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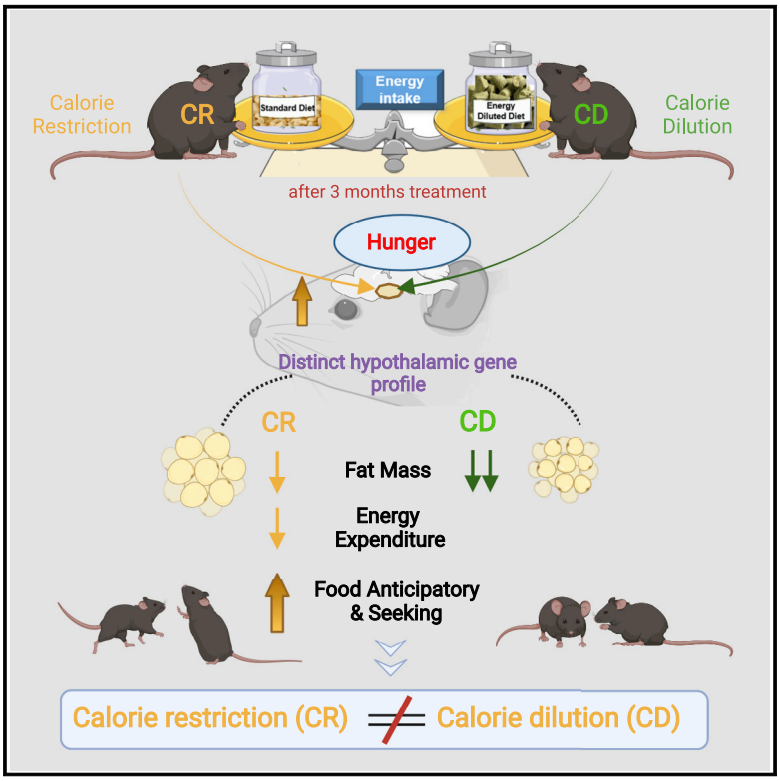
Calorie restriction and calorie dilution have different impacts on body fat, metabolism, behavior, and hypothalamic gene expression

Graphical abstract



Highlights

- CD causes greater loss of fat mass than CR
- CR mice have greater food anticipatory behavior compared with CD mice
- CR leads to greater reduction in energy expenditure and higher RER than CD
- Hypothalamic gene expression is consistent with more hunger after CR than CD

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In brief

Liu et al. compare the impacts of calorie restriction (CR) and calorie dilution (CD) on morphology, metabolism, behavior, and hypothalamic gene expression. Increased hunger under CR suggests that hunger signaling is a key process involved in mediating the benefits of CR for lifespan. CR and CD are not equivalent procedures.

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Calorie restriction and calorie dilution have different impacts on body fat, metabolism, behavior, and hypothalamic gene expression

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SUMMARY

Caloric restriction is a robust intervention to increase lifespan. Giving less food (calorie restriction [CR]) or allowing free access to a diluted diet with indigestible components (calorie dilution [CD]) are two methods to impose restriction. CD does not generate the same lifespan effect as CR. We compare responses of C57BL/6 mice with equivalent levels of CR and CD. The two groups have different responses in fat loss, circulating hormones, and metabolic rate. CR mice are hungrier, as assessed by behavioral assays. Although gene expression of Npy, Agrp, and Pomc do not differ between CR and CD groups, CR mice had a distinctive hypothalamic gene-expression profile with many genes related to starvation upregulated relative to CD. While both result in lower calorie intake, CR and CD are not equivalent procedures. Increased hunger under CR supports the hypothesis that hunger signaling is a key process mediating the benefits of CR.

INTRODUCTION

Calorie restriction (CR) increases life- and healthspans (Speakman and Mitchell, 2011; Weindruch and Sohal, 1997) in a wide variety of both ectotherms and endotherms. This includes among the ectotherms *Caenorhabditis elegans* (Braeckman et al., 2006; Lakowski and Hekimi, 1998) and *Drosophila melanogaster* (Bross et al., 2005; Burger et al., 2010). Among endotherms, it includes mice *Mus musculus* (Weindruch, 1996; Weindruch and Walford, 1982; Yousefi et al., 2018), rats *Rattus norvegicus* (Masoro et al., 1982; McCay et al., 1935; Merry, 2002), macaques (Colman et al., 1999; Mattison et al., 2017), and gray mouse lemurs (Pifferi et al., 2018). Comparisons across inbred strains of mice (Liao et al., 2010; Mitchell et al., 2016b; Rikke et al., 2010), flies (Jin et al., 2020; Spencer et al., 2003), and worms (Lakowski and Hekimi, 1998) show that CR generates a variety of outcomes dependent on genotype, including prolongation and shortening of lifespan. Studies in humans show improvements in several biomarkers related to health outcomes (Dorling et al., 2021; Heilbronn et al., 2006), suggesting that the positive impacts in animals may translate to humans.

One aspect of CR feeding protocols is that inevitably one or more macronutrients (fat, carbohydrate, and protein) are also reduced. This led to the question of whether the impact of CR was due to a reduced supply of protein rather than energy. In *Drosophila*, exposure to a range of diets of different macronutrient compositions and levels of restriction suggested that the impacts of CR on lifespan could be explained by the restriction of protein (Piper et al., 2005, 2011). The reason for this effect, however, could be that the demands for energy among ectotherms (like flies) are lower than in endotherms (like mammals). For example a 30 g endothermic mouse expends about 20× more energy than a 30 g ectothermic lizard (Nagy, 1983, 1987). Thus, restricting energy in an endotherm may be more significant than restricting it an ectotherm, where protein effects may dominate.

It was surprising then that after exposing mice to a matrix of different diets and levels of restriction, a study concluded that the main impact on lifespan was due to restriction of protein

