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The central melanocortin system mediates the benefits of time-restricted feeding on energy balance

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ABSTRACT

Objective: Recent decades have seen a marked increase in the prevalence of obesity and its associated co-morbidities. This increase correlates with greater access to calorie-dense food that is often consumed later in the active phase of the day. Studies in high-fat diet-induced obese (DIO) mice indicate that restricting food access to their active (dark) phase is sufficient to reduce obesity. However, the specific mechanisms mediating these beneficial metabolic effects of dark restricted feeding (DRF) remain unknown.

Methods: We examined the impact of DRF on the response to peripheral signals regulating the central melanocortin system of DIO mice and on *Mc4r*$^{-/-}$ mice.

Results: The body weight loss following DRF has an acute onset that is sustained over time. This effect is contributed by a reduction on food intake that requires a functional central melanocortin system. Specifically, DRF impacts the circadian expression of melanocortin system genes in the arcuate nucleus of the hypothalamus (ARC). Consistent with this, DRF significantly increases the effectiveness of the fasting-feeding signals ghrelin and leptin that interact with the melanocortin system to regulate energy balance. Importantly, DRF did not reduce or prevent obesity in *Mc4r*$^{-/-}$ mice.

Conclusions: Taken together, our data reveal a critical role of brain melanocortin signaling in mediating the beneficial effects of timed feeding on metabolic control, supporting potential meaningful benefits in combining timed feeding with pharmacological targeting of the melanocortin signaling for the treatment of obesity.

1. Introduction

Despite significant progress in understanding the factors and molecular mechanisms playing a role in the worldwide increase in its incidence, obesity remains one of the greatest global health threats of the 21st century [1]. A common denominator of all anti-obesity treatments is the need for simultaneous implementation of changes in lifestyle. Traditionally those have been focused in decreasing calorie intake and increasing calorie expenditure via promoting exercise. In both cases, sustained compliance is hard to achieve, limiting the long-term efficacy of the interventions.

Controlling body weight by limiting caloric intake via chronic dieting has proven difficult or impossible for most people. In contrast, evidence suggests that optimizing the timing of daily food availability may be a viable lifestyle strategy to treat or prevent obesity and improve overall health [2–4]. Compelling evidence indicates that the synchrony between the timing of meals and the sleep-wake cycle plays a critical role in the control of energy balance. This notion arises from the observation that diet-induced obese (DIO) mice that have had continuous (24 h/d) access a high-fat diet (HFD) exhibit increased total daily caloric intake and alteration of normal circadian feeding rhythms, largely due to increased feeding during the light (resting) phase [5]. This change in the feeding pattern correlates with a reduced amplitude of the circadian expression of molecular clock and metabolic genes in tissues involved in the control of metabolism [5]. However, maximizing synchrony between fasting-feeding and sleep-wake cycles by restricting feeding to only the active phase (dark-phase restricted feeding, DRF) promotes sustained body weight loss and improved metabolic control in DIO mice. Importantly, these beneficial effects of DRF on metabolic control are associated with an increased synchrony and amplitude of the circadian rhythms of clock and metabolic genes in key tissues including liver, white adipose tissue (WAT) and brown adipose tissue

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(BAT).

In spite of the fact that unlimited food is available during the animals' active phase, DRF also promotes a sustained reduction in caloric intake that contributes to the BW loss [6–8]. However, the molecular mechanisms whereby DRF reduces calorie intake remain unknown. Given the importance of the hypothalamic melanocortin system in integrating feeding-fasting signals to influence food intake, we performed studies to examine the impact of DRF on the activity of the central melanocortin system in the medio-basal hypothalamus of DIO mice.

2. Material and methods

2.1. Animals

All studies were approved by the Institutional Animal Care and Use Committees at the University of Cincinnati in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Male mice (C57BL/6J, Jackson Laboratories, Bar Harbor, ME) were housed on a 12-h/12-h light-dark cycle at 22°C with free access to a standard chow diet (Teklad LM-485, Envigo; 3.1 kcal/g) and water. To promote diet-induced obesity (DIO), some of the mice were fed a high-fat diet (HFD, #D12331; Research Diets, New Brunswick, NJ. 58% kcal from fat, 25.5% kcal from carbohydrates, and 16.4% kcal from protein) beginning at 8 wk of age for a minimum of 16 wk. LoxTbMc4r (#006414; Jackson laboratory; KO) mice were crossed with the aforementioned C57Bl/6J mice to generate homozygous KO and wild-type littermate controls (WT).

2.2. Body composition measurements

Body composition (fat and lean mass) was measured using nuclear magnetic resonance technology (EchoMRI, Houston, TX).

2.3. Estimation of energy expenditure

Energy expenditure was estimated using the following energy balance equation: TEEbal = Assimilated Energy − (Δ somatic Fat Energy + Δ somatic Fat−Free Energy), assuming 9.4 kcal/gm and 1.0 kcal/gm for fat and fat-free mass gained or lost, respectively, as verified elsewhere [9].

2.4. Glucose tolerance test

Mice were intraperitoneally injected with 1.5 g glucose per kg of BW [20% (wt/v) D-glucose (Sigma) in 0.9% (wt/v) saline]. Tail blood glucose concentrations (mg/dl) were measured before (0 min) and at 15, 30, 60, and 120 min after injection using a handheld glucometer (Freestyle, Abbot).

2.5. Ghrelin and leptin administration

Active, octanoyl-ghrelin (CS9108, CS Bio Co, CA) was administered ip as described in the results section. For Intracerebroventricular Infusion of mouse leptin (AFP1775, NHPP-NIH), mice were anesthetized with 2% isoflurane in oxygen. The tips of stainless steel cannulas were stereotaxically placed in the lateral cerebral ventricle (coordinates from bregma: 0.7 mm posterior, 1.2 mm lateral, and 2.5 mm ventrally from the surface of the brain). The cannula was connected to an osmotic minipump (1007D; Alzet, Cupertino, CA) placed subcutaneously through a polyvinyl tube filled with vehicle (PBS) or leptin, infused at 1 ug/day. The cannulas were secured to the skull and the skin was closed using Vetbond (3M. St. Paul, MN). The animals received a single s.c. dose of 5 mg/kg of meloxiocam (Metacam, Boehringer Ingelheim, Ingelheim, Germany).

2.6. Plasma measurements

Blood was collected from tail laceration or decapitation in EDTA-coated tubes and immediately chilled on ice and centrifuged at 5,000g at 4°C. Plasma was stored at -20°C The following hormones were measured using commercially available kit accordingly to the manufacturers' instructions: FGF21 (Milipore #EZRMFGF21-26K), Insulin (CrystalChem, #90080), Leptin (CrystalChem, #90030) and total Ghrelin (Milipore #EZRGRT-91K).

2.7. Gene expression analysis

Tissue containing the suprachasmatic (SCN) or the arcuate (ARC) nucleus was dissected from the ventral side of the hypothalamus under a microscope from brains preserved in RNALater (ThermoFisher) prior RNA extraction (RNAqueous-Micro kit, #AM1931, ThermoFisher). cDNA was synthetized with SuperScript® III First-Strand Synthesis kit (ThermoFisher) after DNAse I treatment (ThermoFisher) and qPCR was performed using commercially available, gene-specific FAM-labeled Taqman® probes following manufacturer instructions (Invitrogen, Life Technologies). Water-blank samples from the cDNA synthesis were included in the qPCR reaction. The housekeeping gene was Actb, measured simultaneously using a VIC-labeled Actb Taqman probe (ThermoFisher). The relative quantification was performed using the delta Ct method.

