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ARTICLE

Contribution of brown adipose tissue activity to the control of energy balance by GLP-1 receptor signalling in mice

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Abstract
Aims/hypothesis We assessed the contribution of glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) signalling to thermogenesis induced by high-fat diet (HFD) consumption. Furthermore, we determined whether brown adipose tissue (BAT) activity contributes to weight loss induced by chronic subcutaneous treatment with the GLP-1R agonist, liraglutide, in a model of diet-induced obesity.
Methods Metabolic phenotyping was performed using indirect calorimetry in wild-type (WT) and *Glp1r*-knockout (KO) mice during chow and HFD feeding at room temperature and at thermoneutrality. In a separate study, we investigated the contribution of BAT thermogenic capacity to the weight lowering effect induced by GLP-1 mimetics by administering liraglutide (10 or 30 nmol kg^{-1} day^{-1} s.c.) to diet-induced obese (DIO) mice for 6 or 4 weeks, respectively. In both studies, animals were subjected to a noradrenaline (norepinephrine)-stimulated oxygen consumption ($\dot{V}O_2$) test.
Results At thermoneutrality, HFD-fed *Glp1r*-KO mice had similar energy expenditure (EE) compared with HFD-fed WT controls. However, HFD-fed *Glp1r*-KO mice exhibited relatively less EE when housed at a cooler standard room temperature, and had relatively lower $\dot{V}O_2$ in response to a noradrenaline challenge, which is consistent with impaired BAT thermogenic capacity. In contrast to the loss of function model, chronic peripheral liraglutide treatment did not increase BAT activity as determined by noradrenaline-stimulated $\dot{V}O_2$ and BAT gene expression.
Conclusions/interpretation These data suggest that although endogenous GLP-1R signalling contributes to increased BAT thermogenesis, this mechanism does not play a significant role in the food intake-independent body weight lowering effect of the GLP-1 mimetic liraglutide in DIO mice.

Keywords Brown adipose tissue · Diet-induced thermogenesis · GLP-1 · High-fat diet · Liraglutide

Abbreviations

ARC	Arcuate nucleus
BAT	Brown adipose tissue
BW	Body weight
CD	Control diet
CNS	Central nervous system
DIO	Diet-induced obese
DIT	Diet-induced thermogenesis
EE	Energy expenditure
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
HFD	High-fat diet
iBAT	Interscapular brown adipose tissue
iWAT	Inguinal white adipose tissue
KO	Knockout
PPG	Preproglucagon
UCP	Uncoupling protein
VMH	Ventromedial hypothalamic nucleus

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$\dot{V}O_2$	Oxygen consumption
WT	Wild-type

Introduction

Glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) mimetics are effective therapeutics for type 2 diabetes mellitus (reviewed in [1]). In addition to experiencing improved glucose homeostasis, many patients lose weight during treatment with GLP-1-based therapies. Multiple clinical studies suggest that weight loss induced by GLP-1 is mainly attributed to reduced food intake (reviewed in [2]).

We and others have demonstrated that acute activation of central nervous system (CNS) GLP-1Rs increases the activity of brown adipose tissue (BAT) [3, 4]. Given the contribution of BAT to energy expenditure (EE), GLP-1-induced activation of BAT could potentially be an additional mechanism by which GLP-1R activation causes weight loss. BAT is a metabolically active tissue that oxidises, fuels and dissipates energy in the form of heat (reviewed in [5]). Animals lacking functional BAT have decreased EE and are more susceptible to developing obesity [6, 7]. High-fat diet (HFD) consumption increases thermogenesis and EE, which is referred to as diet-induced thermogenesis (DIT; reviewed in [8] and [9]). The mechanisms regulating DIT are not completely understood but seem to be partially due to increased sympathetic innervation of BAT [10, 11], which is assessed in animals at thermoneutral temperatures [7].

Recent studies have demonstrated the existence of functionally active BAT in adult humans [12–14], suggesting that agents that induce BAT thermogenesis may be attractive candidates for treating obesity [15]. Given the evidence of GLP-1R regulation of BAT as well as body weight (BW) loss induced by GLP-1R analogues, we aimed to investigate whether endogenous GLP-1R signalling contributes to increased BAT thermogenesis induced by high-fat feeding. Next, we aimed to determine whether BAT thermogenesis contributes to BW loss induced by chronic peripheral treatment with the GLP-1R agonist, liraglutide.

Methods

Animals All studies were approved and performed following the guidelines of the institutional animal care and use committees of the University of Cincinnati. *Glp1r*-knockout (KO) and age-matched wild-type (WT) male mice on a C57/BL6J background were generated as previously described [16] and bred at the University of Cincinnati. Mice were maintained on a 12:12 h light–dark cycle at 22°C with free access to water and to either a standard chow control diet (CD; 5.6% fat; LM-485, Teklad, Harlan; Indianapolis, IN, USA) or a high-sucrose diet containing 58% energy from fat (HFD; Research Diets #D12331, New Brunswick, NJ, USA), as indicated. Mice were 12–14 weeks and 16–18 weeks of age for CD and HFD diet experiments, respectively.

