. (**e**, **f**) Cumulative energy intake and (**g**, **h**) cumulative total energy expenditure (TEE). (**i**-**l**) Energy intake, (**m**-**p**) TEE, (**q**-**t**) dark phase EE, (u-x) light phase EE, (**z**-**ab**) respiratory exchange ratio (RER). In each set, the time course is followed by the change from baseline (mean of days −3 to −1) to the mean of days 1-3, 7-9, and 18-20, as indicated. Data are mean ± SEM, n = 10 (V) or n = 11 (CS and WM); P values from one-way ANOVA with Tukey post hoc testing.

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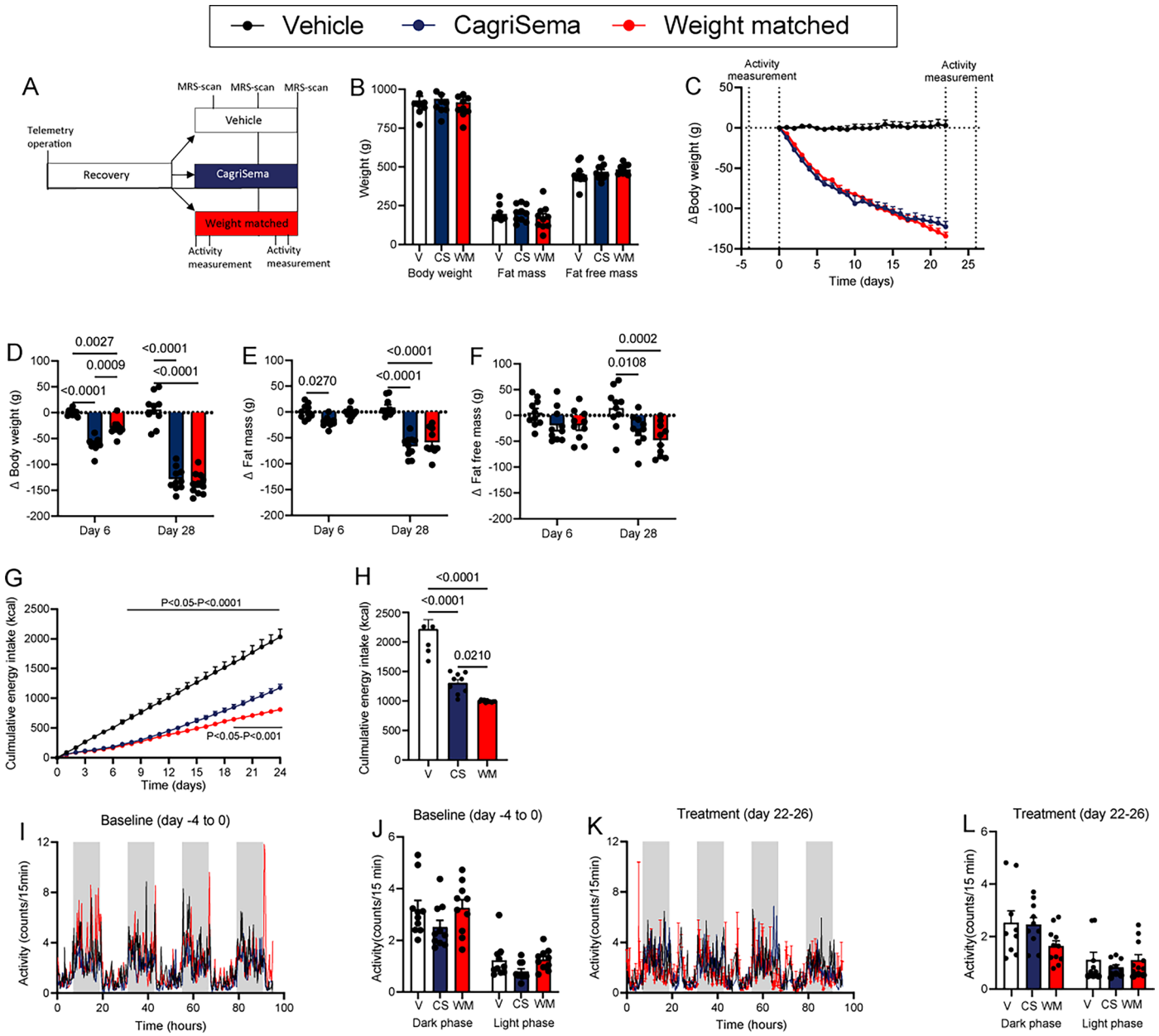