2.8. Immunoblot

UCP-1 protein levels in intrascapular brown adipose tissue (iBAT) were determined as detailed previously [10]. Briefly, flash frozen tissue was homogenized in RIPA lysis buffer containing PMSF, sodium orthovanadate, protease inhibitor (Santa Cruz Biotechnology) and phosphatase inhibitor cocktail (Sigma-Aldrich) using a Tissuelyser (Qiagen). 70 μg of protein solution were separated on 4-15% polyacrylamide gels (Bio-Rad Laboratories), and transferred to nitrocellulose membranes (GE Healthcare). The membranes were blocked and incubated overnight at 4°C with rabbit antibodies against UCP-1 (1:1000, Cell Signaling, # 14670) or beta actin (1:2000, Cell Signaling, #4967) in 5% BSA (Sigma-Aldrich). Membranes were then washed and incubated with secondary antibodies (antirabbit-horseradish peroxidase coupled, 1:5,000; Cell Signaling), washed and developed by chemiluminescence. Films were scanned and densitometry was assessed using ImageJ 1.48v (http://imagej.nih.gov/ij).

2.9. Statistics

Results are expressed as mean ± SEM. Statistical analysis was performed using t-tests for comparison of two groups and two or three-way ANOVA for multiple comparison tests using GraphPad Prism 8 (GraphPad Software, San Diego, California, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Reduction of caloric intake and body weight at the onset of DRF

Obesity in mice resulting from chronic maintenance on a HFD diet (DIO) is associated with increased caloric intake and disruption of normal circadian feeding rhythms, due to increased feeding during the light (resting) phase. In contrast, when food availability is restricted to only in the active phase (dark phase-restricted feeding, DRF) sustained body weight loss occurs. We asked whether the BW loss during DRF is secondary to reduced caloric intake. First, we monitored the acute impact of fasting during the light phase on daily caloric intake during the onset of DRF. DRF reduced daily feeding in DIO mice acutely, within the first day of intervention, compared to AL-fed controls

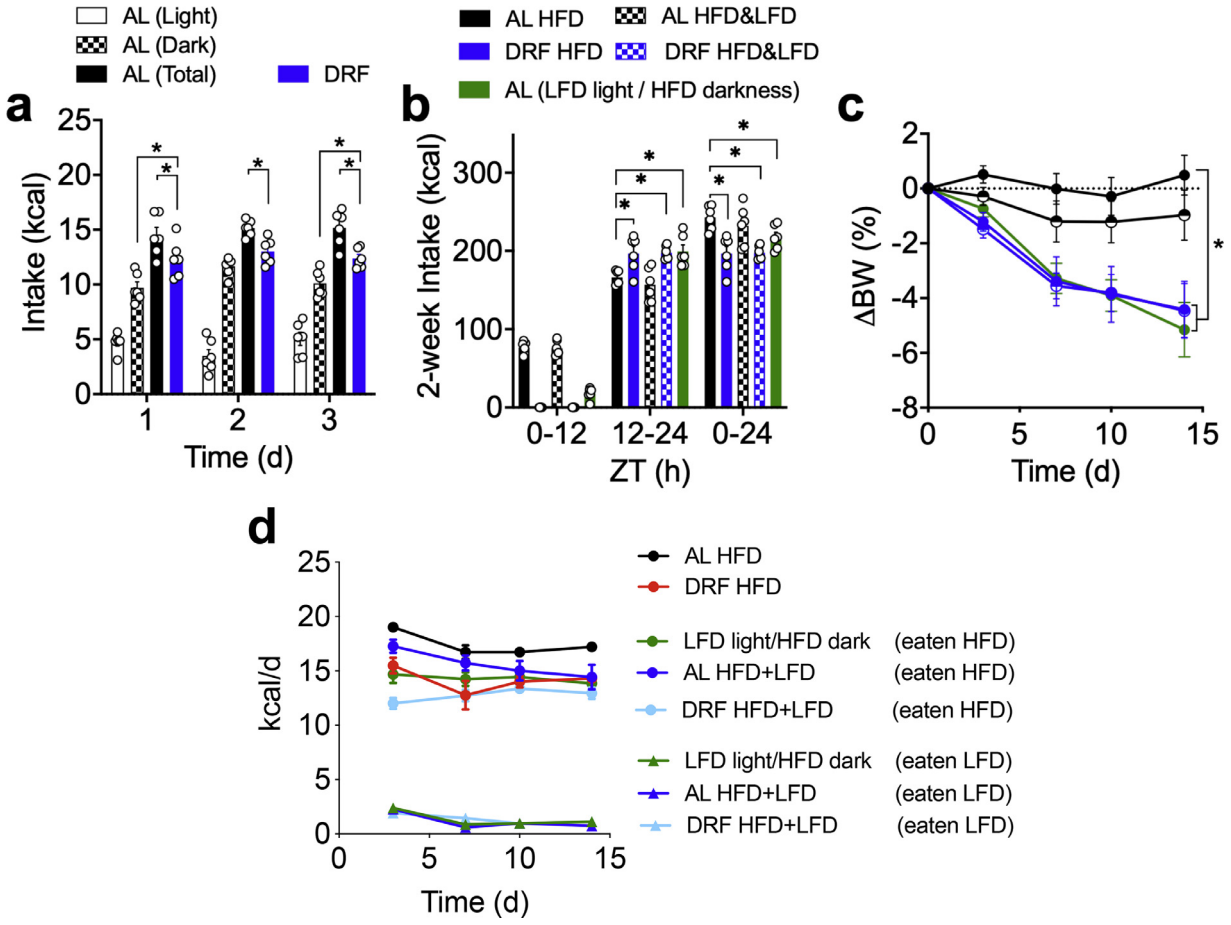

Fig. 1. Acute reduction in caloric intake and body weight following DRF in DIO mice. All mice were simultaneously exposed to HFD and LFD for 4 d before the study began to minimize diet neophobia. The mice were then assigned to the various groups. (a) Caloric intake during the initial 3 d of access to only the HFD (black bars). Intake of mice with ad lib (24-h) access to the HFD is depicted as light-phase, dark-phase and total light plus dark phase periods. Intake of DRF mice (blue bar) was greater than dark-phase intake of the ad lib-fed mice but lower than the total 24-h intake of the ad lib mice. (b) Caloric intake of DIO mice (initial BW = 65.6 ± 0.7g) over 2 wk depicted as light-phase (ZT 0-12), dark-phase (ZT 12-24) and total 24-h intake. Mice fed the HFD ad lib (black bars) consumed more calories in the dark than in the light, and remained the case for a group of mice given simultaneous access to both HFD and LFD. DRF mice (blue bars) had significantly greater intake than ad lib-fed controls during the dark phase, but significantly lower intake than controls over the entire day, and having simultaneous access to HFD plus LFD made no difference. A group of mice with HFD available only during the dark and LFD available during the light were comparable to DRF mice throughout. (c) Compared to ad lib-fed mice (black lines), DRF mice lost significant body weight over the 2 wk (blue lines), and this also occurred even if LFD was available during the light phase. Thus, the DRF effect was unaffected by exposure to LFD. (d) The reduction in HFD feeding following DRF does not result in an increase in LFD feeding in DIO mice. HFD fed DIO mice (2 per cage) were maintained AL or food deprived during the light phase (DRF) for 2 weeks. In another group (LFD light/HFD dark) HFD was removed during the light phase and received instead a matched LFD. Two additional control groups were simultaneously exposed to HFD and LFD, either AL or during DRF. All mice receiving LFD were simultaneously exposed to HFD and LFD for 4 days before the study to minimize diet neophobia. Data shown as mean ± SEM. * = P < 0.05 as indicated (a,c) or vs AL HFD (b). 2-way ANOVA, Sidak post hoc test. n = 6 (a,b, d) or 12 (c). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 1a). This reduction was due to incomplete compensation for the calories not eaten during the light phase since DRF mice consumed similar amounts to that of AL mice during the dark phase. A separate experiment indicated that this lack of compensation persists over time (2-wk) (Fig. 1b), leading to a sustained and significant reduction in cumulative caloric intake that was associated with significant BW loss (Fig. 1c). These data suggest that the increased caloric intake during the light phase supports the maintenance of DIO, and that the body weight lowering effect following DRF is contributed, at least in part, by a reduction in caloric intake.