Male C57BL/6J mice (The Jackson Laboratory; Bar Harbor, ME, USA) were fed HFD (Research Diets #D12331, New Brunswick) starting at 10 weeks of age and were then maintained on the diet for 24 weeks prior to liraglutide treatment. Mice were randomly assigned to groups using Microsoft Excel. Experimenters were not blind to group assignment and outcome assessment. All the data collected are presented. In the case of the gene expression analysis, a subset of samples per group was randomly chosen using Microsoft Excel.

Indirect calorimetry Mice were housed in chambers with integrated control of ambient temperature and simultaneous measurement of food intake, locomotor activity and EE by indirect calorimetry (TSE Systems, Chesterfield, MO, USA). Mice were monitored at 22°C or 31°C to compare energy balance at standard room temperature or at thermoneutrality, respectively.

Noradrenaline (norepinephrine) stimulation of oxygen consumption Animals were adapted to 31°C overnight (14–16 h) prior to experiments. Oxygen consumption ($\dot{V}O_2$) was analysed in response to s.c. (1 mg kg^{-1}) injection of noradrenaline (norepinephrine; Sigma-Aldrich, St Louis, MO, USA).

Body composition measurements Whole-body composition (fat and lean mass) was measured using NMR technology (EchoMRI-100; Echomedical Systems, Houston, TX, USA) [17].

Chronic liraglutide treatment Liraglutide was synthesised at Indiana University as previously described [18], and injected s.c. at a dose of 10 nmol kg^{-1} day^{-1} or 30 nmol kg^{-1} day^{-1} in diet-induced obese (DIO) mice. To determine the contribution of hypophagia to the BW lowering effect of liraglutide, a vehicle-treated pair-fed group was fed the same amount of food consumed by the corresponding liraglutide-treated group. Animals were housed in conventional cages at 22°C then placed in an indirect calorimetry system as indicated.

RNA extraction and quantitative PCR RNA from interscapular BAT (iBAT), inguinal white adipose tissue (iWAT), quadriceps muscle and soleus muscle was extracted using a commercially available kit (RNeasy; Qiagen, Valencia, CA, USA) following the manufacturer's instructions. After DNase I treatment (Invitrogen, Carlsbad, CA, USA), cDNA was synthesised using SuperScript-III (Invitrogen), and gene expression was determined by

quantitative PCR using gene-specific TaqMan assays (Life Technologies, Carlsbad, CA, USA). Gene expression was evaluated using the $\Delta\Delta C_t$ method. The housekeeping gene for iWAT and iBAT gene analysis was 18S, and that for muscle gene analysis was *Rpl32*.

Statistical analysis Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was determined by unpaired Student's *t* test, one-way ANOVA followed by Tukey's multiple comparison post hoc test or two-way ANOVA followed by Bonferroni's multiple comparison post hoc test. All results are given as means±SEM. Results were considered statistically significant when $p<0.05$.

Results

Metabolic phenotype of *Glp1r*-KO mice at room temperature (22°C) and thermoneutrality (31°C) under chow-fed and HFD-fed conditions First, we investigated the contribution of GLP-1R signalling in regulating energy balance in WT and *Glp1r*-KO mice fed a low-fat, CD at standard room temperature (22°C) or at thermoneutrality (31°C; Fig. 1a–g). By observing animals at both temperatures, we can dissect out differences in EE resulting from changes in BAT activity, given that this is greatly reduced at thermoneutral temperatures [19]. As we [3] and others [16] have previously reported, BW was similar between the WT and *Glp1r*-KO mice on a CD (Fig. 1a). Animals were placed in an indirect calorimetry system for 72 h to simultaneously assess $\dot{V}O_2$, EE and food intake. Both genotypes had similar $\dot{V}O_2$ at 22°C and 31°C (Fig. 1b, c). Average EE, food intake and net energy balance (calculated by subtracting caloric intake from EE) were significantly higher in animals maintained at an ambient temperature of 22°C vs 31°C, yet similar between genotypes at both temperatures (Fig. 1d–f). In contrast, locomotor activity was significantly lower at 22°C vs 31°C and significantly lower in *Glp1r*-KO compared with the WT controls (Fig. 1g). Overall, these data suggest that GLP-1R signalling does not play a major role in regulating energy metabolism in lean chow-fed mice.

Then, we aimed to determine whether endogenous GLP-1R signalling plays a role in regulating DIT. Therefore, a second group of mice was maintained on HFD for 4 weeks (Fig. 1h–n). Confirming previous observations [20, 21], the BW of HFD-fed *Glp1r*-KO mice was significantly lower than that of WT animals (Fig. 1h). $\dot{V}O_2$ was significantly lower in HFD-fed *Glp1r*-KO mice compared with WT controls when animals were housed at 22°C (Fig. 1i). However, when HFD-fed animals were investigated at 31°C, to reduce BAT activity, $\dot{V}O_2$ did not differ between the genotypes (Fig. 1j). Similarly, EE was significantly lower in *Glp1r*-KO mice compared with WT controls at 22°C, but no difference between the genotypes was observed at 31°C (Fig. 1k). Food intake and net energy balance did not differ between genotypes at either temperature, but was significantly lower in both groups at 31°C (Fig. 1l, m). Locomotor activity was similar between genotypes and remained unchanged regardless of the change in environmental temperature (Fig. 1n).