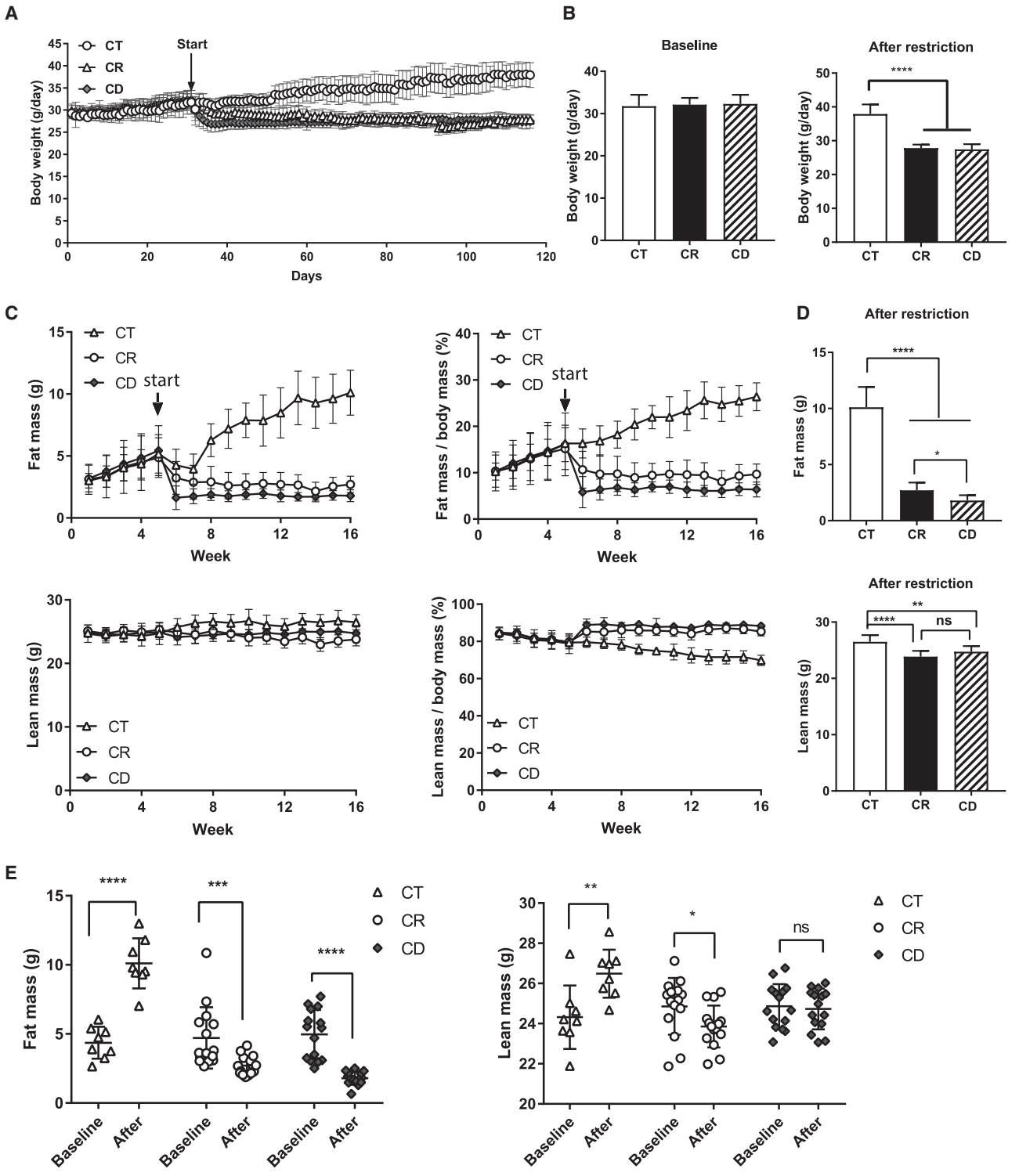

Figure 1. Calorie dilution (CD) caused more fat loss than calorie restriction (CR)

(A) Day 0–116 body-weight changes (g). Baseline (day 0–31): CT: n = 8 and CR and CD: n = 15; treatment period (day 32–116): CT: n = 8 and CR and CD: n = 19.

(B) Average body weight of control (CT), CR, and CD groups at baseline and at the end week of the treatment. Analyzed by one-way ANOVA followed by Tukey's multiple comparisons test; compare each group with every other group.

(C) Weekly changes in fat mass and lean mass.

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(Solon-Biet et al., 2014). Indeed, in this latter study, lower calorie intake was associated with a life-shortening effect. This result was surprising because a number of CR studies in mice have adjusted the dietary protein so that protein was not restricted (Davis et al., 1983; Lopez-Dominguez et al., 2015), and these studies showed that under calorie, but not protein, restriction, the lifespan extension is identical to that when both are restricted (Speakman et al., 2016). Moreover, the responses of mice to protein, but not calorie, restriction do not generate most of the phenotypic responses to CR that are believed to mediate the beneficial impacts (Mitchell et al., 2015a, 2015b, 2015c, 2016a).

One interesting difference between the multi-diet mouse study (Solon-Biet et al., 2014) and other studies of CR in mice was that the methodology to generate restriction was to dilute the diet with different levels of an inert indigestible component (e.g., cellulose). Hence, the mice had *ad libitum* access to the diet but still consumed fewer calories. This contrasts the usual method where simply less food is provided. This could be a problem because one of the proposed mechanisms by which CR generates its beneficial effects is via stimulation of the hunger-signaling pathways in the brain (Derous et al., 2016a, 2016b). The anomalous results of Solon-Biet et al. (2014) might be because diluting the diet (calorie dilution [CD]) rather than restricting it (CR) may have fundamentally different impacts on these pathways. The goal of the present study was to compare the responses of the same strain of mice (C57BL/6J) with equivalent levels of CR and CD to evaluate this hypothesis.

RESULTS

Food intake and assimilation efficiency

Mice were fed *ad libitum* during a baseline period with a standard low-fat (10%) diet (D12450B) (Figure S1A). There was no significant difference among groups in mean energy intake during baseline: control (CT), 63.01 ± 4.50 KJ/day (n = 8); CR, 63.06 ± 4.04 KJ/day (n = 15); and CD, 62.13 ± 3.97 KJ/day (n = 15) (Figure S1B). From day 31 onward, mice were exposed to CR and CD protocols using a pair-feeding design for 85 days. The CD group were fed *ad libitum* with a custom designed diet comprising 50% cellulose (D16061505). Mice from the CR group were pair fed a day later to match the energy absorption of the CD group. Under CD, food intake increased, but this was insufficient to offset the indigestible portion of the diet. Hence, net energy intake of CD mice decreased dramatically on the first day of feeding. Over the next 9 days, food intake gradually increased until it stabilized (Figure S1A). Mean energy intake over the whole treatment period was 62.34 ± 9.65 KJ/day (n = 8) for CT, 51.56 ± 3.81 KJ/day (n = 19) for CR, and 52.74 ± 5.25 KJ/day (n = 19) for CD (Figure S1B). Energy intake of the CT group was significantly higher than both CR and CD groups, which were not significantly different from each other. The overall achieved restriction was 14.6% ± 3.89% for CD and 16.5% ± 4.67% for CR relative to their own baseline intakes. The fecal-pellet size of the CD group was much larger than that of CT and CR groups during treatment (Figure S1C), and the daily feces production of the CD group (3.6 ± 0.3 g) was much heavier than CT and CR mice, at 0.31 ± 0.02 and 0.30 ± 0.04 g, respectively, on day 70. Similar differences in daily feces production were observed at days 45 and 54 (Figure S1D). However, calorie content of feces changed more slowly than fecal-production levels after treatment. There was no significant difference in fecal calorie content among groups on day 45, but a significant difference was observed on days 54 and 70. Feces of CD mice contained more calories than that of CT and CR groups, and feces from the CR group also contained more calories than that from the CT group. On day 70, feces produced by CD mice contained 16.1 ± 5.4 kJ/g compared with 13.5 ± 4.5 kJ/g for CT and 15.1 ± 5.0 kJ/g for CR (one-way ANOVA followed by Tukey's multiple comparisons test, CT versus CR: $p = 0.005$, CT versus CD: $p < 0.0001$, and CR versus CD: $p = 0.0478$) (Figure S1E). Assimilation efficiency was calculated from fecal-energy production compared with the simultaneous food intake. There were no significant changes in assimilation efficiency over the dietary treatment period among the different treatment groups (two-way ANOVA test). The assimilation efficiency of CR and CT groups was not significantly different, but both were much higher than CD (two-way ANOVA followed by Tukey's multiple comparisons test, CT versus CR: $p > 0.05$, CT versus CD: $p < 0.0001$, CR versus CD: $p < 0.0001$). The assimilation efficiencies of the CR group were 90.0% ± 2.7%, 91.6% ± 0.6%, and 91.0% ± 0.8% and in CT mice were 90.2% ± 1.3%, 93.4% ± 0.4%, and 94.1% ± 0.7% on days 45, 54, and 70, respectively. In comparison, the assimilation efficiency of the CD group was 46.7% ± 9.3%, 49.9% ± 4.2%, and 46.5% ± 1.3% on the same days (Figure S1F).