We reasoned that if the increase in caloric intake during the light phase is solely driven by impaired homeostatic control due to DIO, then substituting HFD with an alternative caloric source such as a micronutrient-matched low-fat diet (LFD) available only during the light phase should result in a total daily calorie accrual similar to that exhibited by the AL control mice. We tested this possibility and included control groups exposed to both HFD and LFD either AL or in the DRF paradigm, to determine whether the reduction in caloric intake due to DRF is accompanied by changes in dietary preference favoring LFD intake. DRF reduced feeding and BW in DIO mice offered simultaneous access to both a HFD and LFD (Fig. 1b,c) without increasing the preference for LFD compared to the AL HFD + LFD group (Fig. 1d). Replacing HFD for LFD only during the light phase led to a marginal increase in caloric intake during that period that was insufficient to match the intake exhibited by the AL HFD counterparts. In fact, mice receiving LFD only during the light phase exhibited a significant reduction of caloric intake during that period that mimicked the effect of imposed fasting during DRF and led to significant BW loss (Fig. 1b,c). These data imply that the increased energy consumption exhibited by DIO mice during light phase is not solely due to an impaired homeostatic control, and rather is mostly due to the opportunity to consume palatable food. These data also indicate that caloric restriction during the light phase (either exogenously imposed via DRF or voluntarily imposed via avoidance of LFD) leads to sustained reduction in caloric intake without

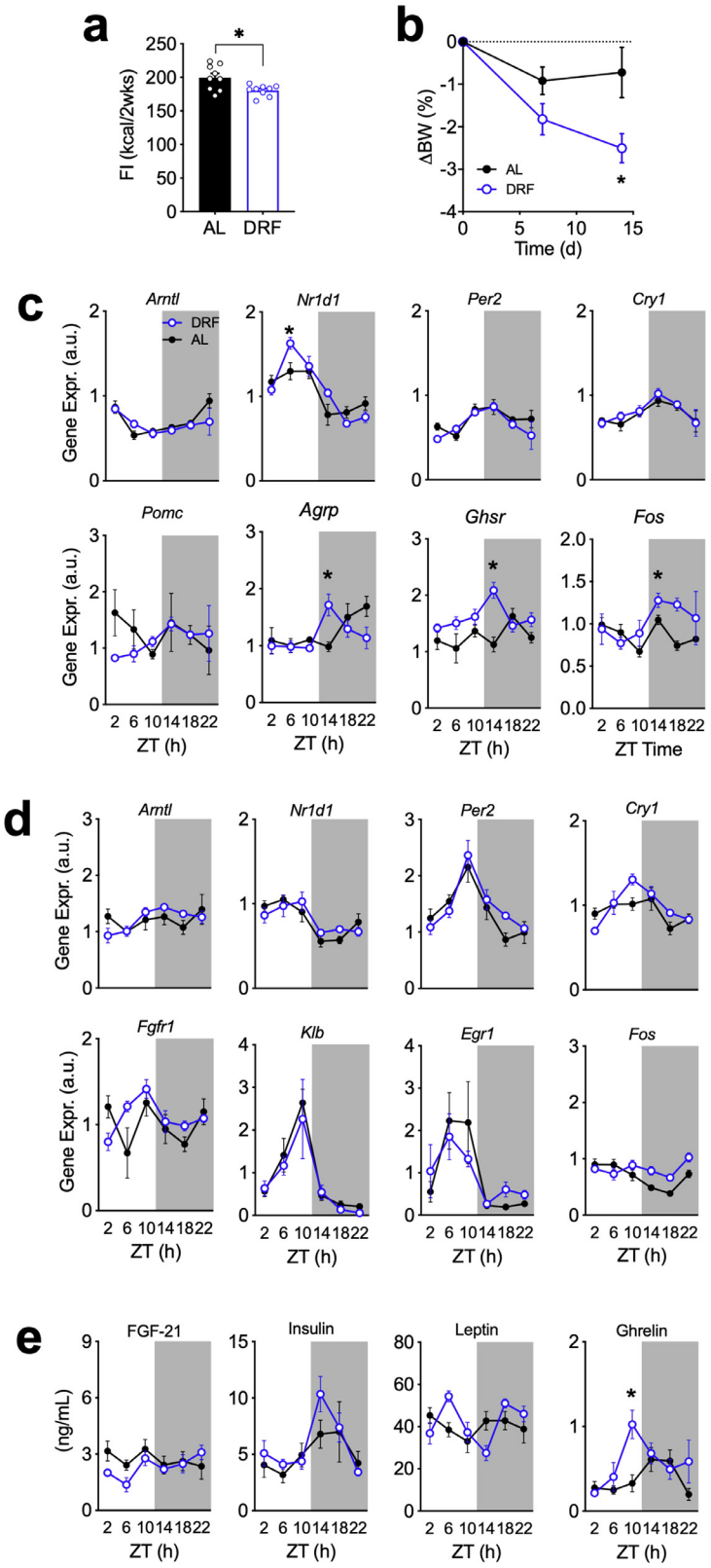

Fig. 2. Hypothalamic gene expression and plasma hormone levels in DIO mice following 2-wk DRF. DIO mice (initial BW = 66.3 ± 0.7g) were housed 4/cage and fed *ad libitum* (AL) or maintained on dark-phase restricted feeding (DRF) for 2 wk. (a) Caloric consumption and (b) body weight change during that period. (c) Circadian gene expression in the ARC of *Arntl, Nrd1d1, Per2* and *Cry1* in the SCN (top row, left to right); and *Pomc, Agrp, Ghsr, Fos* (bottom row, left to right). (d) Circadian gene expression in the SCN of *Arntl, Nrd1d1, Per2* and *Cry1* (top row, left to right); and *Fgfr1, Klb, Egr1, Fos* (bottom row, left to right). (e) Circadian plasma levels of (left to right) FGF21, Insulin, Leptin and Ghrelin. Data are depicted as mean ± SEM. (a) n = 9/group, (b) n = 36/group, (c-e) n = 5-6. * = P < 0.05 AL vs. DRF. t-Student test (a-b) or 2-way ANOVA followed by Sidak multiple comparisons test (c, e).

changing dietary preferences and that this contributes to BW loss.

