Physiological and behavioral responses to intermittent starvation in C57BL/6J mice

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The dual intervention point model states that body mass is controlled by upper and lower intervention points, above and below which animals (and humans) intervene physiologically to bring their body mass back into the acceptable range. It has been further suggested that the lower intervention point may be defined by the risk of starvation, while the upper intervention point may be defined by the risk of predation. The objective of the present study was to test whether the risk of starvation determines the lower intervention point and to examine the physiological and behavioral mechanisms that underpin the regulation of body mass, when the risk of starvation is increased. Sixty-four mice were exposed to random days of complete fasting or 50% food restriction and their body mass and fat mass responses were measured. Food intake, physical activity and body temperature were measured throughout the experiment. In addition, plasma leptin and insulin, triglyceride and non-esterified fatty acids, along with hypothalamic neuropeptides gene expression in the arcuate nucleus were assessed after 13 and 42 days of treatment. We found that C57BL/6J mice increased body mass and fatness in response to a short-term (13 days) intermittent fasting, which was restored to baseline as the treatment was prolonged. In contrast, intermittently 50% food restricted mice showed no significant changes in body mass or fatness. Over the first 13 days of treatment the data were consistent with the dual intervention point model as the mice showed both increased body mass and adiposity over this period. Over the more protracted period of 42 days the effect waned and was therefore inconsistent with the model. The body mass and fat mass gains in intermittently fasted mice were mainly accounted for by increased food intake. Elevated NPY gene expression after 13 days (three 24 h fasting events) may have driven the increase in food intake. However, no changes were observed in such neuropeptides as POMC, CART, AgRP, Ob-Rb and SOCS 3 or circulating levels of leptin, insulin, NEFA and TG. Hypothermia during fasting days may have also contributed to the increase in body mass. Over 42 days of treatment (nine 24 h fasting events) cumulative food intake was not affected by intermittent starvation. However physical activity, mainly activity during the light phase was lowered suggesting an adaptation to unpredictable starvation. Overall, mice exhibited different behavioral and physiological responses to intermittent starvation depending on the duration of treatment.

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1. Introduction

1.1. Body mass regulation

The global epidemic of obesity has raised the need for a better understanding of the mechanisms that regulate body and fat mass. A balance between energy intake and energy expenditure is necessary to maintain a stable body mass. It is well established that the central nervous system (CNS) regulates food intake and energy expenditure in response to neuronal, hormonal and nutrient signals [1–3]. The hypothalamus is the most studied area in CNS with respect to the regulation of energy homeostasis. The arcuate nucleus in particular is known to play an important role in energy homeostasis because it contains two distinct populations of neurons: pro-opiomelanocortin (POMC) neurons that express the endogenous anorectic melanocortin receptor agonist (α-melanocyte stimulating hormone, α-MSH) along with cocaine-and-amphetamine-regulated transcript (CART), and neurons that express neuropeptide Y (NPY) and also the endogenous orexigenic melanocortin receptor antagonist, agouti-related protein (AgRP) [4–6]. These neuronal populations respond to peripheral signals, such as leptin, insulin and gastrointestinal hormones by modifying the production of the above neuropeptides that modulate energy balance [7]. Moreover, leptin signaling within neurons is dependent on the presence of the long form of the leptin receptor (Ob-Rb) [8], which signals principally via the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway [9]. The suppressor of cytokine signaling 3 (SOCS3) is a negative regulator of the leptin receptor which is stimulated by the JAK/STAT2 signal transduction pathway.
pathway [10–13]. Gene expression levels of Ob-Rb and SOCS3 are markers of leptin sensitivity [14].

There have been several theoretical models that have attempted to conceptualize the mechanisms involved in the regulation of body mass and fatness. Kennedy (1953) proposed the lipostatic set point model which suggested that the size of body fat depots is sensed by a ‘lipoast’, which adjusts food intake and energy metabolism to maintain the body mass and fat masses at a set-point [15]. Although leptin has been often interpreted as the molecular manifestation of the lipostatic fat signal [16], this model is in conflict with the evidence in patterns of changes in animal and human body mass [17,18]. An alternative interpretation emerged suggesting that body mass is not regulated by a set-point, but rather is controlled by upper and lower intervention points, above and below which animals (and humans) intervene physiologically to bring their body mass back into the acceptable range [19,20]. In humans the upper intervention point may be located at different positions in different individuals explaining why some individuals become obese when exposed to environments with readily available food supplies, but others are able to regulate their body weights at normal levels. Based on data from small mammals and birds, Speakman (2007) has further suggested that the lower intervention point may be defined by the risk of starvation while the upper intervention point may be defined by the risk of predation [20]. In this scenario, one would expect that an increased risk of starvation would increase the lower intervention level and animals would gain fat and body mass.