CD mice lost more fat than CR mice

There was no significant difference in mean body weight during the baseline among three groups (Figures 1A and 1B). The mean baseline body weights of CT were 31.76 ± 2.7 g (n = 8), of CR were 32.13 ± 1.6 g (n = 15), and of CD were 32. 27 ± 2.2 g (n = 15) (Figure 1B). Across the whole experiment, body weight varied significantly over time (repeated measures-generalized linear model [RM-GLM], $F_{(168,3318)} = 8.16$, $p < 0.0001$) between groups (RM-GLM, $F_{(2,3064)} = 3201$, $p < 0.0001$), with a significant diet-by-day interaction (RM-GLM, $F_{(84,3318)} = 4.26$,$p < 0.0001$). Body weight decreased rapidly in CD mice from day 32 to 41 then remained stable. Body weight of CR mice deceased more slowly than that of CD mice (Figure 1A). At the end of treatment, the mean body weights of the CR (27.8 ± 1.1 g, n = 19) and CD (27.44 ± 1.5 g, n = 19) groups were significantly lower than that of the CT mice (37.91 ± 2.8 g, n = 8), but there was no significant difference between CR and CD mice (one-way ANOVA followed by Tukey's multiple comparisons test, CT versus CR: $p < 0.0001$, CT versus CD: $p < 0.0001$, and CR versus CD: $p = 0.79$) (Figure 1B).

Fat mass and lean mass were recorded weekly. Fat mass declined significantly over time (RM-GLM, $F_{(15,\ 611)} = 10.5$,

(D) Fat mass and lean mass after 11 weeks of treatment. Analyzed by one-way ANOVA followed by Tukey's multiple comparisons test; compare each group with every other group.

(E) Changes in lean and fat mass between baseline and 11 weeks of of treatment. Analyzed by paired t test for each group.

For (B)–(E), CT: n = 8 and CR and CD: n = 15. All data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

$p < 0.0001$) and was affected by dietary treatment (RM-GLM, $F_{(2,564)} = 413.83$, $p < 0.0001$) and by the interaction of time and treatment (RM-GLM, $F_{(30,611)} = 21.65$, $p < 0.0001$). There were no significant differences in the mean body fat mass and lean mass among CT, CR, and CD groups at baseline, with fat mass being 5.2 ± 1.5, 4.9 ± 1.6, and 5.4 ± 2.0 g and lean mass being 24.7 ± 1.3, 25.3 ± 1.1, and 24.8 ± 1.2 g, respectively (Figures 1C and 1E). After treatment, mice from both CR and CD groups showed marked fat-mass reduction relative to CT. Moreover, mice from the CD group had significantly lower fat mass compared with mice from the CR group (one-way ANOVA followed by Tukey's multiple comparisons test, CT versus CR: $p < 0.0001$, CT versus CD: $p < 0.0001$, and CR versus CD: $p = 0.037$), but lean mass was not significantly different between CR and CD in the end of the experiment (one-way ANOVA followed by Tukey's multiple comparisons test, CT versus CR: $p < 0.0001$, CT versus CD: $p = 0.0017$, and CR versus CD: $p = 0.075$) (Figure 1C). Compared with baseline, fat mass at the end of experiment in both CD and CR groups was significantly reduced (paired t test, CT: $p < 0.0001$, CR: $p = 0.0079$, and CD: $p < 0.0001$). Lean mass was significantly reduced compared with baseline in the CR group; however, no significant difference was observed in the CD group (paired t test, CT: $p = 0.0026$, CR: $p = 0.0223$, and CD: $p = 0.4259$) (Figure 1E).

Mice responded differently in organ utilization under CR and CD treatments

Wet weights of adipose tissue, spleen, pancreas, kidneys, tail, liver, skin, and carcass were reduced under restriction/dilution relative to CT (Table S1). Adipose tissue including subcutaneous white adipose tissue (Sub. WAT), retroperitoneal WAT (Retro. WAT), epididymal WAT (Ep. WAT), mesenteric WAT (Mesent WAT), and brown adipose tissue (BAT) had greater reductions compared with structural components but were not significantly different between CR and CD groups. For the vital organs, brain, testes, lungs, and heart in both CR and CD groups were not significantly different from the CT group. The wet weight of gastrointestinal tract tissues in CD mice was much heavier than in CR and CT mice. In addition, there was a significant difference in wet weight of caecum (one-way ANOVA followed by Tukey's multiple comparisons test, CT versus CR: $p = 0.2430$, CT versus CD: $p < 0.0001$, and CR versus CD: $p < 0.0001$) and small intestine (one-way ANOVA followed by Tukey's multiple comparisons test, CT versus CR: $p = 0.0148$, CT versus CD: $p < 0.0001$, and CR versus CD: $p = 0.0057$) between CD and CR (Figure 2A; Table S1).

Adipose tissue and the pancreas preferentially contributed to the weight loss after treatment in both CR and CD groups ($\beta > 1$). Adipose tissues were the most utilized under both CR and CD, but most fat stores were utilized more under CD than CR. Carcass and skin were preferentially utilized tissues in CD mice as well ($\beta > 1$) but not in CR mice ($0 < \beta < 1$). However, in contrast, the spleen and liver were protected tissues in the CD group but not in the CR group. Kidneys, tail, lungs, brain, and testes were in protected status under both CD and CR treatments and were more protected in CR, except for testes ($0 < \beta < 1$). Small intestine, caecum, colon, and stomach were invested in ($\beta < 0$), substantially so in the CD group compared with the CR group (Figure 2B; Table S2). These data indicated that organs were utilized differently under CR and CD treatments. In particular, fat stores were more depleted under CD than CR.

Differential hormonal responses to CR and CD treatments

After both CR and CD treatments, leptin levels were significantly lower in CT mice, but there was no significant difference between CR and CD (one-way ANOVA followed by Tukey's multiple comparisons test, CT versus CR: $p < 0.0001$, CT versus CD: $p < 0.0001$, CR versus CD: $p = 0.4784$) (Figure S2A). Fat mass in the CR group was positively related with leptin levels (Pearson correlation, $r = 0.911$, $p = 0.0043$) but unrelated to leptin in CT and CD groups (Pearson correlation, $r = 0.87$, $p = 0.13$ and $r = -0.081$, $p = 0.88$, respectively). There were no significant correlations between fat mass and ghrelin, insulin, insulin growth factor (IGF)-1, glucose-dependent insulinotropic polypeptide (GIP), and gut hormone peptide YY (PYY) (Figure S2B).

One of the major suggested mechanisms by which caloric restriction mediates its effects is via the reduction in insulin and IGF-1 levels. Mice from both CR and CD groups had significantly decreased insulin levels compared with CT mice. However, CR mice had lower insulin compared with the CD group (one-way ANOVA followed by Tukey's multiple comparisons test, CT versus CR: $p < 0.001$, CT versus CD: $p = 0.002$, and CR versus CD: $p = 0.087$). There was no significant difference among CT, CR, and CD groups in levels of GIP and PYY (Figure S2B). Ghrelin in CR mice was significantly higher than in CD mice (one-way ANOVA followed by Tukey's multiple comparisons test, CT versus CR: $p = 0.98$, CT versus CD: $p = 0.022$, and CR versus CD: $p = 0.010$) (Figure S2A).

CR mice showed greater reduction of metabolic rate and higher respiratory exchange ratio (RER) than mice on CD

Mice from CR and CD groups during baseline showed similar total oxygen consumption (mL O2/6-min time interval) during light and dark cycle (two-way ANOVA, $F_{(2,\ 13)} = 0.63$, $p = 0.548$) (Figures S3A and S3B). After 34 days of treatment, the oxygen consumption in both CD and CR groups was reduced compared with the CT mice (Figures S3C and S3D). After 40 days of treatment (day 70), a significant difference was observed between CR and CD in the average light-cycle oxygen consumption, with CR = 66.3 ± 5.2 mL/h and CD = 73.1 ± 7.2 mL/h (ANCOVA, $p = 0.0004$) (Figures S3E and S3F). At day 95, CD mice consumed more oxygen than CR mice throughout the 24 h cycle (ANCOVA, $p = 0.0043$), during the light phase CR = 60.8 ± 14.2 mL/h and CD = 71.1 ± 15.6 mL/h (ANCOVA, $p = 0.00037$), and during the dark phase CR = 92.3 ± 20.5 mL/h and CD = 97 ± 14 mL/h (ANCOVA, $p = 0.031$) (Figures S3G and S3H).