3.2. Impact of 2-week DRF on hypothalamic gene expression in DIO mice

Since DRF leads to an increase in the amplitude of the circadian expression of molecular clock genes in peripheral tissues, we asked whether DRF also regulates the circadian expression of molecular clock genes in nuclei of the medio-basal hypothalamus involved in the circadian control of energy balance. To this end, we limited the access of DIO mice to HFD to the ZT13 to 23 hours within the dark phase for 14 days. Consistent with our previous data (Fig. 1), this period was sufficient to promote a significant reduction in food intake (Fig. 2a) and BW

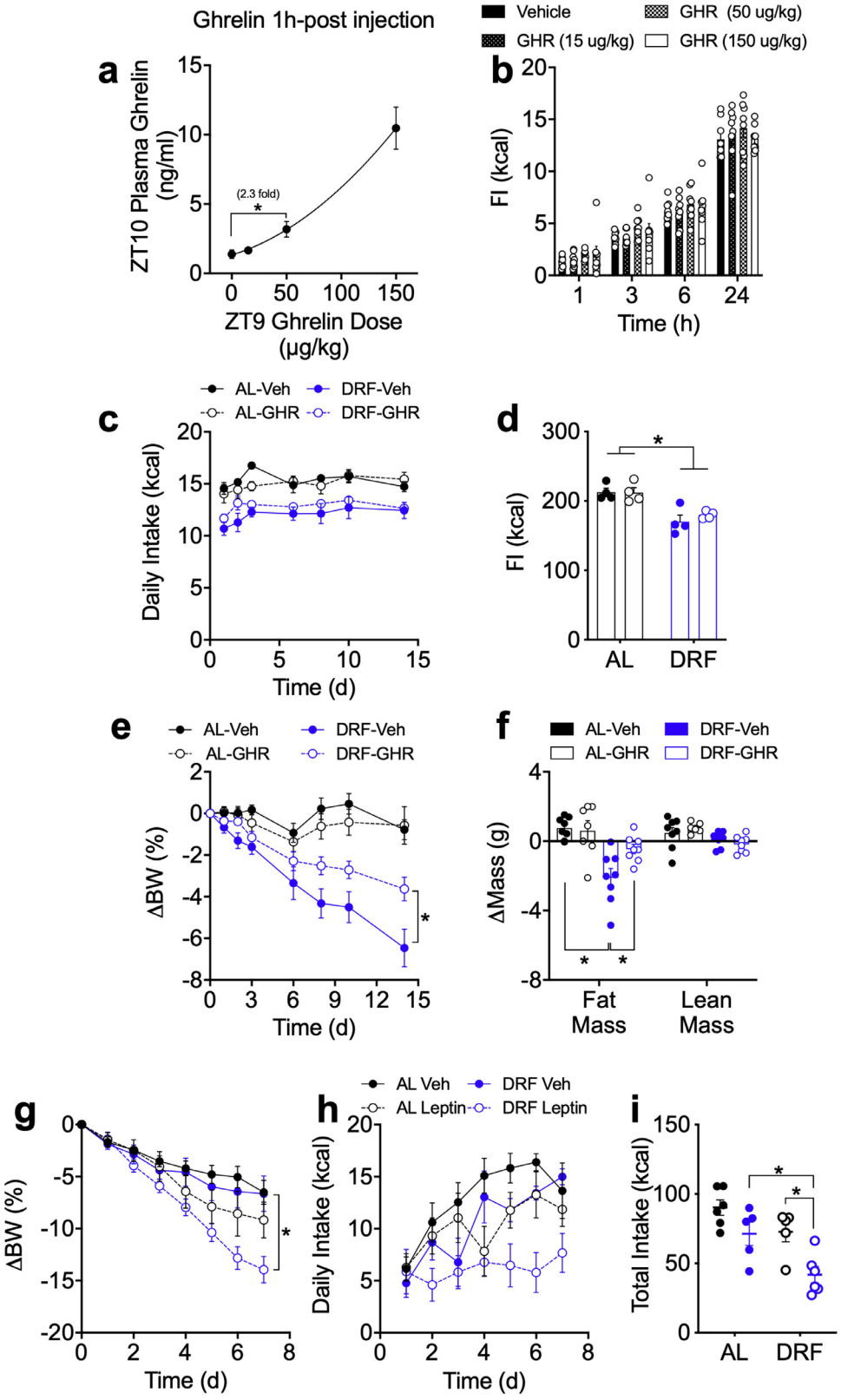

Fig. 3. DRF enhances the ability of ghrelin and leptin to regulate body weight in DIO mice. (a) Plasma ghrelin at ZT10 following exogenous s.c. injection of increasing ghrelin doses at ZT9 in AL-fed DIO mice. (b) Impact of the s.c. ghrelin on caloric intake following injection at ZT9. A different cohort of single-housed DIO mice received ghrelin at ZT9, the food was removed for the next 3 h and returned at the onset of the dark phase at ZT12, and consumption was measured after 1, 3, 6 and 24 h. (c-f) Effect of AL or DRF on double-housed DIO mice (initial BW = 54.3 ± 0.5g) fed while receiving ghrelin (50 ug/kg, s.c.) daily at ZT9 for 14 days. Daily (c) and cumulative (d) caloric intake, and change on body weight (e) and on body mass composition (f). (g-i) DRF increases leptin's reduction of BW in DIO mice (initial BW = 64.6 ± 0.9g). Body weight change (g), daily (h) and cumulative caloric intake (i) of single-housed DIO mice fed AL or DRF while receiving a continuous leptin infusion into the lateral cerebral ventricle (1 ug/day) using osmotic minipumps for 7 d. Data are depicted as mean ± SEM. (a) n = 4/group, (b) n = 8/group, (c-d) n = 4, (e-f) n = 8, (g-i) n = 5/6group. * = $P < 0.05$ AL vs. DRF or as indicated by bracket. t-Student test (a) or 2-way ANOVA followed by Sidak multiple comparisons test (d-g, i).

(Fig. 2b) compared to the AL control mice that had unrestricted access to HFD. A subset of mice from each group was then euthanized every 4 h and the brain processed for microdissection of the hypothalamic Arcuate (ARC) and Suprachiasmatic Nucleus (SCN) to measure the expression of several molecular clock genes, namely *Arntl, Nr1d1, Per2* and *Cry1*.