Extended Data Fig. 2 | No effect of treatment with CagriSema on physical activity. (**a**) Study design. Treatments are vehicle (V), CagriSema (CS, 2 + 2 nmol/kg), and calorie-restricted to match the weight of the CS rats (weight matched, WM). (**b**) Body weight, fat mass and fat free mass at day −6. (**c**) Body weight time course, (**d**-**f**) change in (**d**) body weight, (**e**) fat mass, and (**f**) fat free mass from day −6 to day 6 and day 28. (**g**, **h**) Cumulative energy intake. (**i**) Baseline physical activity time course (days −4 to 0). Dark phase is indicated by shading. (**j**) Baseline dark and light phase activity levels. (**k**) Activity level time course on treatment days 22 to 26. (**l**) Treated dark and light phase activity levels. Data are mean ± SEM, n = 10/group, P values from one-way ANOVA with Tukey post hoc testing.

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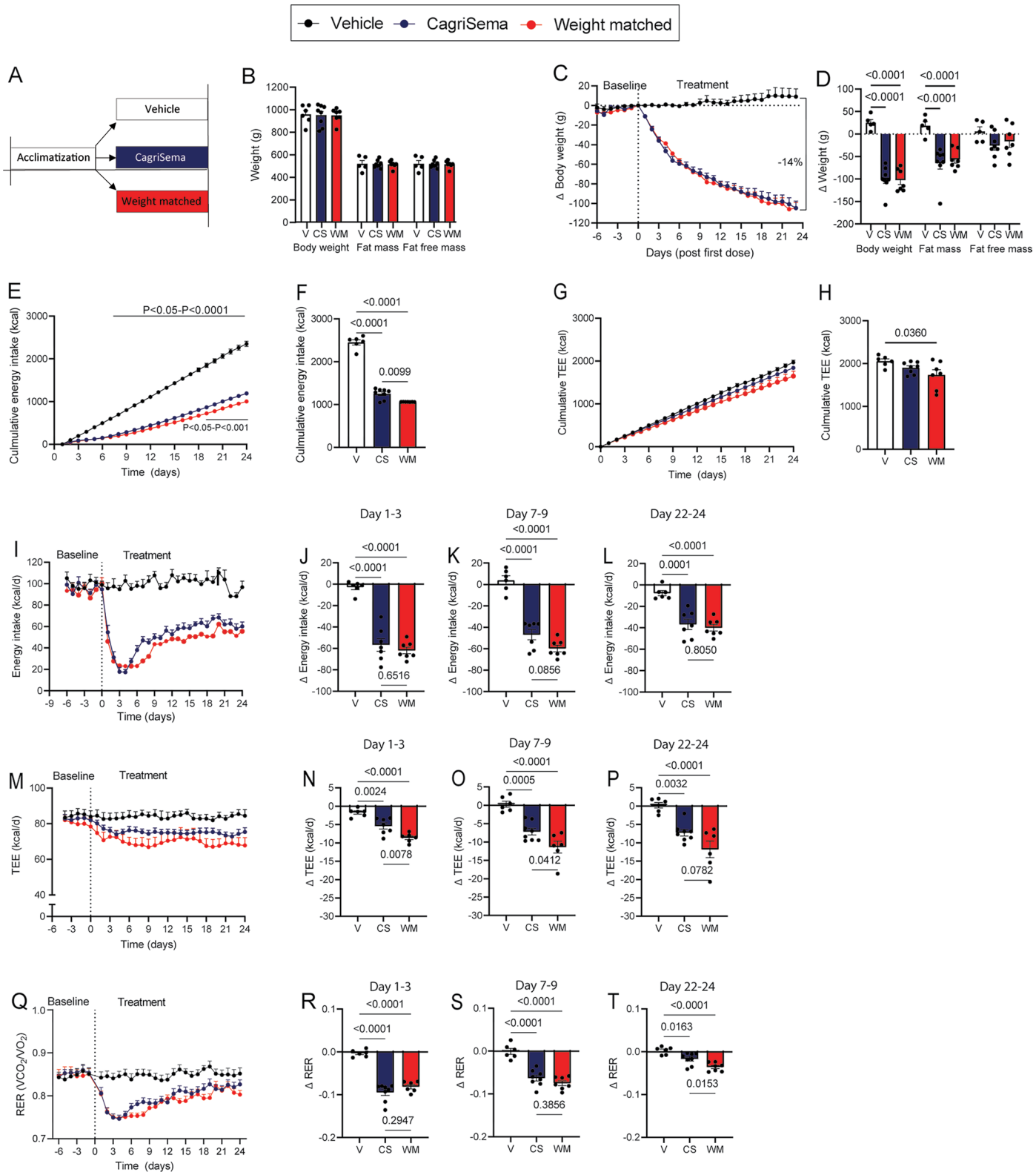