There was no difference among CT, CR, and CD groups at baseline in the RER. During the light cycle, the average RERs in CT, CR, and CD groups were 0.84 ± 0.045, 0.82 ± 0.081, and 0.87 ± 0.07 and in the dark cycle were 0.89 ± 0.086, 0.89 ± 0.085, and 0.92 ± 0.084 (Figures S4A and S4B). After dietary treatment, the RER in the 2 h before lights off in the CR group continued to fall, whereas in the CD and CT groups, it was slightly increased. The RER of CD mice

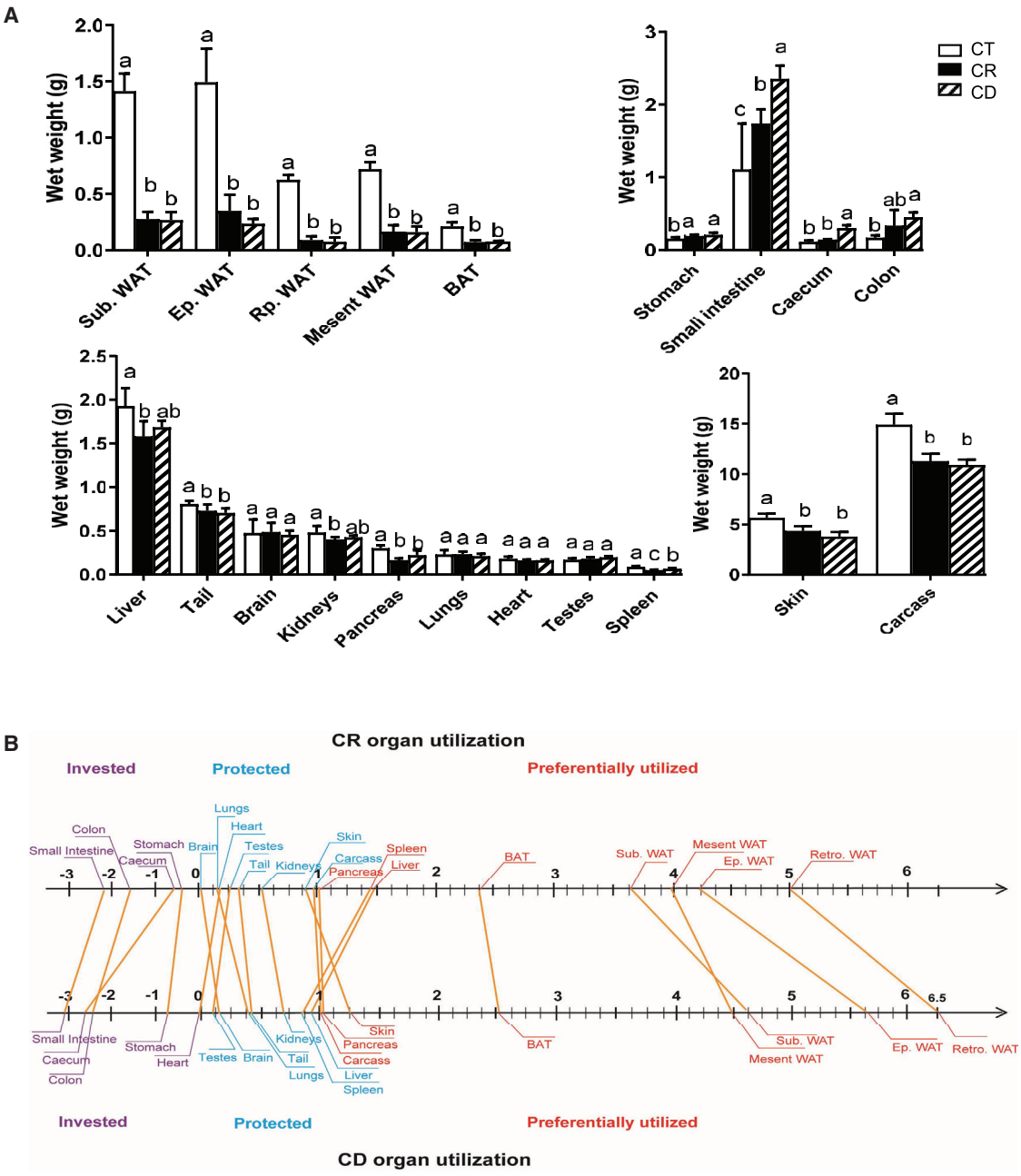


Figure 2. Differential individual organ responses under CR and CD relative to CT

(A) Tissue wet weight from CT, CR, and CD. CT: n = 4, CR: n = 8, and CD: n = 7. Each tissue was separately analyzed by one-way ANOVA followed by Tukey's multiple comparisons test; compare each group with every other group, and differences are indicated by the labeling letters a, b, and c. Groups with significant differences were labeled with different letters. Groups containing the same letter indicate no difference between each other.

(B) Organ-utilization patterns. The gradient of linear least-squares regression equation between logged final body weight and the final organ weight to express the relative utilization of each tissue in the CR and CD groups. Values less than 0 reflect tissues that were invested in. Values >0 and <1 reflect protection of the tissue, and values >1 indicate preferential utilization during weight loss (CR: n = 11 and CD: n = 13). All data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

was much higher than CT and CR groups during this period (Figures S4C, S4E, and S4G). On day 70, during the first 3 h after lights off, the RER of CR group was significantly higher than that of CT and CD groups (two-way ANOVA followed by Tukey's multiple comparisons, $p < 0.05$) (Figure S4E). The same changes in RER at day 75 were observed on day 95 (two-way ANOVA followed by Tukey's multiple comparisons, $p < 0.05$) (Figure S4G).

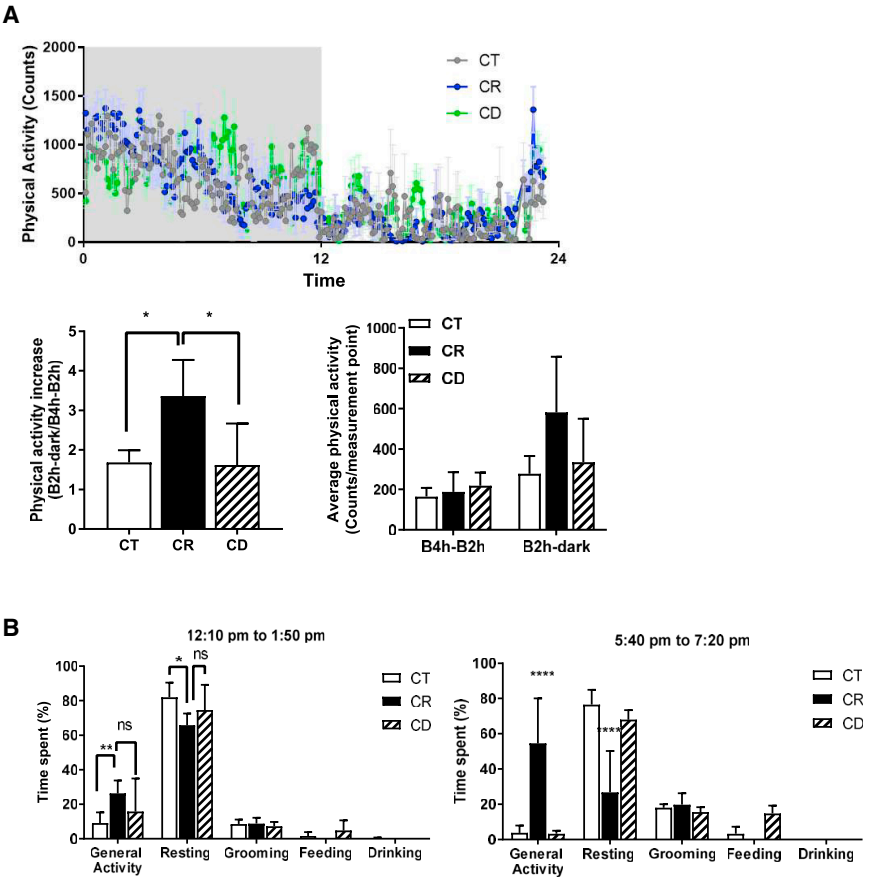


Figure 3. Mice under CR showed stronger food-anticipatory behavior than mice under CD