In contrast to the effect on circadian expression of clock genes in peripheral tissues [11], DRF did not significantly impact the expression of these genes in the ARC (Fig. 2c) when compared to controls, except for ARC-*Nr1d1* expression ($P < 0.05$, interaction ZT x DRF), which exhibited a statistically significant difference at ZT6h (Fig. 2c). These results suggest that mechanisms other than those dependent on the

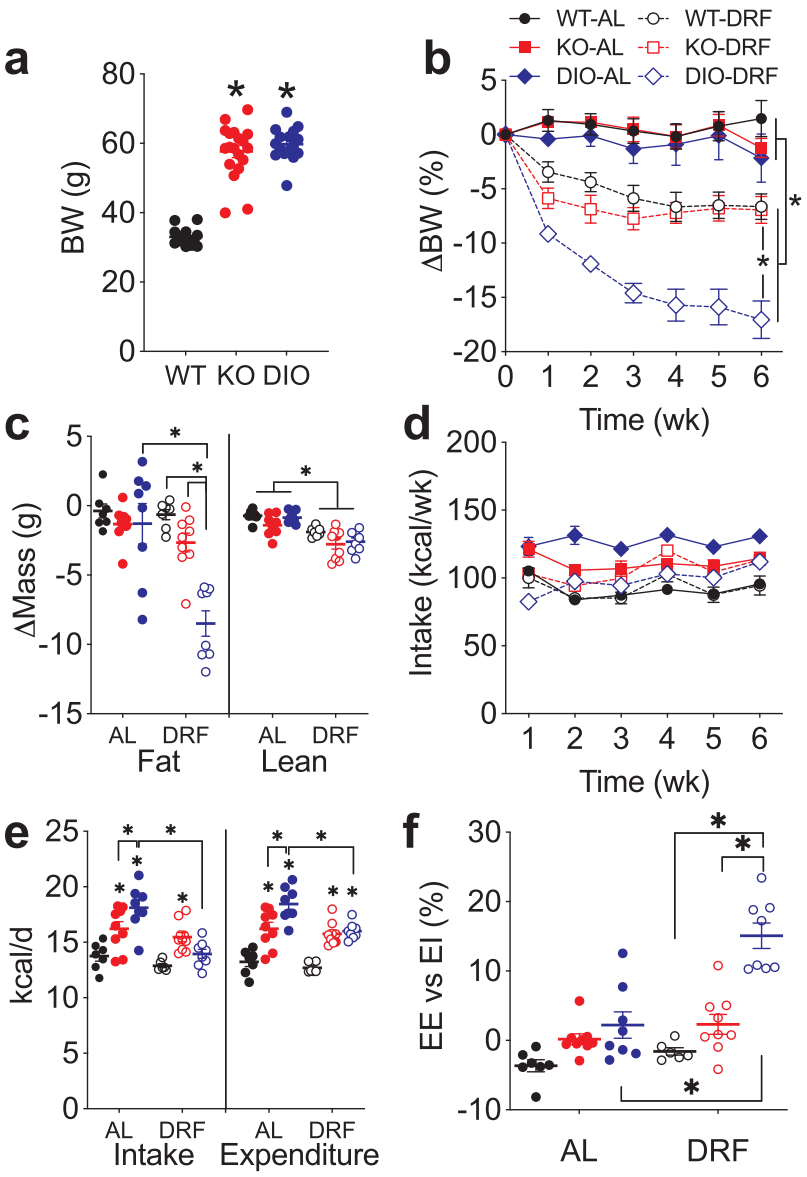

Fig. 4. Decreased efficacy of DRF to reduce obesity and caloric intake in *Mc4r*$^{-/-}$ mice. (a) Body weight of male DIO mice, BW-matched *Mc4r*$^{-/-}$ mice and their wildtype littermates prior to DRF feeding for 6 wk. (b) BW change, (c) change of fat and lean mass and (d) weekly caloric intake of single housed mice following AL or DRF feeding for 6 wk. (e) Average daily energy intake and calculated energy expenditure during the experimental period. (f) Energy expenditure relative to energy intake expressed as a percentage. Data are depicted as mean ± SEM. n = 7/10 group. * = P < 0.05. 2-way ANOVA followed by Sidak multiple comparisons test.

changes in the expression of the molecular clock play a role in the reduction of food intake and BW following DRF (Fig. 2a). We therefore assessed the circadian expression of genes regulated by metabolic signals associated with fasting and feeding in the same nuclei. We examined the expression of genes associated with the activity of the melanocortin system, a neuronal circuit that plays a critical role in the control of feeding. *Pomc* (Fig. 2c), as well as *Lepr* gene expression (data not shown), were unaffected by DRF. In contrast, DRF mice had significantly increased expression of *Agrp* and *Ghsr* at ZT14 compared to AL controls (Fig. 2c), and this difference preceded a higher *Fos* gene expression throughout the dark phase in the DRF group, that reached statistical significance at ZT18 (Fig. 2c).

The expression of the aforementioned clock genes in the SCN did not differ significantly between AL and DRF mice (Fig 2d). Likewise, genes associated with FGF21 signaling *(Fgfr1* and *Klb)*, known to regulate metabolic control at the level of the SCN [12], did not differ significantly (Fig. 2d). Likewise, the expression of *Egr1,* a marker of acute activation of FGF21/KLB signaling [13], did not differ significantly between the DRF and AL groups (Fig. 2d). Consistent with this, *Fos* gene expression in the SCN, a marker of overall neuronal activation, did not differ significantly between the AL and DRF mice (Fig. 2d).

Multiple hormones relay information related to energy status to these hypothalamic nuclei. Circulating levels of FGF21, which targets the SCN via the Fgfr1/Klb receptor complex to regulate energy balance [14], did not change significantly with DRF (Fig. 2e). Insulin levels varied significantly throughout the day (P < 0.001, main effect of ZT time) and tended to be higher during the first half of the dark phase in both groups. Although, the levels did not differ significantly between the two groups (Fig. 2e), only the DRF mice displayed significant differences throughout the day (post hoc Sidak's test P < .005, 14h vs. 6h, 10h and 22h), suggesting an increase in the amplitude of the circadian rhythm of insulin in the DRF mice following the initiation of feeding. Leptin levels also exhibited a significant effect of ZT time (P < 0.05, main effect of ZT time) and a significant interaction with DRF

($P < 0.01$), reaching the highest concentration at ZT6 in the DRF group. Interestingly, plasma total ghrelin levels were significantly regulated by DRF, and its plasma concentration was higher at ZT 10h in the DRF mice relative to controls (Fig. 2e). This increase preceded the presentation of food to the DRF mice as well as the increase in the expression of *Ghsr* and *Agrp* expression in the ARC (Fig. 2c).

Collectively, these data contrast with the effect of DRF on the expression of molecular clock genes in peripheral tissues and consequently suggest that the BW-lowering effect of DRF in DIO does not appear to be due to changes in the circadian expression of molecular clock genes in these hypothalamic nuclei. In contrast, differential regulation of genes and changes in circulating levels of hormones important for the function of the melanocortin system suggest a pivotal role for the melanocortin system mediating metabolic benefits of DRF.

3.3. DRF increases the fat preservation activity of ghrelin in DIO mice

To determine the contribution of the increased total plasma ghrelin at ZT10 to the effect of DRF (Fig. 2), we first sought a dose of exogenous active ghrelin (i.p.) that would increase plasma ghrelin at ZT10 comparably to that induced by 2 wk of DRF. A dose of 50 ug/kg of active ghrelin administered at ZT9 increased total plasma ghrelin 2.3 fold at ZT10 in AL DIO mice (Fig. 3a). Neither that nor higher doses of ghrelin (up to 150 ug/kg) increased food intake acutely when given to AL DIO mice (Fig. 3b). Likewise, daily injection of 50 ug/kg of active ghrelin at ZT9 for 14 d had no effect on caloric intake (Fig. 3c,d) in AL or DRF DIO mice. Intriguingly, this low dose of ghrelin was sufficient to significantly blunt the efficacy of DRF to reduce BW (Fig. 3e). Analysis of body composition revealed that the prevention of BW loss was due to significant preservation of fat mass in the DRF mice (Fig. 3f).