Extended Data Fig. 3 | Characteristics of rats treated with CagriSema for body temperature measurement. (**a**) Study design during indirect calorimetry. Treatments are vehicle (V), CagriSema (CS, 2 + 2 nmol/kg), and calorie-restricted to match the weight of the CS rats (weight matched, WM). (**b**) Body weight, fat mass and fat free mass at day −5. (**c**) Body weight time course, (**d**) changes in body weight, fat mass, and fat free mass from day −5 to 26. (**e**, **f**) Cumulative energy intake and (**g**, **h**) cumulative total energy expenditure (TEE). (**i**-**l**) Daily energy intake, (**m**-**p**) daily TEE, and (**q**-**t**) respiratory exchange ratio (RER). Body temperature dark or light phase (12 h bins) are in main Fig. 1u-ab. Data are mean ± SEM, n = 8/group. P-values from one-way ANOVA with Tukey post hoc testing.

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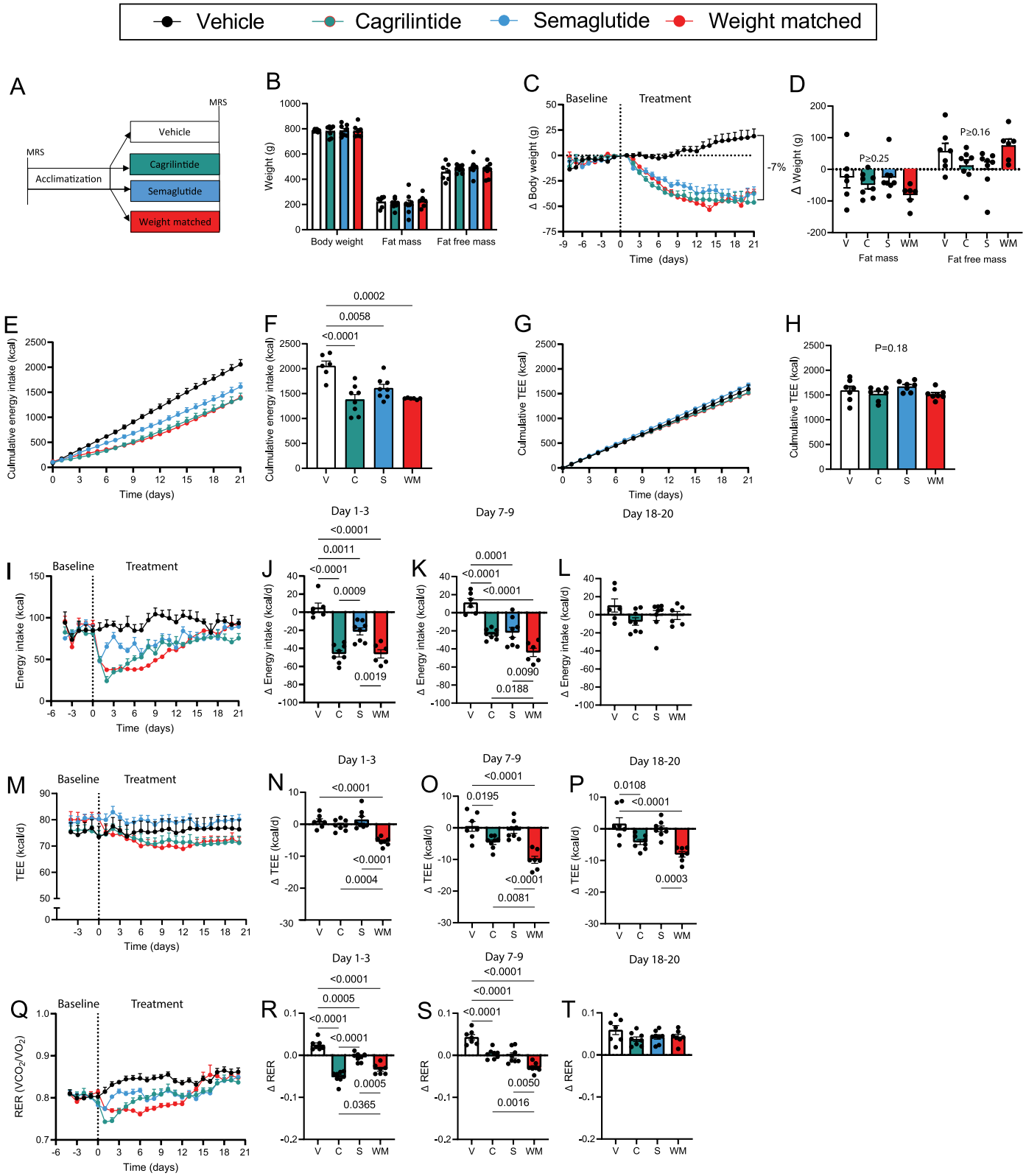