Noradrenaline-stimulated $\dot{V}O_2$ in chow or HFD-fed *Glp1r*-KO mice The different $\dot{V}O_2$ at 22°C but not at 31°C, suggested a different contribution of BAT activity in the control of EE in HFD-fed WT and *Glp1r*-KO mice. To further evaluate the contribution of endogenous GLP-1R signalling to the BAT thermogenic capacity, we assessed the acute change in $\dot{V}O_2$ in response to s.c. injection of noradrenaline in WT and *Glp1r*-KO animals that were previously housed overnight at thermoneutrality [19]. As expected, noradrenaline injection elicited a significant increase in $\dot{V}O_2$ in CD-fed mice that was similar between both genotypes (Fig. 2a). The maximum $\dot{V}O_2$ was similar in WT and *Glp1r*-KO animals, whereas AUC was slightly but significantly higher in CD-fed *Glp1r*-KO animals (Fig. 2b, c). In contrast, HFD-fed *Glp1r*-KO mice exhibited significantly reduced $\dot{V}O_2$, maximum $\dot{V}O_2$ as well as AUC $\dot{V}O_2$ when compared with HFD-fed WT controls (Fig. 2d–f). These data suggest that BAT activity may be impaired due to lack of GLP-1R signalling under HFD-fed conditions.

Food intake-independent effects of liraglutide on BW and fat mass To determine whether chronic GLP-1R activation increases BAT activity, we treated DIO mice s.c. with 10 nmol kg^{-1} day^{-1} (Fig. 3a–c) or 30 nmol kg^{-1} day^{-1} of liraglutide (Fig. 3d–f) for 6 or 4 weeks, respectively. A pair-fed group was included in each study to determine food intake-independent effects of liraglutide. Mice treated with 10 nmol kg^{-1} day^{-1} had a transient reduction in food intake, which was similar in all groups at the end of the treatment period (Fig. 3a). Consistent with reduced feeding, BW and adiposity (Fig. 3b, c) in mice treated with 10 nmol kg^{-1} day^{-1} of liraglutide and those in the vehicle-PF group were significantly decreased compared with vehicle-treated animals fed ad libitum. However, BW and adiposity were significantly lower in 10 nmol kg^{-1} liraglutide-treated mice compared with vehicle-PF mice (Fig. 3b, c). When the dose of liraglutide was increased to 30 nmol kg^{-1} day^{-1}, a marked reduction in food intake remained evident for the entire treatment duration (Fig. 3d). The pair-feeding necessary to match this enhanced hypophagic effect led to a significant reduction in BW in vehicle-PF animals (Fig. 3e), which was similar in magnitude to that observed in liraglutide-treated mice. Nevertheless, the 30 nmol kg^{-1} dose of liraglutide reduced adiposity to a greater extent compared with vehicle-PF animals (Fig. 3f). In both experiments, liraglutide and vehicle-PF mice lost significantly