Given the increased obesogenic effect of ghrelin, and that leptin also targets the Agrp neurons in the ARC to reduce body weight, we investigated whether DRF could improve energy balance, at least in part, by modulating the efficacy of leptin regulating energy balance. To test this, we infused chronic leptin (icv, 1 μg/day for 7 d) or vehicle (p.b.s.) into DIO mice (BW = 65 ± 1g) on a DRF schedule. As expected, leptin failed to reduce BW or food intake in the AL-DIO control mice (Fig. 3g-i). In contrast, leptin promoted significant BW loss in DRF-DIO mice compared to vehicle controls (Fig. 3g), an effect secondary to a significant and near-immediate reduction of food intake (Fig. 3h,i). These data indicate that the imposed DRF schedule enhances the catabolic action of leptin in the brain.

Taken together, the increase in ghrelin and leptin action suggest that the imposed DRF schedule improves the control of energy balance by enhancing the response of the melanocortin system to afferent fasting-feeding signals.

3.4. Role of Mc4r signaling in the reduction of obesity by DRF

We next asked whether the efficacy of DRF to reduce obesity requires intact melanocortin signaling by comparing the efficacy of DRF in DIO mice and obese, BW-matched *Mc4r*$^{-/-}$ (KO) mice, as well as in their age-matched WT littermates (Fig. 4a). Both KO and WT groups were fed standard chow since the exaggerated BW gain exhibited by KO mice when fed a HFD precludes equalizing BW and age. The mice were singly-housed and one cohort of each genotype underwent DRF for 6 weeks while the other cohorts (AL) had free access to food. There was a significant BW loss in all three DRF groups when compared to the corresponding AL groups ($P < 0.05$, main effect of DRF, Fig. 4b). DRF promoted significantly greater BW loss in DIO mice when compared to lean DRF-WT mice, and also when compared to DRF-KO mice, which failed lose BW beyond the small effect seen in their lean DRF-WT littermates (Fig. 4b). DRF resulted in highly significant fat mass loss DIO in mice (Fig. 4c). In contrast, DRF failed to significantly reduce fat mass in lean WT or in obese Mc4r-/- mice (Fig. 4c). DRF did promote a small but significant reduction in lean mass that was similar between groups (Fig. 4c) and which is consistent with the BW loss exhibited by the DRF WT and KO mice (Fig. 4b). Consistent with our previous findings, DRF led to an immediate and significant reduction in weekly energy intake in DIO mice that persisted throughout the experiment (Fig. 4d). In contrast, DRF did not impact significantly weekly caloric intake in WT and KO compared to their AL counterparts, with the exception of a transient but significant reduction in calorie intake during the first week in the DRF-KO mice compared to the AL controls (Fig. 4d). DRF had no impact on total 2-wk caloric intake in lean WT or obese hyperphagic KO mice (Fig. 4e, left). In contrast, DRF significantly reduced the hyperphagia exhibited by DIO mice down to the levels of both lean WT groups (Fig. 4e, left). We estimated energy expenditure throughout the experiment by taking into account the caloric consumption and the changes in fat and lean mass. 2-way ANOVA detected a significant effect of genotype and DRF on estimated energy expenditure, as a well as a significant interaction between both variables ($P < 0.05$, Fig. 4e, right). Notably, a *post-hoc* analysis revealed that energy expenditure in the DIO-DRF group was significantly reduced compared to that of DIO-AL controls. Nonetheless, the ratio of energy expenditure to energy intake was significantly elevated in DIO-DRF mice compared to those of the DRF groups or their AL counterparts (Fig. 4f). This suggests that despite the marked reduction in energy intake, DIO-DRF exhibited a lesser reduction in energy expenditure and that this could contribute to the larger BW loss.

3.5. Role of Mc4r signaling in the prevention of diet-induced obesity bestowed by DRF

DRF not only reduces [11] but can also prevent DIO [15]. We next investigated the contribution of MC4R to the efficacy of DRF by offering the same HFD, either AL or as DRF, to male MC4R KO and their WT littermates with similar starting BW. As expected [15], DRF prevented excessive BW gain in WT mice (Fig. 5a) by specifically reducing fat mass gain (Fig. 5b). This was associated with a significant reduction of energy intake in DRF-WT mice compared to AL-fed WT controls which, on the other hand, exhibited a progressive and statistically significant development of hyperphagia (Fig. 5c,d). HFD-fed KO mice gained significantly more BW fat and lean mass than their WT littermates (Fig. 5a,b), and although DRF had an overall statistically significant effect on the BW of KO mice ($P < 0.05$), this difference lost statistical significance after the initial 45 d (Fig. 5a). Consistent with this, fat and lean mass gain in DRF and AL-KO mice did not differ by the end of the study (Fig. 5b). The initial attenuation of BW gain in DRF-KO mice was correlated with a transient but significant reduction of their hyperphagia compared to AL-KO controls (Fig. 5c). Indeed, total energy intake at the end of the study did not differ between DRF-KO mice and their AL counterparts (Fig. 5d, left). In contrast, DRF resulted in a significant reduction in energy intake in the WT controls, as expected (Fig. 5d, left). Total energy expenditure throughout the experiment was significantly higher in KO mice compared to WT controls (Fig. 5d, right). Consistent with the results described earlier, DRF did not increase energy expenditure. In fact, DRF mice exhibited significantly lower energy expenditure than their AL controls, although *post hoc* analysis failed to detect differences within genotypes (Fig. 5d, right). Since non-shivering thermogenesis via uncoupling protein 1 (UCP-1) in brown adipose tissue (iBAT) is a main determinant of energy expenditure in mice, we measured UCP-1 protein in the iBAT at the end of the study. UCP-1 levels were significantly reduced in KO mice compared to WT controls, whereas DRF had no significant impact on UCP-1 levels on either genotype (Fig. 5e), suggesting that increased non-shivering thermogenesis did not contribute to the reduction in BW gain by DRF in WT mice. Despite the total reduction of energy expenditure, WT-DRF mice had significantly higher energy expenditure relative to energy intake compared to their WT-AL controls (Fig. 5f). This is consistent with the data above (Fig. 4f) and supports the possibility that DRF prevents excessive reductions on energy expenditure that