Extended Data Fig. 4 | Low efficacy of the individual CagriSema active agents. (**a**) Study design during indirect calorimetry. Treatments are vehicle (V), cagrilintide (C, 2 nmol/kg), semaglutide (S, 2 nmol/kg), and calorie-restricted to match the weight of the CS rats (weight matched, WM). (**b**) body weight, fat mass and fat free mass at day −5. (**c**) Body weight time course. (**d**) change in fat mass and fat free mass from day −5 to 21. (**e**, **f**) Cumulative energy intake and (**g**, **h**) cumulative total energy expenditure (TEE). (**i**-**l**) Daily energy intake, (**m**-**p**) daily TEE, and (**q**-**t**) respiratory exchange ratio (RER). In each set, the time course is followed by the change from baseline (mean of days −3 to −1) to the mean of days 1-3, 7-9, and 18-20, as indicated. Data are mean ± SEM, n = 6-8/group.

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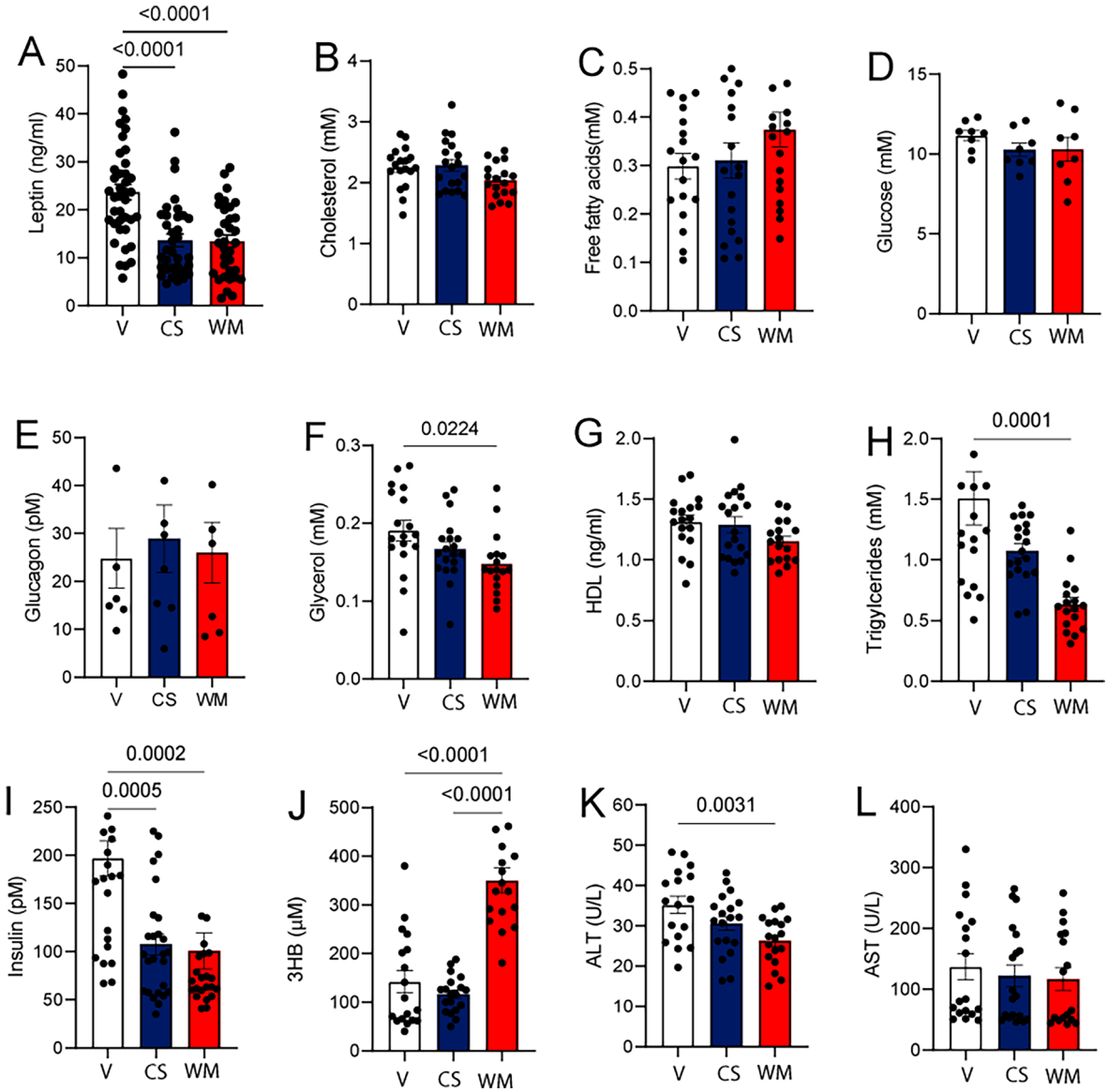

Extended Data Fig. 5 | Plasma concentrations ketone-bodies, glucose, glucagon, lipids, insulin, leptin, and liver inflammation markers. Concentrations after 5-6 h fast. (**a**): Leptin, (**b**): Cholesterol, (**c**): Free fatty acids, (**d**): Glucose, (**e**): Glucagon, (**f**) Glycerol, (**g**): High-density lipoprotein (HDL), (**h**): Triglycerides, (**i**): Insulin, (**j**): 3-hydroxybuturate (3HB), (k) alanine transaminase (ALT), and (**l**) aspartate transaminase (AST). Data (all except leptin) are pooled from the experiments reported in main Fig. 1 and Extended Data Fig. 1). Leptin are from main Fig. 1, Extended Data Figs. 1, 2, 3, and 4). Data are mean ± SEM, n (all except leptin: V = 18, CS and WM = 19. n (leptin): V = 40, CS = 35, and WM = 34. Statistical significance was tested by One-way ANOVA followed by Tukey post hoc test. Colours: White: vehicle, Dark blue: CagriSema, and grey: weight matched.