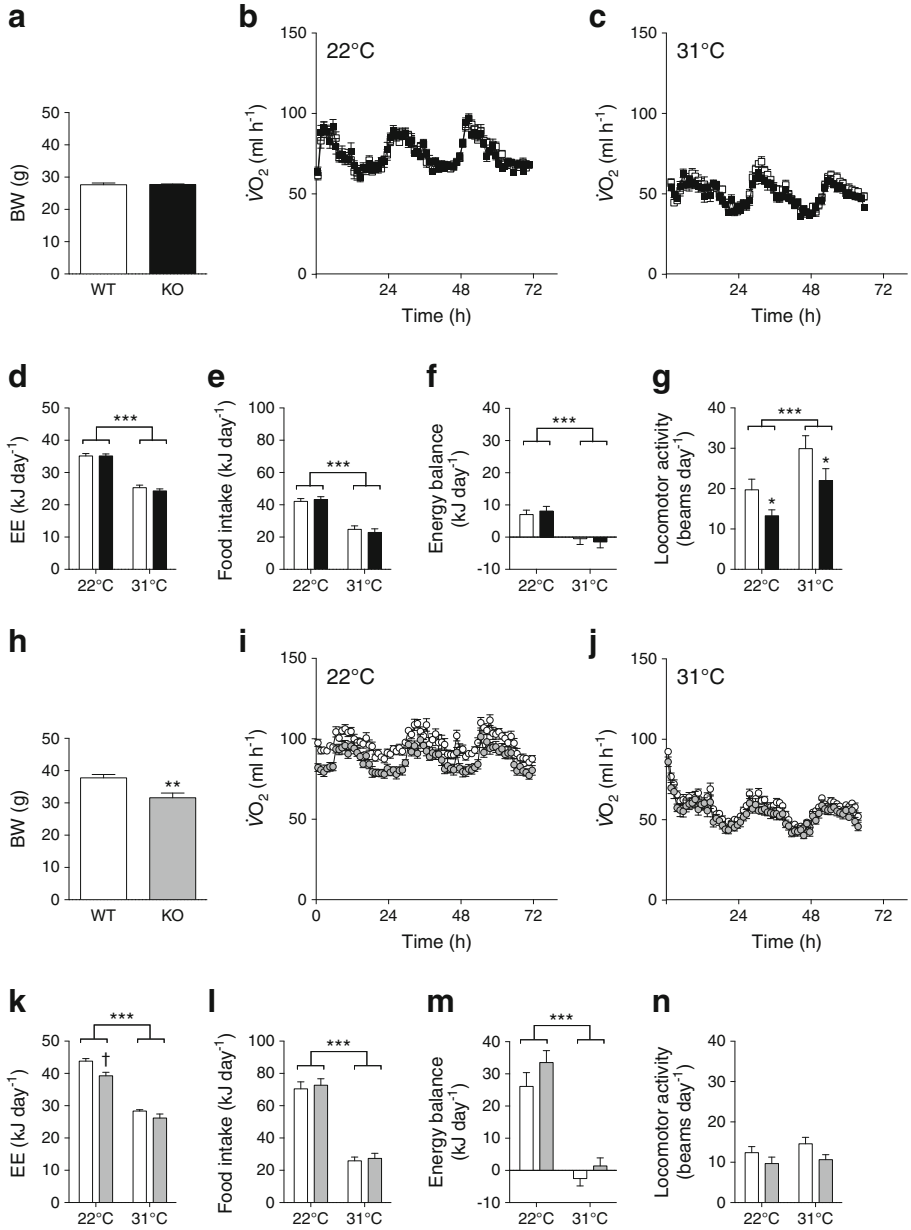

Fig. 1 Energy metabolism in CD-fed and (HFD)-fed WT and *Glp1r*-KO mice maintained at 22°C and 31°C. CD-fed WT (white bars, white squares) and *Glp1r*-KO (black bars, black squares) mice exhibited similar BW (**a**), $\dot{V}O_2$ at 22°C (**b**) and $\dot{V}O_2$ at 31°C (**c**). No differences between the genotypes were detected for average EE (**d**), food intake (**e**) or energy balance (**f**) at 22°C or 31°C, but *Glp1r*-KO mice exhibited significantly lower daily locomotor activity (**g**) (*$p<0.05$; two-way ANOVA, main effect of genotype). Significantly different EE, food intake, energy balance and locomotor activity were observed in both genotypes at 31°C compared with 22°C (**d**–**g**; ***$p<0.001$; two-way ANOVA, main effect of temperature). BW of HFD-fed *Glp1r*-KO (grey bars) mice was significantly lower than that of HFD-fed WT (white bars) mice (**h**; **$p<0.01$; *t* test). When maintained at 22°C, *Glp1r*-KO mice (grey circles and bars) had lower $\dot{V}O_2$ (**i**; $p<0.001$; two-way ANOVA, main effect of genotype) and EE (**k**; $^{\dagger}p<0.05$; two-way ANOVA with Bonferroni post hoc test) compared with WT controls (white circles and bars). When maintained at 31°C, both HFD-fed groups had a similar $\dot{V}O_2$ (**j**) and EE (**k**). Food intake (**l**) and energy balance (**m**) in HFD-fed WT compared with *Glp1r*-KO mice were similar regardless of the ambient temperature. EE (**k**), food intake (**l**) and energy balance (**m**) were significantly lower in both groups at 31°C compared with 22°C (***$p<0.001$; two-way ANOVA, main effect of temperature), while locomotor activity remained unchanged due to temperature (**n**). ($n=12$)

more lean mass than the ad libitum fed control mice; the loss in the liraglutide-treated mice with 10 nmol kg^{-1} and 30 nmol kg^{-1} being significantly larger (10 nmol kg^{-1} vs vehicle-PF; −1.99±0.14 g vs −1.44±0.17 g, $p<0.05$ Tukey post hoc test) and significantly smaller (30 nmol kg^{-1} vs vehicle-PF; −1.74±0.17 g vs −2.56±0.2 g, $p<0.05$), respectively, than that loss in corresponding vehicle-PF control mice.

Noradrenaline-stimulated $\dot{V}O_2$ in DIO mice chronically treated with liraglutide To investigate the potential