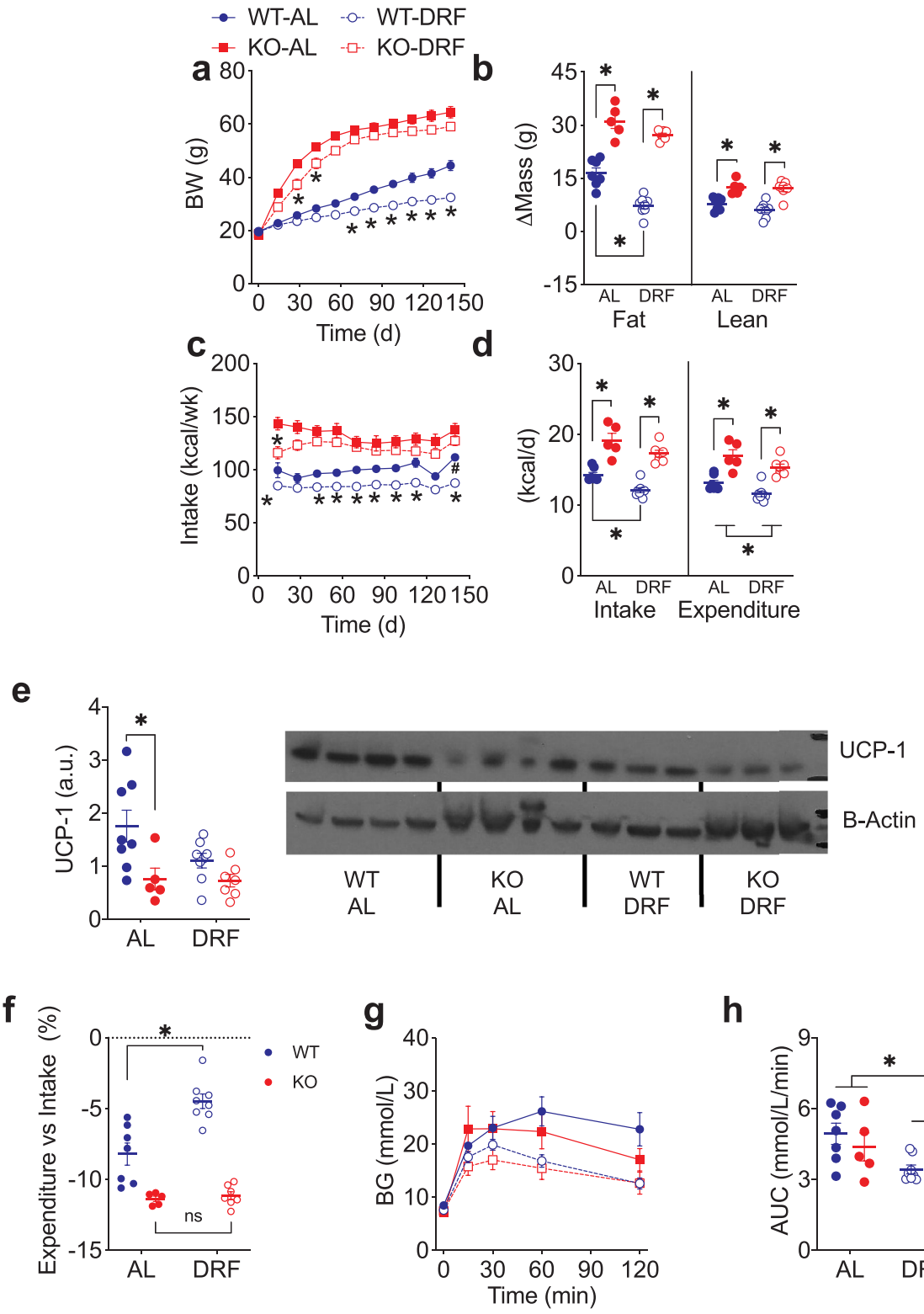

Fig. 5. Decreased efficacy of DRF to reduce DIO and caloric intake in *Mc4r*$^{-/-}$ mice. (a) Body weight of male *Mc4r*$^{-/-}$ mice (KO) and their wildtype (WT) littermates fed a HFD either *ad libitum* (AL) or as dark-phase restricted feeding (DRF). (b) Change of fat and lean mass during the period of HFD feeding. (c) Weekly caloric intake. (d) Average daily energy intake and expenditure for the duration of the experiment. (e) Uncoupling protein-1 (UCP-1) levels in intrascapular brown adipose tissue at the end of the study. (f) Energy expenditure relative to energy intake expressed as a percentage. (g) Blood glucose levels during a glucose tolerance test during the final week of the experiment. (h) Calculated area under the curve (AUC) of the data presented in (g). Data are depicted as mean ± SEM. n = 5/8 group. * = P < 0.05. 2-way ANOVA followed by Sidak multiple comparisons test.

contribute to BW in wildtype mice. Consistent with their similar increases in fat and lean mass, both groups of KO mice exhibited comparably lower energy expenditure relative to energy intake, irrespective of DRF (Fig. 5f). Interestingly, and in contrast to the effect on BW, DRF improved glucose tolerance both in WT and KO mice (Fig. 5g,h). These data further suggest that signals generated due to DRF target the melanocortin system to improve energy balance. In contrast, mechanisms other than Mc4r signaling play a predominant role in mediating the beneficial effects of DRF on other aspects of metabolic control, such as whole-body glucose homeostasis.

4. Discussion

The high incidence of obesity is a pervasive negative contributor to overall health that remains in need of effective treatment. Recent studies in DIO mice suggest that restricting food availability to specific periods of time, specifically dark-phase restricted feeding (DRF), is sufficient to reduce or prevent DIO leading to improvements in glycemic and lipid control. This change has been associated with increased synchrony and amplitude of circadian rhythms of gene expression in metabolically relevant peripheral tissues [11,15,16]. Our data suggest that the full extent of metabolic benefits following DRF require a functional brain-melanocortin system.

The activities of multiple metabolic pathways have marked circadian rhythms, and the importance of such rhythms to maintain appropriate energy balance is illustrated by the metabolic abnormalities exhibited by mutant mouse models lacking expression of specific molecular clock genes [17]. In agreement with this, metabolic dysfunction, including diet-induced obesity (DIO), is associated with impaired circadian rhythms. Specifically, DIO mice have a decreased amplitude of circadian locomotor activity and altered feeding rhythms due to an increase in caloric intake during the inactive, light phase [5]. Furthermore, compared to lean controls, DIO mice have reduced amplitude in the circadian expression of molecular clock genes and others that regulate metabolic activity in multiple peripheral tissues. This impaired circadian control of gene expression has been linked to the development of metabolic disease associated with obesity [5,11].

In contrast to initial findings that DRF prevents or reduces DIO independently of caloric intake [11,15], we consistently documented a significant reduction of total daily caloric intake by removing food during the light phase (DRF mice) compared to ad lib-fed (AL mice) DIO mice. These data are consistent with other reports of DRF using different high-fat diets, mouse strains and sex [6–8]. The reduction of total daily intake occurs acutely and persists over time. Despite this, the feeding of DRF mice during the dark phase exceeds that of AL mice. This relative overconsumption is consistent with an effective sensing of the caloric deprivation that is a consequence of the imposed fasting during the light phase. Intriguingly, providing access to low-fat calories during the light phase did not increase total caloric intake of DRF mice to that of AL mice. These data suggest that the increased feeding during the light phase, a feature characteristic to DIO mice [5], may be opportunistic and driven by hedonic behavior rather than the result of unrestrained feeding due to impaired homeostatic control of energy balance.

DRF has been linked to increased amplitude of circadian oscillations of the expression of molecular clock genes in peripheral tissues such as fat and liver [11], oscillations known to be blunted by DIO [5]. Despite this and the impact of DRF on feeding, we failed to detect consistent effects of DRF on the circadian expression of molecular clock genes in SCN, PVN or ARC, hypothalamic nuclei known to be involved in the control of energy balance. The one exception was a significant increase in amplitude of *Nr1d1* gene expression in the ARC following DRF. *Nr1d1* gene expression in AGRP neurons of the ARC is significantly downregulated by food deprivation [18], and Nr1d1 agonists promote BW loss in DIO mice, an effect that correlates with changes in the expression of molecular clock genes in the hypothalamus [19]. The extent to which these and our observations link increased circadian oscillation of Nr1d1 expression in the ARC to the beneficial effect of DRF remains to be demonstrated. However, this effect appears to be more the exception than the norm and, in contrast to the effect on peripheral tissues, the expression of other molecular clock genes remained largely unaffected by DRF. It should be noted that, in contrast to the effect seen in peripheral tissues, DIO does not impair the circadian expression of molecular clock genes in the hypothalamus [5], including discrete nuclei involved in the control of energy balance including the dorsomedial nucleus of the hypothalamus, ARC and SCN [20]. Considering this, lack of improvement of circadian expression of the molecular clock gene following DRF is not surprising. These data alone do not exclude the possibility that the control of the molecular clock function in specific neurons of the hypothalamus contributes to the metabolic benefits derived from chronic DRF. However, when considered with the reports that DRF promotes the same metabolic benefits effects in various mouse models with impaired molecular clock function [16,21,22], the implication is that engagement of other mechanisms must account for such benefits.