(A) 24 h physical activities recorded in TSE metabolic cages on day 95. The ratio of average physical activities (counts per measurement point-6min) in 2 h phase, B2h-dark, and B4-B2, and the average physical activity (counts) per measurement point-6min in this 2 h phase. CT: n = 4 and CR and CD: n = 6.

(B) Time distribution on different behavior from 12:10 to 1:50 p.m. and 5:40 to 7:20 p.m. on week 15 (day 105). CT, CR, and CD: n = 10. All data are presented as mean ± SD. Analyzed by one-way ANOVA Tukey's multiple comparisons test; compare each group with every other group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

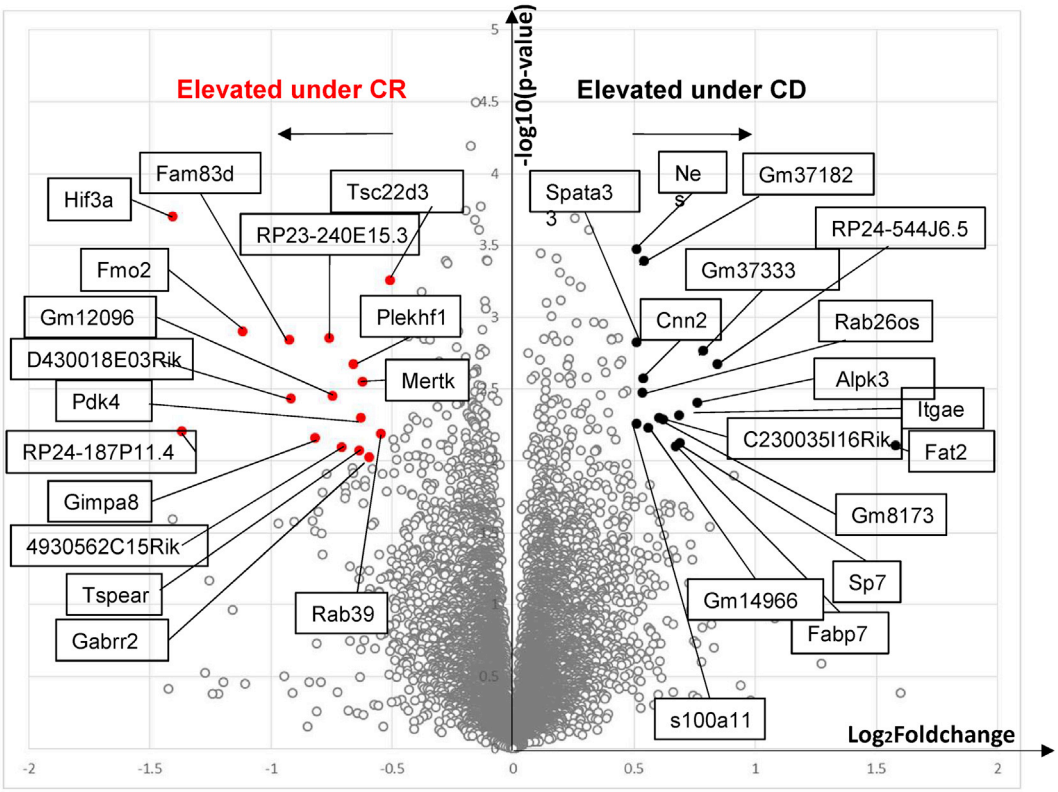
Mice under CR had greater food-anticipatory behavior compared with mice on CD

All mice from CT, CR, and CD groups showed similar physical-activity patterns during baseline. They maintained around 5 h of high-level physical activity after the lights went off. Afterward, their activity levels reduced, and then they increased again during the hour before lights on. During the day, mice of all groups had lower levels of activity than at night (Figure S5A). A significant difference was observed of their daily physical-activity pattern among the three treatment groups on day 70 (two-way ANOVA, $F_{(2, 692)} = 12.79$, $p < 0.0001$) (Figure S5E). The average physical activity of CD mice during both light and dark cycles were higher than that of CR mice (two-way ANOVA followed by Tukey's multiple comparisons test, light: CR versus CD: p = 0.0443, and dark: CR versus CD: p = 0.0167) (Figure S5F). Several small physical-activity peaks of CD mice were observed during the day (Figures S5E, S5G, and 3A), probably reflecting feeding behavior.

We evaluated food-anticipatory behavior of CT, CR, and CD groups by calculating average physical-activity counts in two phases, 4 to 2 h before lights off (B4h–B2h, 15:20–17:20) and 2 to 0 h before lights off (B2h–dark, 17:20–19:20) based on metabolic cage (TSE Systems) measurements. We then calculated the ratio between the period immediately before lights out and that 2 h earlier as a measure of the increase due to food anticipation. The ratio was significantly different among the three groups, with the ratio of the CR group increasing dramatically compared with the CT and CD groups (one-way ANOVA followed by Tukey's multiple comparisons test, CT versus CR: p = 0.0275, CT versus CD: p > 0.05, and CR versus CD: p = 0.0111) (Figure 3A; Table S3). This suggested that the CR mice had stronger food-anticipatory behavior than the other groups that had food constantly available.

During focal behavior observations, the total percentage of time each mouse spent on each physical-activity category was calculated in two 100 min time periods: 12:10 to 1:50 p.m. and 5:40 to 7:20 p.m. During 12:10 to 1:50 p.m., all three groups spent their most time resting (CT: 82.0% ± 8.4%, CR: 65.7% ± 6.8%, and CD: 74.5% ± 14.7%). Mice from the CR group spent much more time on general activity (one-way ANOVA followed by Tukey's multiple comparisons test, p = 0.0067) and less time on resting behavior (one-way ANOVA followed by Tukey's multiple comparisons test, p = 0.011) relative to the CT group, which had similar activity to the CD group (one-way ANOVA followed by Tukey's multiple comparisons test, p > 0.05) (Figure 3B). From 5:40 to 7:20 p.m., immediately before lights off, resting behavior still was the main behavior for CD and CT mice; however, CR mice were considerably more active in this period, spending 54.5% ± 25.1% of their time on general activity, about 15 times higher than CD and CT mice (CT: 3.75% ± 6.3%, and CD: 3.25% ± 8.4%) (one-way ANOVA followed by Tukey's multiple comparisons test, p = 0.0026 and 0.0024). Time spent on resting of CR mice was correspondingly significantly lower compared with CT and CD mice (CR: 26.7% ± 23.4%, CT: 76.5% ± 8.4%, and CD: 68.0% ± 5.5%) (one-way ANOVA followed by Tukey's multiple comparisons test, p = 0.0026 and 0.0083). In this period, CD mice performed more feeding behavior than the other two groups (one-way ANOVA followed by Tukey's multiple comparisons test, p = 0.0029 and 0.0005) (Figure 3B).