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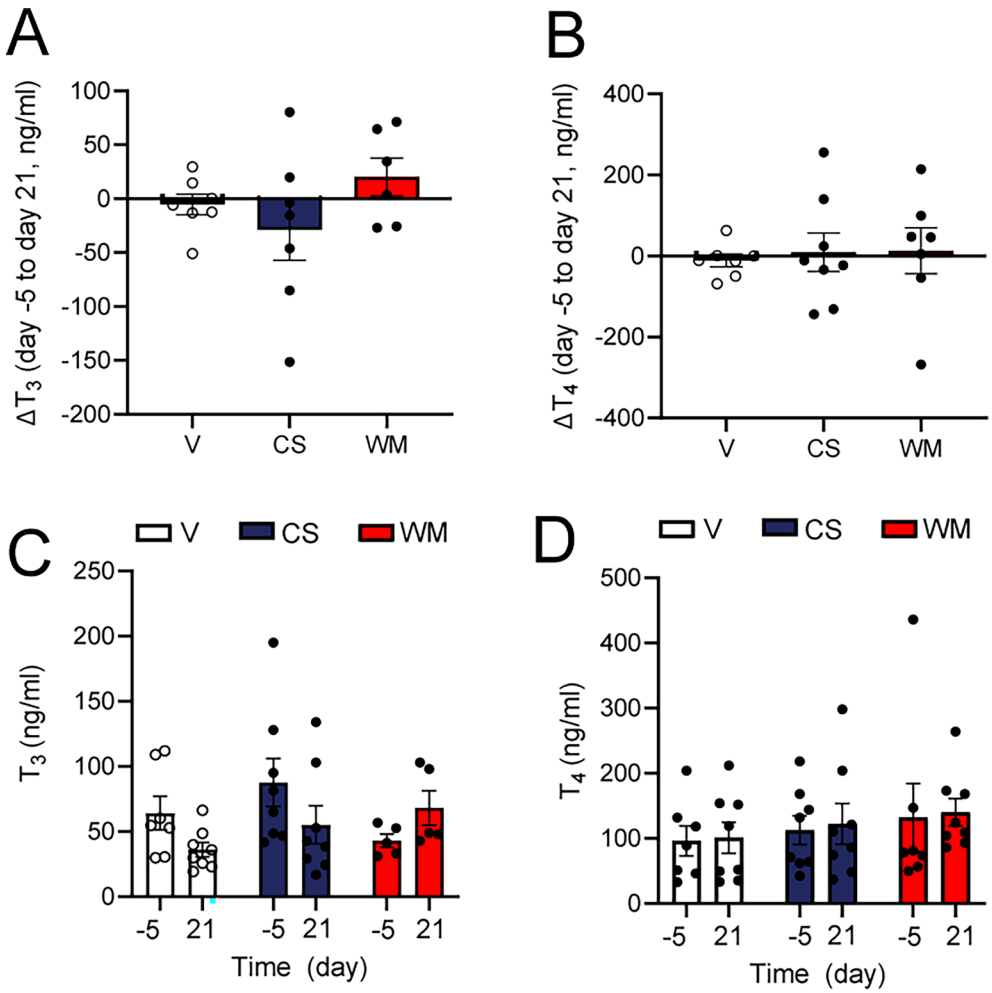

Extended Data Fig. 6 | Changes in plasma concentrations triiodothyronine (T_3) and thyroxine (T_4). Samples are from rats in main Fig. 1. **a**: T_3 and **b**: T_4, **c** and **d**: T_3 and T_4 at baseline (day −5) and by end of treatment (day 21), Data are shown as means ± SEM. Dots are individual rats. n = 6-8/group. Statistical significance was tested by Oneway ANOVA. ANOVA-value was in both cases non-significant ($P > 0.05$). Abbreviations: V: vehicle, CS: CagriSema, and WM: Weight matched.