We also investigated the impact of DRF on the circadian expression of genes regulated by metabolic signals. FGF21 in the SCN is an important modulator of the control of energy balance. This capacity is supported by the relatively specific expression of the FGF21 co-receptor *Klb* [23] and the expression of *Fgfr1* in the SCN [12]. We detected *Fgfr1* and *Klb* expression in the SCN. Interestingly, *Klb,* as well as *Egr1* expression which is an indicator readout of acute KLB activation by FGF21 [13], exhibited a robust circadian rhythm. However, DRF impacted neither circulating FGF21 levels nor the circadian rhythm of SCN-*Egr1* expression, suggesting a limited contribution of FGF21 signaling directly at the SCN to the changes induced by DRF.

In contrast to the lack of differential engagement of the hypothalamic molecular clock or FGF21-regulated signaling in the SCN, our results point toward an involvement of the brain melanocortin system as a critical contributor to the effects of DRF on energy balance. This notion is supported by DRF-elicited changes in *Fos* expression in ARC and PVN. Feeding-induced changes in *Fos* expression in those nuclei are largely attributed to first- and second-order neurons of the melanocortin system [24,25]. Further, increased fos activity occurs simultaneously with increases in the amplitude of *Agrp* and *Ghsr* circadian gene expression in ARC. Consistent with previous findings in rats [26], we observed maximal expression of *Agrp* expression after the onset of the feeding phase, and that DRF further increases the amplitude of *Agrp* expression levels. This timing is also consistent with increased activity of Agrp neurons nearing the onset of the dark phase [27] and suggests that DRF may promote an increase in the circadian activity of Agrp neurons.

Consistent with a previous report [28], an increase of circulating ghrelin preceded the presentation of anticipated food as well as an increase in *Agrp, Ghsr* and *Fos* gene expression in the ARC. All of these point toward a contribution of ghrelin, a peptide known to activate Agrp neurons [29], to the increased circadian activity of Agrp neurons during DRF. Interestingly, DRF mice had maximal leptin concentrations during the fasting period (light phase).F Taken together, these data suggest that DRF may change the activity of the melanocortin system by regulating the levels of its afferent hormones. Interestingly, our pharmacological studies involving exogenous administration of leptin or ghrelin suggest that DRF not only impacts their circadian levels but also increases their efficacy regulating energy balance in a manner consistent with their actions on the central melanocortin system. Increased efficacy of leptin to suppress feeding and regulate gene expression specifically in Agrp neurons during the light phase has recently been reported [30]. This suggests that DRF may potentiate the circadian response of the Agrp neuron to leptin. In the case of ghrelin, it is noteworthy that pharmacological supplementation with doses leading to circulating ghrelin levels similar to those exhibited by DRF mice were ineffective at regulating caloric intake but were sufficient to

significantly attenuate the BW loss induced by DRF in DIO mice, largely due to preservation of fat mass. The inability of such doses to affect feeding is consistent with previous studies which determined that eliciting ghrelin-induced hyperphagia in mice requires concentrations more than two orders of magnitude above physiological [31]. That report proposed that the predominant physiological role of circulating ghrelin levels is protection from hypoglycemia during severe starvation. DRF reveals the protection from excessive depletion of energy stores as another physiological role of circulating ghrelin. Given the critical role of ghrelin receptor expression in the Agrp neurons mediating ghrelin-induced adiposity [32] and that factors like high fat diet feeding impair ghrelin signaling at the Agrp neurons [33], our data suggest that DRF may increase ghrelin effectiveness preventing fat mass loss by modulating the function of Agrp neurons. Being that the case, pharmacological interventions aimed to reduce ghrelin action may offer additional weight lowering benefits when combined with dietary interventions mimicking the effects of time-restricted feeding. Regardless of that, the increase in leptin and ghrelin action suggest that the imposed fasting during DRF helps maintain catabolic activity by enhancing the response to afferent fasting-feeding signals known to modulate the brain melanocortin system.

The loss of effectiveness of the DRF paradigm to lower BW in Mc4r KO mice confirms the critical contribution of melanocortin system activity to the anti-obesity effects of DRF. This loss of efficacy occurred in both the context of intervention, once the mice were already obese, and that of prevention since it failed to reduce the BW gain upon chronic HFD feeding. This contrasts with the clear benefits of DRF in wildtype mice in both circumstances, consistent with previous reports [11,15]. The loss of anti-obesity efficacy of DRF in Mc4rKO mice was associated with a failure to reduce caloric intake. Furthermore, DRF resulted in decreased total energy expenditure and in a trend toward reduction, certainly not an increase, in UCP-1 protein levels in iBAT, a marker of non-shivering thermogenesis. Previous work reported transient increases in energy expenditure that correlated with increased *Ucp1* gene expression in iBAT at the end of the feeding phase in DRF mice [11]. In that report, energy expenditure was determined by indirect calorimetry during a discrete period of time throughout the experiment, and the data were normalized to body weight. Those factors limit the possibility to accurately determine to which extent those transient increases in energy expenditure or *Ucp1* gene expression impact overall energy balance. On the other hand, the higher ratio of energy expenditure to energy intake observed in HFD-DRF *wildtype* mice suggests that DRF prevents the excessive reduction in energy expenditure associated with BW loss due to reduced caloric intake [34]. Whether non-shivering thermogenesis or other contributor to energy expenditure account for that difference remains to be determined. Nonetheless, these data further emphasize the importance of reductions on energy intake, rather than increases of energy expenditure, as the main contributor to the negative energy balance promoted by DRF to reduce obesity, at least in our model of DIO.

Mc4r signaling is a critical determinant of glucose homeostasis and plays a role in the circadian rhythm of glucose tolerance [35]. Interestingly, DRF improves glucose tolerance in Mc4rKO mice despite the loss of effectiveness at reducing hyperphagia and obesity. The improvement in glucose tolerance in DIO mice following DRF has been well documented [11,15], but the mechanisms mediating such improvements remain to be elucidated. Interestingly, such mechanisms can overcome, not only the loss of MC4R signaling, but also to the loss of a functional molecular clock to promote better glucose control, as recently reported [16].

5. Conclusions

We provide novel insights on the physiological systems and potential mechanisms whereby timed feeding promotes metabolic benefits, placing the control of energy balance by the central melanocortin signaling as a critical mediator of such benefits. Taken together, these results suggest that a combination of meal timing along with pharmacological control of the central melanocortin signaling may provide efficacious and long-lasting benefits in metabolic control.

Disclosure statement

The authors have nothing to disclose.

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