A



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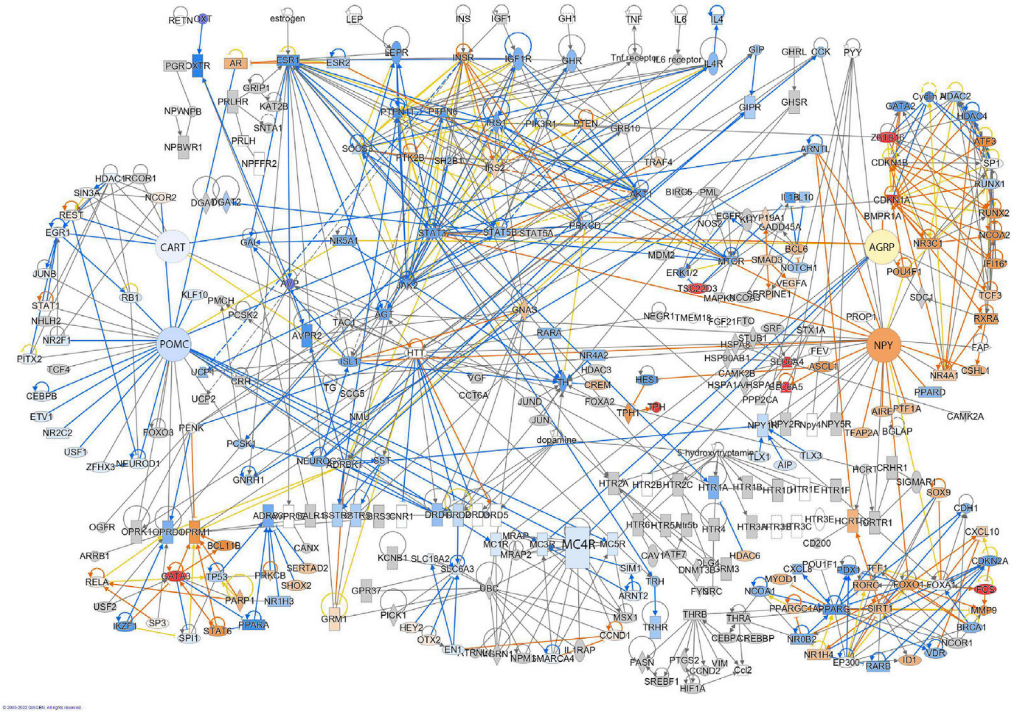

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CR mice had a hypothalamic gene expression consistent with increased hunger relative to CD

We compared the gene expression profiles derived from hypothalamic bulk RNA-seq of CR and CD mice using volcano plots and pathway analysis using the ingenuity pathway analysis program. The volcano plot (Figure 4A) identified 16 genes significantly upregulated under CR relative to CD and 16 genes significantly upregulated under CD relative to CR (Table S4). The most significantly overexpressed genes under CR relative to CD included Hif3 ($p = 0.000198$), fmo2 ($p = 0.00126$), and pdk4 ($p = 0.005$). The most significantly upregulated genes under CD included Spata33 ($p = 0.0003$), Nes ($p = 0.00033$), alpk3 ($p = 0.0026$), fat2 ($p = 0.008$) and fabp7 ($p = 0.0078$).

To determine whether hypothalamic hunger signaling pathways were activated we uploaded the data into the Ingenuity Pathway Analysis program and compared individual genes of significance across all three treatment conditions (Figures 4B and S6). Under both CR and CD there was upregulation of AgRP and NPY, and downregulation of POMC and CART compared to CT mice. In addition, there was upregulation of some of the NPY receptors (specifically Y1 and Y6) and 5-HT receptors 5a and 5b (Figure S6). SLC6a3 and SLC18A2 were both downregulated. These patterns were broadly the same in the CR and CD groups compared to the CT mice (Figures 4B and S6). The main differences between the CR and CD mice in the hunger pathway were that mice under CR showed down-regulation of tyrosine hydroxylase, arginine vasopressin, dopamine receptor D5, and suppressor of cytokine signaling 3 (SOCS3) and upregulation of the leptin receptor, delta and mu opioid receptors, and some components of the insulin signaling pathway (Figure 4B). Mice under CR also had increases in the circadian signaling pathway compared with CD (Figure S7) with significantly elevated PER1 and PER2.

DISCUSSION

We compared the responses of C57BL/6 mice under equivalent levels of CR and CD with unrestricted CT mice to establish the differences between the CD and CR responses. Under the energy imbalance imposed by restricted intake of calories, mice initially draw down their fat reserves (Mitchell et al., 2015c). This is later followed by reductions in lean tissue mass (Mitchell et al., 2015c), which also reduces energy expenditure (Mitchell et al., 2017). Consistent with these earlier findings, mice on both CR and CD treatments exhibited similar reductions in body weight. However, the detailed organ responses were different. CR mice did not lose as much body fat (Figure 2). In contrast, the CD mice showed profound enlargement of sections of the alimentary tract, presumably to aid digestion of the greatly elevated food intake of the diluted diet.

Insulin and leptin levels were reduced under both CR and CD when compared with CT. Although the CD mice lost more fat than the CR mice, they did not have significantly lower leptin levels. Moreover, the levels of insulin were higher under CD than CR. These latter two changes were likely because both insulin and leptin are responsive not only to fat levels but to food intake (Schoeller et al., 1997; Simon et al., 1998), and the CD mice probably had greater intake prior to being sacrificed than the CR mice. This probably also explains why ghrelin levels were significantly reduced in the CD group. Since elevated ghrelin increases hunger, the higher ghrelin in the CR group was consistent with them being hungrier than the CD mice.

Also consistent with our hypothesis that the CR mice were hungrier is that they showed a greater activity toward the lights-out period, which is when they are fed each day. This food-anticipatory behavior response was consistent with our previous work in mice under graded restriction (Derous et al., 2016a). Its absence in the CD (and CT) groups was because these animals had access to food and showed greater feeding behavior than the CR group in the period leading up to darkness. Hunger, reflected in elevated food-anticipation behavior, is considered a key aspect of the life-extending response to CR (Dorling et al., 2020; Doucet et al., 2003; McGuire et al., 1999; Speakman, 2020; Sumithran et al., 2011). The absence of this behavior in the CD mice was thus consistent with them being less hungry and their lack of life extension when compared with CT mice (Solon-Biet et al., 2014).

In the hypothalamus, 16 genes were upregulated and 16 were downregulated under CR relative to CD. Among those upregulated under CR were hypoxia-inducible factor 3a (*Hif3a*), which was previously shown to be upregulated 2-fold in the hypothalamus in response to starvation and is responsive to endogenous leptin levels (Poplawski et al., 2010). *Tsc22d3* encodes a leucine zipper protein and is a transcriptional regulator that is stimulated by glucocorticoids (Ayroldi and Riccardi, 2009). Knockout animals showed a reduction in body weight and resistance to high-fat feeding (Suarez et al., 2012). Previous work shows that *Tsc 22d3* is upregulated about 2-fold during starvation in white adipose, liver, and skeletal muscle (Schupp et al., 2013). Pyruvate dehydrogenase kinase 4 (PDK4) is a mitochondrial protein that inhibits pyruvate-dehydrogenase-reducing conversion of pyruvate to acetyl coenzyme A (CoA). Also regulated by glucocorticoids, it is elevated in the heart during hibernation in squirrels (Andrews et al., 1998) and is believed to be instrumental in metabolic suppression in this state. It is upregulated in multiple tissues during starvation in Wistar rats (Wu et al., 2000) including the brain (Sugden and Holness, 2003), which potentially conserves glucose utilization. Flavin-containing mono-oxygenase 2 (*Fmo2*) is also upregulated in peripheral tissues during starvation by between 2.5- and 3.2-fold (Schupp et al., 2013) and was previously observed to be upregulated in the liver by CR (Fu and Klaassen, 2014). Gaba receptor rho2 (*Gabrr1*) is involved in starvation-induced remodeling of olfactory sensitivity in flies during starvation (Slankster et al., 2020). FAM83D was shown to inhibit

Figure 4. Hypothalamic gene-expression differences between CR and CD mice

(A) Annotated volcano plot showing differentially expressed genes between CR and CD treatments, defined as >2 fold change and $p < 0.05$. Red dots are genes upregulated in CR relative to CD. Black dots are genes upregulated under CD treatment relative to CR.