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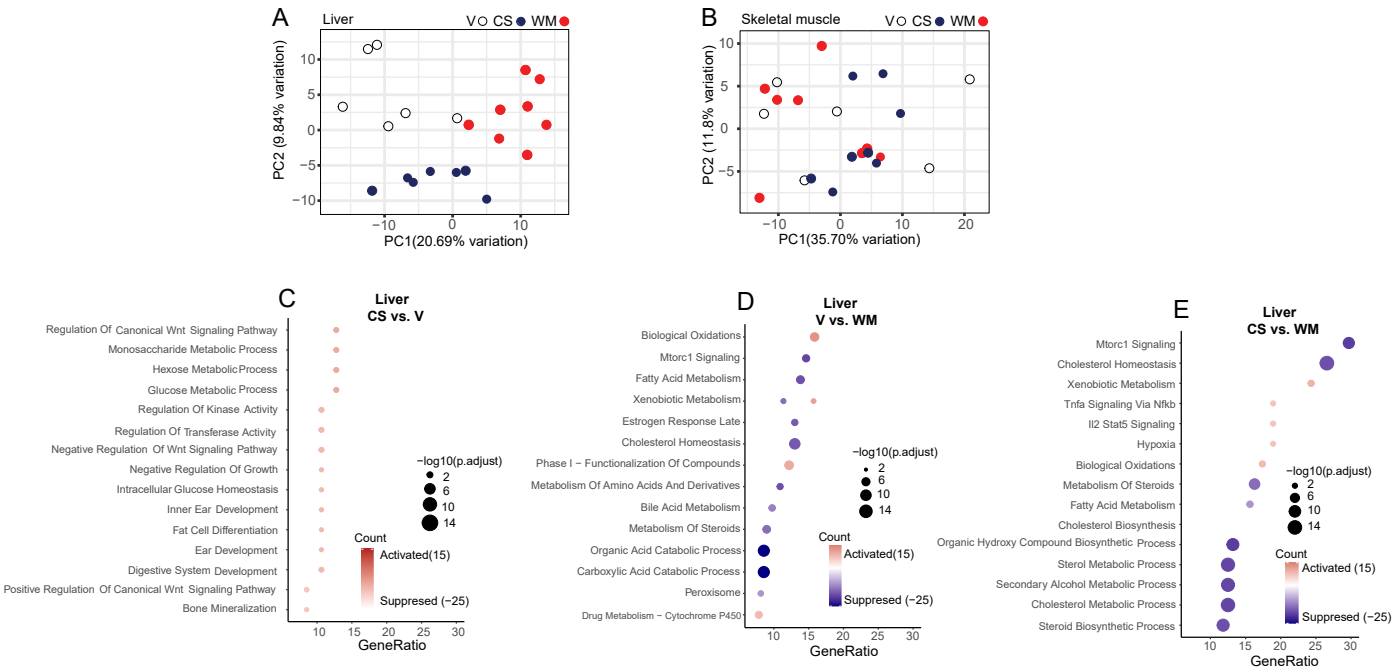

Extended Data Fig. 7 | Principal component and pathway enrichment analysis. Samples are from main Fig. 2. **a** and **b**: Principal component analysis of liver (**a**) and skeletal muscle gene expression (**b**). (**c**-**e**) Pathway enrichment analysis of liver transcriptomes; **c**: V vs. CS, **d**: V vs. WM, and **e**: CS vs. WM (n = 8). Abbreviations: V: vehicle, CS: CagriSema, WM: Weight matched.

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Extended Data Table 1 | Characteristics of rat cohorts

Experiment	Treatments	Initial body weight (g)	Age at start of treatment (wks)	n/group	Exposure (pM)
Main fig. 1	V, CS, PF, WM	882.4 ±16.2	37	8	C: 15,750±1,832 S: 4,735±754.3
Main fig. 2	V, C, S, WM	761.5 ±12.5	36	8	C: 287,444±444 S: 184,750±5,918
Extended fig. 1	V, CS, WM	751.7 ±7.7	42	8	C: 22,809±1,595 S: 14,309±1,261
Extended fig. 2	V, CS, WM	958.0 ±16.8	41	10	-
Extended fig. 3	V, CS, WM	935.5 ±18.2	57	7-8	C: 14,360±3,056 S: 12,138±1,052
Extended fig. 4	V, C, S, WM	792.4 ±6.6	31	7-8	
Main fig. 3	Pool group	Initial body weight (g)	Final body weight (g)	n/group	
	V	818.5 ±17.0	839 ±17	38	
	CS+C+S	807.4 ±12.8	734 ±12	57	
	WM+PF	836.8 ±13.9	761 ±14	50	
	All	820.4 ±8.3		145	

Body weight and age are means±SEM. Exposure data are means±SD. Exposure levels are from 24 h after last administration. Abbreviations: V: vehicle, C: cagrilintide, CS: CagriSema, S: semaglutide, PF: pair-fed, and WM: weight matched.