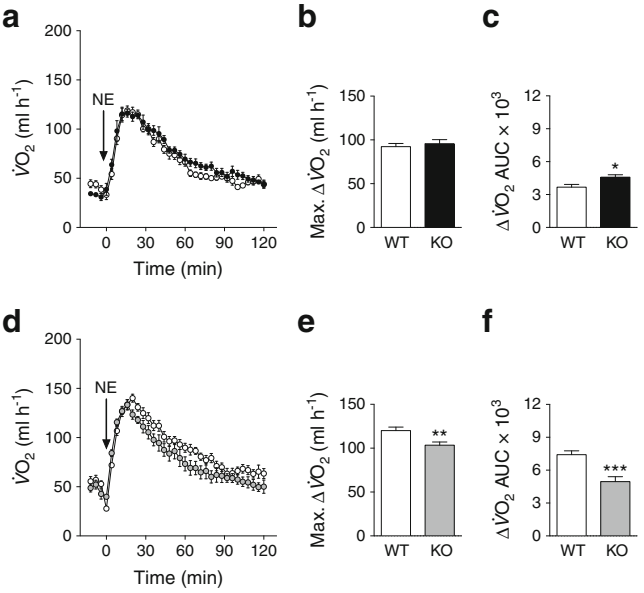

Fig. 2 Noradrenaline-stimulated $\dot{V}O_2$ in WT and *Glp1r*-KO mice on a CD or HFD. $\dot{V}O_2$ (**a**) and maximum $\Delta\dot{V}O_2$ (**b**) following s.c. injection of noradrenaline was similar between *Glp1r*-KO (black circles) and WT (white circles) mice fed a chow diet. AUC of $\Delta\dot{V}O_2$ was significantly higher in *Glp1r*-KO (black bars) mice compared with WT (white bars) mice (**c**; *$p<0.05$, *t* test). In HFD-fed animals, $\dot{V}O_2$ was significantly lower in *Glp1r*-KO (grey circles) compared with WT (white circles) mice following a noradrenaline injection (**d**; $p<0.001$; two-way ANOVA, interaction time×genotype). Maximum $\Delta\dot{V}O_2$ (**e**; **$p<0.01$; *t* test) and AUC $\Delta\dot{V}O_2$ (**f**; ***$p<0.001$; *t* test) were lower in *Glp1r*-KO HFD-fed (grey bars) mice compared with WT HFD-fed (white bars) mice. ($n=12$). Max., maximum

contribution of increased BAT metabolic capacity to the food intake-independent reduction in BW and fat mass exhibited by DIO mice treated with liraglutide, we placed the animals in sealed chambers for detailed analysis of energy balance. Locomotor activity, respiratory exchange ratio and EE were similar in all groups in both the 10 nmol kg^{-1} and 30 nmol kg^{-1} liraglutide study (data not shown). To assess changes in BAT thermogenesis, animals were adapted to 31°C and then monitored for changes in $\dot{V}O_2$ in response to noradrenaline injection. $\dot{V}O_2$ and AUC $\dot{V}O_2$ in response to noradrenaline were similar in all the groups in the 10 nmol kg^{-1} study (Fig. 4a, b). In the study involving administration of 30 nmol kg^{-1} of liraglutide, both the liraglutide-treated and vehicle-PF animals had significantly lower $\dot{V}O_2$ in response to noradrenaline injection compared with vehicle-treated mice (Fig. 4c). When $\dot{V}O_2$ was assessed as AUC, only the data from vehicle-PF animals were significantly lower compared with vehicle-treated animals (Fig. 4d).

We also assessed iBAT and iWAT (Fig. 5a) and skeletal muscle (Fig. 5b, c) for changes in expression of genes involved in regulating non-shivering thermogenesis. Despite the difference in BW and fat mass among the groups we did not detect differences in the expression of genes involved in controlling BAT, iWAT or skeletal muscle activity.

Discussion

Recent evidence demonstrating that central activation of GLP-1R signalling plays a role in the control of BAT activity raised the possibility of a contribution of BAT to the BW lowering effect following chronic administration of GLP-1 mimetics. Our data demonstrate that although direct activation of GLP-1R signalling in the brain is sufficient to increase BAT activity [3], this is not necessary for the BW lowering effects of chronic peripheral treatment with the GLP-1 mimetic liraglutide in DIO mice.

Consumption of an HFD increases EE and thermogenesis, which is referred to as DIT and is attributed to an increase in activity of BAT [22]. HFD consumption leads to an increase in brainstem preproglucagon (PPG) expression [23]. Furthermore, other studies show that the amount of adiposity in rats fed an HFD has a positive correlation with PPG gene expression [24]. This, and the recent evidence revealing a role for GLP-1R to control BAT activity [3, 4], supported the hypothesis that endogenous GLP-1R signalling contributes to increased BAT activity following HFD feeding. Here, we tested this hypothesis by comparing the $\dot{V}O_2$ of WT and *Glp1r*-KO mice in response to physiological and pharmacological stimuli of BAT activity.

As a physiological stimulus, we compared the $\dot{V}O_2$ during housing at standard temperatures of 22°C or at a thermoneutral temperature. Feldman and colleagues elegantly used this approach to unveil the role of uncoupling protein 1 (UCP1) in the protection against diet-induced obesity [7]. With this approach, we found that *Glp1r*-KO mice fed with an HFD for 4 weeks have lower $\dot{V}O_2$ when compared with their WT controls when housed at 22°C but not at 31°C, which supports a role of endogenous GLP-1R in the development of DIT. Noteworthy, HFD-fed *Glp1r*-KO mice had a lower BW in comparison with WT controls. This resistance to diet-induced obesity is consistent with previous observations [20, 21] and its aetiology remains unknown. It may be contributed by a direct role of GLP-1R signalling in regulating adipogenesis [25] or by other compensatory signals developed in response to congenital disruption of the *Glp1r* gene [26]. Importantly, *Glp1r*-KO mice preserved the ability to adjust their energy intake in response to the changes in environmental temperature, indicating that GLP-1R signalling does not play a critical role in the homeostatic control of energy intake.