(B) Pathway diagram for hunger-signaling pathway. Blue indicates genes that were downregulated and red indicates genes that were upregulated in the CD group relative to the CR group. Gray indicates no significance. CT: n = 6, CR: n = 5, and CD: n = 6.

autophagy via stimulation of the insulin signaling pathway involving PI3K/AKT and mTOR (Zhu et al., 2019). A common feature of 5/10 genes with the highest upregulation under CR relative to CD was that these genes are typically upregulated during starvation. This is consistent with the CR mice being hungrier, as reflected in their greater food-anticipation behavior. In contrast, the genes upregulated under CD relative to CR were typically not starvation-induced genes. These genes included fat-like cadherin 2 (*Fat2*), which is involved in cell proliferation and migration (Sadeqzadeh et al., 2014), Nestin (*Nes*), a neuronal stem-cell marker, and Calponin 2 (*Cnn2*), an actin-binding protein involved in the stabilization of actin filaments (Liu and Jin, 2016).

As expected given their lower calorie intake, both CR and CD groups showed significant upregulation of the hypothalamic hunger-signaling pathway relative to the CT group, with *Npy* and *Agrp* upregulated and *Pomc* and *Cart* downregulated (Figure S6), as observed previously under graded CR (Derous et al., 2016a). Surprisingly, however, given the genes identified in the volcano plots linked to starvation responses and the behavioral assays suggesting greater hunger, there was no difference in the four major hunger neuropeptides between CR and CD groups. Nevertheless, CR mice had higher expression of mu and delta opioid receptors, hypothalamic expression of which have been linked to greater food intake in rats (Ardianto et al., 2016). Stimulation of opioid receptors is linked to perception of hunger and food intake in species as diverse as sheep and chicken *Gallus gallus* (Baile et al., 1981; Webster et al., 2013). In contrast, there was a reduction of tyrosine hydroxylase and dopamine D5 receptor gene expression, pointing to a reduction in hypothalamic dopamine signaling in the CR mice compared with the CD mice. Low levels of dopamine also stimulate appetite (Meguid et al., 2000), consistent with elevated hunger in the CR condition.

A recent study (Pak et al., 2021) compared the responses of mice fed with different feeding protocols, including the traditional CR protocol, as well as mice fed using a timer and mice given continuous access to a diluted diet—similar to the pair-fed CD diet used here. They found that many of the phenotypes associated with the CR protocol were not replicated when the food was diluted. Moreover, the CD mice did not live longer. Our data provide details of the neuropeptide responses to these protocols supporting the key finding that these treatments are fundamentally different because they impact hunger signaling in different ways.

Limitations of the study

Our study has several limitations. We only used male mice, and we only studied one strain. This was principally because the previous studies of CR in our own group and in those using the CD protocol used this strain and were focused mostly on males. Hence, the results are germane to the difference observed between these studies in terms of their impacts on lifespan but may not be more widely generalizable. We only reported the differences in gene-expression profiles at one time of day, and these might vary over time in relation to when the CR mice had access to food.

In conclusion, the comparison of mice under identical levels of calorie deficit but imposed via CR or CD showed different responses. In particular, the partitioning of tissue losses and metabolic rates and the levels of circulating hormones were different between the two protocols. Moreover, behavioral assays were consistent with the mice under CR being hungrier than those under CD. This pattern was despite the responses of some of the major hunger-signaling neuropeptides responding as expected relative to CTs but not being significantly different between CR and CD groups. Nevertheless, elevated opioid signaling and depressed dopamine signaling under CR were consistent with increased hunger in the CR animals. These data show that CR and CD at identical levels generate very different phenotypic responses at various levels from gene expression to morphology and behavior. CR and CD generate mice with very different phenotypes and should not be considered equivalent procedures. The different responses of mice under CR and CD treatments are consistent with the hypothesis that hunger signaling is a critical component of the CR response activating changes that extend lifespan (Pak et al., 2021).

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

X.L. performed the experiments, analyzed the data, and co-wrote the manuscript. Z.J. and S.S. performed the experiments. S.S. and D.D. performed the RNA sequencing (RNA-seq) alignment. M.L., B.L., and L.L. helped with data collection. J.R.S. directed the project, conceived and designed the

experiments, contributed to the analysis, and co-wrote the paper. All authors approved the final version prior to submission.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Trizol	Invitrogen	15596018
Critical commercial assays		
RNeasy lipid Tissue Mini Kit	Qiagen	Cat# 74804
Ultrasensitive Insulin ELISA kit	Crystal Chem	Cat# 90080
Leptin ELISA kit	Crystal Chem	Cat# 90030
GIP ELISA kit	Crystal Chem	Cat# 81517
PYY ELISA kit	Crystal Chem	Cat# 81501
Ghrelin (Human/Mouse/Rat) ELISA kit	Novus	Cat# KA1863
IGF-1 ELISA kit	Crystal Chem	Cat# 80574
Deposited data		
RNA-seq data	NCBI GEO	GSE200820
Experimental models: Organisms/strains		
Mouse: C57BL/6J	Charles River Laboratories	RRID: IMSR_CRL:27
Software and algorithms		
Microsoft Excel	Microsoft	N/A
GraphPad Prism 7.0	GraphPad Prism	N/A
Image J 1.5i	NIH	N/A
IPA	Ingenuity Systems	N/A
Adobe Illustrator	Adobe	N/A
Other		
Standard diet	Research Diet	D12450B
Dilution diet	Research Diet	D16061505

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents may be directed to and will be fulfilled by the Lead Contact, John R. Speakman (j.speakman@abdn.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

RNA seq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

The study did not generate any unique code.

Any additional information required to reanalyze the data reported in this work paper is available from the Lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethical statement

All the experiments were approved by the Institutional Review Board, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences: approval number AP2016035.

Mice

All animals used in this study were C57BL/6J male mice that were purchased at age 8 weeks from Charles River Laboratories. The mice were single housed in a specific pathogen free facility at 60% humidity with a 12-h light/12-h dark circle (light, 7:30 am to 7:30 pm). Appropriate housing temperature for studies of mice and how best to mimic the situation in humans is disputed. Here we followed the suggestion in Speakman and Keijer (2012) that when provided with nesting and bedding materials standard room temperature (22–24°C) is appropriate.