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Extended Data Table 2 | Linear regression parameters for Fig. 3

Final total energy expenditure vs. final body weight, Fig. 3C

Pool group	R^2	Slope	Intercept
V	0.474	0.0653±0.0115	21.9±9.7
CS+C+S	0.359	0.0543±0.0098	30.9±7.2
WM+PF	0.344	0.0606±0.0123	17.8±9.5

Final energy intake vs. final body weight, Fig. 3D

Pool group	R^2	Slope	Intercept
V	0.156	0.0469±0.0184	55.4±15.6
CS+C+S	0.038	0.0303±0.0206	48.0±15.2
WM+PF	0.092	0.0353±0.0160	34.2±12.3

Delta total energy expenditure vs. delta body weight, Fig. 3E

Pool group	R^2	Slope	Intercept
V	0.389	0.1416±0.0296	-3.6±0.8
CS+C+S	0.100	0.0946±0.0383	0.5±3.0
WM+PF	0.334	0.1703±0.0355	-1.4±2.8

Delta energy intake vs. delta body weight, Fig. 3F

Pool group	R^2	Slope	Intercept
V	0.001	0.0249±0.1448	1.6±9.7
CS+C+S	0.241	0.3089±0.0746	6.4±5.8
WM+PF	0.225	0.3097±0.0829	-9.0±6.7

Delta energy intake vs. delta total energy expenditure, Fig. 3G

Pool group	R^2	Slope	Intercept
V	0.033	0.0481±0.0441	-0.81±0.74
CS+C+S	0.046	0.1029±0.0635	-4.81±1.50
WM+PF	0.221	0.2100±0.0581	-7.61±2.12

Linear regression parameters for Fig. 3: 1) total energy expenditure vs. final body weight (Fig. 3c), 2) final energy intake vs. final body weight (Fig. 3d), 3) delta total energy expenditure vs. delta body weight (Fig. 3e), 4) delta energy intake vs. delta body weight (Fig. 3f), 5) delta energy intake vs. delta total energy expenditure (Fig. 3g).

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Energy expenditure data were collected and processed by Sable Systems Macro Interpreter v. 2.45
Data analysis	Data are presented as means±SEM. Graphs were made in GraphPad Prism 9 (La Jolla, CA) and figures were edited in Adobe Illustrator (Adobe Systems Incorporated, San Jose, CA). Statistical significance was assessed in GraphPad Prism and tested by one-/two-way ANOVA for repeated measurements followed by Tukey's multiple comparison test, or unpaired one-way ANOVA followed by Tukey multiple comparison test, as indicated in figure legends

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All data in fig. 1-3 are deposited as source data. RNA seq. raw data have been deposited under the accession code GSE295272. Data in extended figures are available from the corresponding author upon request.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	N/A
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Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample sizes were chosen based on pilot experiments
Data exclusions	No data were excluded from the analysis
Replication	The main findings in this study (fig. 1) was replicated in two independent experiments (Extended data Fig. 1 and -3).
Randomization	Rats were allocated into groups matched on body weight, body composition, food intake and energy expenditure.
Blinding	Blinding was not possible in this study as rats were administered different traceable compounds.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	ELISA kits (T3: Antibodies online cat# ABIN6970859 T4: Antibodies online cat# ABIN6970688). used antibodies.
Validation	Provided instructions were followed. Informations regarding dilutions are not disclosed.

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	DIO male Sprague Dawley (SD) rats were used in this study. Rats were 31-57 weeks at start of treatments.
Wild animals	No wild animals were used in this study
Reporting on sex	This study exclusively used male SD DIO rats.
Field-collected samples	No field-collected samples were used in this study.
Ethics oversight	Animal studies were conducted with permission from the Danish Animal Experiments Inspectorate (2020-15-0201-00683) in accordance the National Institutes of Health (publication number 85-23) and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1985).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	No seed stocks were used in this study
Novel plant genotypes	No novel plant genotypes were used in this study
Authentication	Not relevant