As a pharmacological stimulus of BAT activity, we performed noradrenaline injections in mice previously housed at thermoneutrality [19]. Consistent with the lower $\dot{V}O_2$ at 22°C, HFD-fed *Glp1r*-KO mice exhibited a lower $\dot{V}O_2$ compared with WT controls when challenged with noradrenaline,

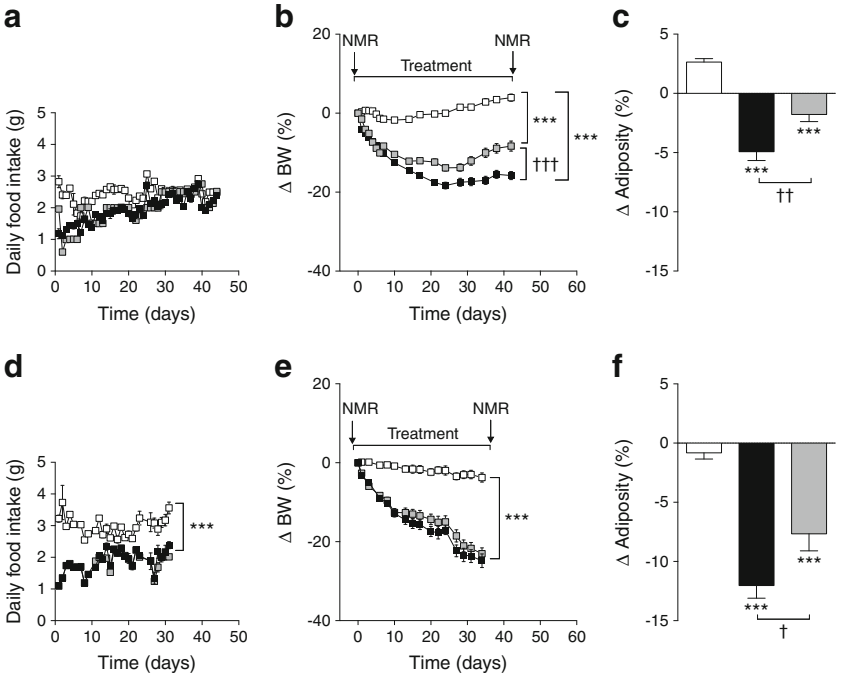

Fig. 3 Food intake, BW and adiposity in DIO mice chronically treated with liraglutide. Mice were given daily s.c. injections of vehicle (white squares and bars), liraglutide at a dose of 10 nmol kg^{-1} day^{-1} (**a**–**c**; black squares, black bars) or 30 nmol kg^{-1} day^{-1} (**d**–**f**; black squares, black bars), or vehicle and then pair-feeding to corresponding liraglutide-treated animals (grey squares, grey bars). Food intake in mice treated with 10 nmol kg^{-1} day^{-1} of liraglutide was transiently decreased (**a**) whereas BW (**b**; ***$p<0.001$ vs vehicle; $^{†††}p<0.001$ vs vehicle-PF, two-way ANOVA) and adiposity (**c**;***$p<0.001$ vs vehicle; $^{††}p<0.01$ vs vehicle-PF, one-way ANOVA with Tukey's post hoc test) remained significantly lower compared with both vehicle-treated controls and vehicle-PF mice. s.c. injection of liraglutide at a dose of 30 nmol kg^{-1} day^{-1} reduced feeding during the entire treatment period (**d**; ***$p<0.001$, 30 nmol kg^{-1} day^{-1} liraglutide and vehicle-PF vs vehicle, two-way ANOVA). BW was reduced in liraglutide and vehicle-PF treated mice (**e**; ***$p<0.001$, 30 nmol kg^{-1} day^{-1} liraglutide and vehicle-PF vs vehicle, two-way ANOVA). Adiposity was also decreased in liraglutide and vehicle-PF mice and the decrease in adiposity was significantly greater in liraglutide-treated mice compared with vehicle-PF mice (**f**; ***$p<0.001$ vs vehicle; $^{†}p<0.05$ vs vehicle-PF, one-way ANOVA with Tukey's post hoc test). ($n=16$, **a**–**c**; $n=8$, **d**–**f**)

which strengthens the potential involvement of endogenous GLP-1R signalling in the development of BAT thermogenesis in response to high-fat feeding. Intriguingly, chow-fed, lean *Glp1r*-KO mice exhibited a slight but significant increase in $\dot{V}O_2$ compared with WT controls, following noradrenaline injection. The cause of this difference in the noradrenaline-stimulated $\dot{V}O_2$ under chow-fed conditions remains to be determined.