All the mice were under *ad libitum* access to water and standard diet (D12450B) in the first 30 days as the baseline period. During this period the data of baseline body weight, food intake, body composition (fat mass and lean mass), basal metabolism (RER, respiratory exchange rate, Oxygen consumption, and carbon dioxide production) and physical activity from each mouse were collected. Afterward, mice were divided into 3 groups - control group (CT), calorie restriction group (CR) and dietary dilution group (CD) were exposed to the corresponding experimental diets for 3 months. CR mice were pair fed the baseline diet to the absorbed intake of ad lib fed CD mice fed the high cellulose diet. The energy intake of CD group was calculated daily by weighing the food that went missing from the hopper (plus any food items recovered from the bedding). This was calculated for each mouse. We then provided paired mice from the CR group with the corresponding amount of standard diet adjusted for the absorption efficiency and dilutent. The food was provided just before lights off on that day in the evening. CT mice were under ad libitum supply of the baseline diet throughout. We did second cohort mice (CR and CD group 4 vs 4) to confirmed the body weight responses to diet. In this cohort we provide CR mice with 50% of daily food intake of CD mice all the time. In this cohort body weight and food intake were daily recorded only after caloric restriction (starting feeding with cellulous diet).

METHOD DETAILS

Experimental diets

Standard diet (D12450B): 16.11 KJ/g including 20% protein, 70% carbohydrate, 10% fat. Dilution diet (D16061505): custom designed, adding 50% cellulose that is indigestible for mice based on D12450B diet including 7.95 KJ/g, 10% protein, 35% carbohydrate, 5% fat. Dilution diet contains high water absorption property. Before starting experiment, diet package was opened in advance for several days. Both diets were purchased from Research Diets. Component information of each diet can be obtained from the website of Research Diets (www.researchdiets.com).

Energy intake, body weight and body composition measurement

Body weight and energy intake of mice from each group were measured daily. Live body fat mass and lean mass were quantified weekly by an EchoMRI Body Composition Analyzer.

Energy intake (KJ/day) for the CD and CT groups was calculated as the diet energy content (KJ/g) measured by bomb calorimetry multiplied by the daily food intake (g/day). Food intake was assessed from the food that went missing from the hopper at the same time each day plus any food spilled into the cages. To get the assimilated energy intake we then multiplied this by the measured assimilation efficiency. It was calculated by measuring the weight of feces produced over a period of 3 days. Feces were dried for 14 days at 60°C and their energy content were measured by bomb calorimetry. Assimilation efficiency was then calculated from the relative fecal energy production compared to the simultaneous food intake. During the first 2 weeks, we provided the CR group with 47.5% amount of CD group daily food intake. So CR group daily food intake = CD daily food intake*47.5%. After the second measurement of assimilation efficiency of the CD group we adjusted this value to 46.5%.

Tissue and serum collection

Mice were sacrificed by CO_2 overdose in the late afternoon near lights off time. CT and CD mice were not fasted before sacrifice. CR mice already ran out of food at that time, so CR mice were in a fasting state before sacrifice. Wet weights of all tissues were taken after the mice were killed at the end of the experiment, including brain, heart, lungs, liver, pancreas, spleen, kidneys, adiposity tissues, stomach, small intestine, caecum, colon, testis, tail, skin and carcass. Before weighing intestinal tract tissues, the contents were removed and the intestine washed with phosphate-buffered saline (PBS) and dry it on filter paper.

Feces energy content measurement

Feces were collected over 3 days periods. Feces were dried at 60°C for 14 days. along with samples of two diets. Energy content of feces and diet were measured by bomb calorimetry (Parr 1281).

Assimilation efficiency (AE) analysis

We measured the AE at 4 time points: baseline, and days 45, 70 and 95. AE was calculated as 1- (daily feces production* energy content of feces/daily food intake* energy content of diet).

Energy expenditure

Metabolic rates and behavior of the mice were monitored using a TSE PhenoMaster/LabMaster system at four time points, baseline, and treatment days 45, 70 and 95. We measured mice for 3 days at each time point which has been previously shown sufficient to obtain an accurate measure of energy metabolism (Li et al., 2019, 2021; Speakman, 2013). After calibrating the system with the

reference gases (20.950%/0.05% for O_2/CO_2), the oxygen (O_2) consumption (mL/min), carbon dioxide (CO_2) production (mL/min), respiratory exchange ratio (RER = VCO_2/VO_2) and locomotor activity (Counts), food intake (g) and energy expenditure were recorded. Measurements were taken at 1-min intervals for the whole period.

Physical activity measurement

Physical activity was continuously recorded with metabolic performance by the TSE system. Besides, the total percentage of time each mouse spent on each of 5 physical activity category were calculated by focal observation: resting (where the animal remained stationary including sleeping), general activity (walking around the cage or climbing), grooming (mouse performing preening activities), feeding (either directly from the food hopper or from food that has fallen through the hopper) and drinking (drinking from the water bottle) (Speakman and Rossi, 1999). The focal observation experiment was carried out across all mice on a rotation basis over a period of 5 days to analyze behavioral patterns.

All observations were carried out at 12:10–13:50 and 17:40–19:20 two phases for each day of focal observations. Measurements were carried out within the animal house facility and each session involved observing the activity of 6 mice, all within their cages, for a total period of 100 min. For every minute, 1 mouse from each of the 6 cages was observed for 10 s intervals and the last behavior exhibited at the end of each interval was recorded. For each session, 2 individuals were selected from each group (CT, CR and CD) based on paired-feeding.

Hormone level measurement

Blood samples were collected immediately from the jugular vein after mice was killed for dissection. Blood samples were put on the ice for 30 min. Afterward, samples were centrifuged for 30 min at 3500 rpm. Serum insulin, leptin, GIP, PYY and IGF-1 levels were determined using Crystal Chem ELISA kits according to the manufacturer's instructions. Ghrelin level was measured by ELISA kit (Novus, cat#KA1863).

RNA isolation and transcriptome analysis

Hypothalamus were carefully dissected and RNA isolated by the RNeasy lipid Tissue Mini Kit (QIAGEN, cat# 74804). Sequencing was performed by the Beijing Genomic Institute (BGI) and for a detailed description see Derous et al., 2016 (Derous et al., 2016a). In short, samples were prepared for library preparation by enriching total RNA with oligo(dT) magnetic beads. The library products were sequenced using an BGI-seq 500 sequencer, resulting in 50 bp single end reads (standard protocol BGI). Standard primers and barcodes developed by BGI were used and these were removed prior to receiving the sequencing files. Resulting FASTQ files underwent quality control using fastQC and no files needed trimming based on phred score cut-off of >28 (www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Alignment was performed by using HISAT2 (Kim et al., 2019) and prebuild indexes were used (GRCm38 release 81 version of *M. Musculus*). On average 94% of the read successfully aligned to the reference genome and read were quantified using featureCounts (v1.5.1) (Liao et al., 2014). Differential gene expression was modeled using the edgeR package in R (Robinson et al., 2010). To remove any genes that exhibited no or a very low number of mapped reads only genes that had more than 1 count per million in at least 2 samples across all treatments were retained for further analysis. This resulted in a total of 15,707 unique genes. Read counts were normalized using the trimmed mean of M values (TMM normalization) to account for highly expressed genes consuming substantial proportion of the total library size. Pairwise comparisons were conducted between the different treatment groups and Benjamini Hochberg adjusted p value was calculated (FDR). Significantly affected pathways were observed by Ingenuity pathway (IPA) program (Ingenuity Systems; http://www.ingenuity.com/).

QUANTIFICATION AND STATISTICAL ANALYSIS

All values are displayed as mean ± SD. General linear model with repeated measures (GLM-RM) was used to compare the effects of time and diet and was performed using Minitab 17. One-way ANOVA was used to compare different effects among three diet treatment groups. Subsequently Tukey's multiple comparisons tests were used to identify differences among three groups. Paired t test was used for repeated measures from same individual but at different time points. two-way ANOVA were used in TSE data to compare the different groups throughout time. These statistical analyses were performed using GraphPad Prism 7.0. Significantly affected pathways in hypothalamus were observed by Ingenuity pathway (IPA) program (Ingenuity Systems; http://www.ingenuity.com/). Graphical Abstract was generated using BioRender.