The impaired DIT in mice lacking endogenous GLP-1R signalling contrasts with the increased EE and increased UCP1 and beta-3 adrenergic receptor gene expression in iBAT exhibited by HFD-fed dipeptidyl peptidase 4 deficient (*Dpp4*$^{-/-}$) mice, a model with increased endogenous GLP-1 levels [27]. Consistently, direct CNS administration of GLP-1R ligands—including liraglutide [3, 4]—activates BAT. However, the characterisation of the control of BAT by central GLP-1R signalling was conducted in lean rodents fed standard low-fat diets undergoing relatively short periods of treatment (under 7 days) [3, 4]. These conditions differ significantly from those in which GLP-1 mimetics promote meaningful BW loss in humans. Thus, we aimed to determine the contribution of BAT thermogenesis to the weight lowering effect of chronic (4–6 weeks) pharmacological gain of GLP-1R function in a standard model of obesity such as the DIO-C57Bl6 mouse. Our data clearly demonstrate the ability of liraglutide to reduce BW and adiposity to values that exceed what could be attributable to the reduced caloric intake, likely by engaging additional physiological mechanisms that contribute to further a negative energy balance, one of which could be BAT thermogenesis. Surprisingly, we detected neither enhanced BAT activity, as measured by $\dot{V}O_2$ in response to noradrenaline injection, nor increased expression of genes associated with increased BAT activity or 'browning' of WAT. We also measured thermogenic genes in quadriceps and soleus muscle and noted no changes in gene expression in response to chronic liraglutide treatment. To discard that the absence of a significant increase in BAT activity was the result of a subthreshold dose with liraglutide, we repeated the study but this time with a higher dose (30 nmol kg^{-1} day^{-1}). With the higher dose, mice experienced sustained hypophagia during the treatment period and a more pronounced BW loss. In this case, BAT thermogenesis, as measured by noradrenaline-stimulated $\dot{V}O_2$, did not increase but was actually reduced, likely as a consequence of excessive BW loss. It could be argued that

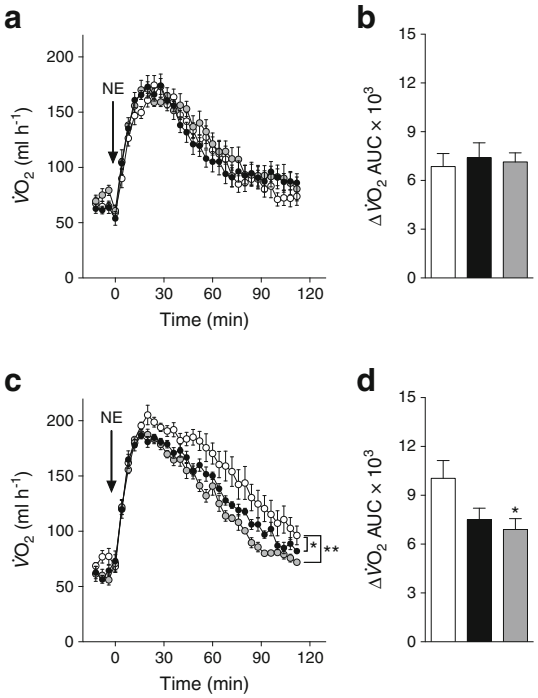

Fig. 4 Noradrenaline-stimulated $\dot{V}O_2$ in DIO mice chronically treated with liraglutide. Following 6 weeks of vehicle (white circles, white bars), liraglutide treatment at a dose of 10 nmol kg^{-1} day^{-1} (**a**–**b**; black circles, black bars), or 4 weeks with 30 nmol kg^{-1} day^{-1} (**c**–**d**; black circles, black bars), or vehicle and pair-feeding to corresponding liraglutide-treated group (grey circles, grey bars), mice were subjected to a noradrenaline s.c. injection and monitored for $\dot{V}O_2$. No differences in $\dot{V}O_2$ (**a**) or $\Delta\dot{V}O_2$ AUC (**b**) were detected among the three groups in the 10 nmol kg^{-1} day^{-1} liraglutide study. In the 30 nmol kg^{-1} day^{-1} study, the 30 nmol kg^{-1} day^{-1} liraglutide-treated and vehicle-PF animals had lower $\dot{V}O_2$ compared with vehicle-treated animals (**c**; $*p<0.05$, $**p<0.01$, two-way ANOVA with Bonferroni's post hoc test). $\Delta\dot{V}O_2$ AUC was significantly lower in vehicle-PF animals compared with the vehicle-treated animals (**d**; $*p<0.05$; one-way ANOVA). (n=7–10). NE, norepinephrine (noradrenaline)

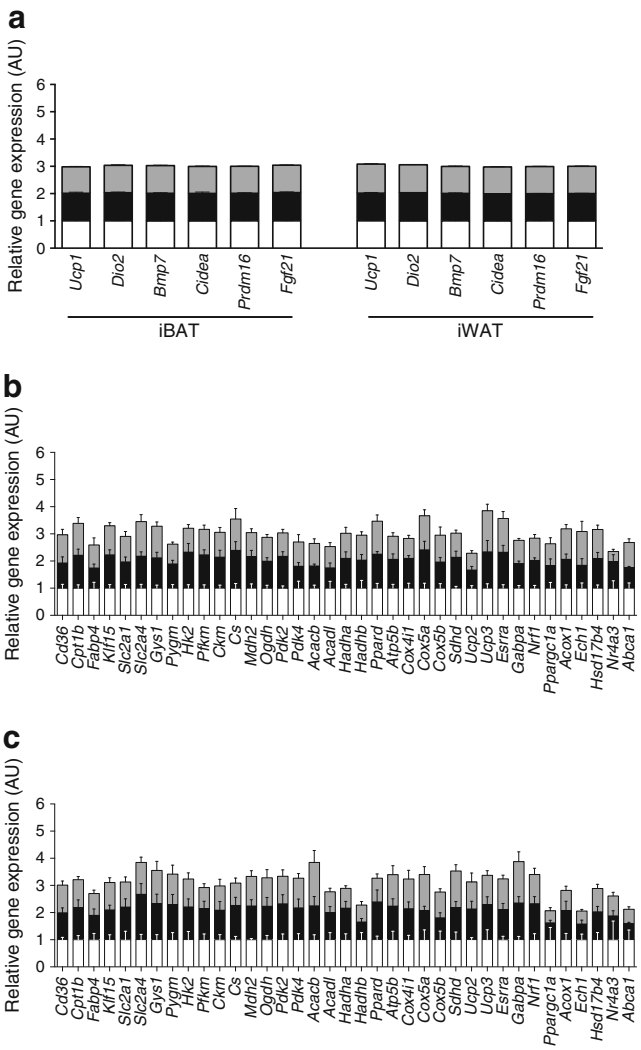

Fig. 5 Relative expression of genes involved in thermogenesis in iBAT, iWAT quadriceps and soleus of DIO mice following 6 weeks of treatment with vehicle, liraglutide (10 nmol kg^{-1} day^{-1}) or vehicle-PF. Following 6 weeks of vehicle (white bars), liraglutide treatment at a dose of 10 nmol kg^{-1} day^{-1} (black bars), or vehicle and pair-feeding to the liraglutide-treated group (grey bars), gene expression was assessed in iBAT and iWAT (**a**) as well as in quadriceps (**b**) and soleus (**c**). Gene expression in all tissues was similar among all three groups. (n=5–6). AU, arbitrary units

lack of effect of liraglutide on BAT activation in DIO mice was the result of intrinsically elevated DIT, but certainly, the pharmacological activation of GLP-1R signalling does not prevent its reduction associated with weight loss.

A study examining the appearance of peripherally administered liraglutide into the brain indicated that the majority of liraglutide appears in the arcuate nucleus (ARC) [28]. Lower amounts were found in the paraventricular nucleus (PVN), and little to no liraglutide appears in the ventromedial hypothalamic nucleus (VMH) or dorsomedial hypothalamic nucleus (DMH), which are the major centres regulating BAT thermogenesis [28]. Importantly, Beiroa et al gave intranuclear brain injections of liraglutide to rats and demonstrate that the VMH, not the ARC, is the site of GLP-1R-mediated activation of BAT [4]. Poor access of subcutaneously injected liraglutide to hypothalamic nuclei involved in regulating BAT activity may be contributing to the discrepancy in results of peripheral vs central administration of GLP-1R agonists on BAT activation.

Our data strongly suggest that mechanisms other than the regulation of feeding or BAT activity play a significant role in the BW lowering effect following chronic administration of GLP-1 mimetics. Another potential mechanism could be enhanced thermogenesis in skeletal muscle. UCP2 and UCP3 in skeletal muscle have been shown to play a role in adaptive thermogenesis (reviewed in [29]). Similar to upregulation of *Ucp1* in BAT in response to HFD feeding, both *Ucp2* and *Ucp3* in quadriceps are upregulated by high-fat feeding in

C57Bl6 mice, suggesting a role for skeletal muscle in mediating DIT [30]. Liraglutide-treated mice had a similar expression level of both *Ucp2* and *Ucp3* in skeletal muscle compared with both vehicle and vehicle-pair-fed mice, suggesting that enhanced muscle thermogenesis is not the mechanism driving the superior weight loss in the liraglutide-treated mice. The physiological and molecular basis underlying the mechanisms for enhanced weight loss remains to be determined but may be of significant importance to understand the efficacy on weight loss of therapies involving GLP-1R agonists.

In summary, our results demonstrate that although endogenous GLP-1R signalling contributes to the increase in BAT thermogenesis [3, 4], and may contribute to DIT, this is not seminal for the BW lowering effect observed with chronic peripheral treatment with the GLP-1 mimetic, liraglutide, or congenital loss of function of GLP-1R signalling. Importantly, our data demonstrate that mechanisms other than increased BAT thermogenesis play a role in the food intake-independent BW lowering effect mediated by the increase in GLP-1R signalling. Unveiling such mechanisms should provide alternative targets for the treatment of obesity.

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