1.2. Intermittent starvation

Periods of negative energy balance arising from restricted feeding or total starvation are common events [21]. Throughout their lives, small mammals must face periodic food shortages interspersed by periods of food abundance.

Stochastically imposed intermittent periods of fasting (complete absence of food) or periodic food restriction (involving reduced levels relative to habitual intake, but not complete absence) may mimic the unpredictable food availability in the wild. However, few studies have investigated the physiological and behavioral responses to stochastic food exposure. Swiss mice showed increased food intake and decreased energy expenditure on days that intervened between 24 h fasting events over 4 weeks treatment (including 3 or 4 fasting days and 4 or 3 feeding days each week), and they decreased overall body mass [22]. However in another study the same group found that 4 weeks treatment including 3 fasting days and 4 feeding days each week had no effect on overall body mass [23]. Alternate day fasting (ADF) is a similar and widely studied regimen, however, most of the studies have been focused on the beneficial effects on aging and the effect of ADF on body weight is less often reported. Body mass has been shown to be highly variable in response to ADF in both humans and animal models. In Fisher rats, when ADF regimens were applied in the short term, no effect on body weight was observed [24]. whereas gains in body mass were noted in C57BL/10 mice after 8 weeks [25]. However, in other studies, when ADF was administered for 12 weeks, body mass decreased in both C57BL/6 mice and rats. This variability in response is confusing and its relationship to the dual-intervention model is uncertain because in ADF the fasting days come at predictable intervals, and hence the uncertain risk of starvation is not altered by this treatment. Even in the studies performed by Zhao and colleagues [22,23] the occurrence of fasting days is so frequent that it may be predictable — leading to different responses from those predicted by the dual-intervention point model.

In the present study we aimed firstly to test whether starvation risk defines the lower intervention point for body mass and adiposity as suggested in the dual intervention point model, by experimentally increasing starvation risk over a period of 42 days, by exposing mice to nine random days of complete fasting or food restriction, and recording their body mass and fat mass responses. Second we investigated the physiological and behavioral mechanisms that underpin the regulation of body mass during intermittent fasting or food restriction, by measuring food intake, physical activity and body temperature throughout the experiment. In addition, NPY, AgRP, POMC, CART, Ob-Rb and SOCS3 gene expression in the arcuate nucleus of the hypothalamus were assessed after 13 days and 42 days of treatment. We also measured plasma leptin and insulin, as well as triglyceride (TG) and non-esterified fatty acids (NEFA). We hypothesized that if the dual intervention point model is correct randomly imposed intermittent fasting and food restriction would cause body mass and fat mass to increase as a result of an increase in the lower intervention point. Secondly, we hypothesized that physiological and behavioral compensation mechanisms would be employed to adjust body mass and fatness within the range and thirdly, that leptin, insulin and other potential signals would change in response to intermittent fasting and food restriction and neuropeptides sensitive to leptin would be involved in the body mass response.

2. Material and methods

2.1. Animal housing and intermittent starvation regimen

Sixty four female C57BL/6 mice aged 6–8 weeks were purchased from Charles River (Charles River UK Ltd, Kent, UK) and housed in single cages (M3 cage 48 × 15 × 13 cm, NRP Cages, Kent, UK) in a temperature controlled room (21 ± 1 °C) under a 12:12-hour light:dark photoperiod with the lights coming on at 06:00 and a “dawn/dusk” period of 20 min at either end of the light period. Wood shavings and shredded paper bedding were provided for enrichment. At around age 9–10 weeks mice were implanted with transmitters that measured their body temperatures and physical activity levels (details below) and were then fed a standard control diet (D12450B, 10% kcal/fat, Research Diet, New Brunswick, NJ, USA) ad libitum until 22 weeks of age when they were randomly assigned into three groups: a control group AL, fed ad libitum (n = 20); IF, intermittently fasted (n = 22); IR, intermittently 50% food restricted (n = 22). All mice had free access to water throughout the study. All procedures were reviewed by a local ethical committee and performed in accordance with UK home office regulations under license PPL 60/3707 held by JRS.

Mice were divided into two cohorts containing 32 mice in each AL (n = 10); IF (n = 11); IR (n = 11). Note the sample sizes in each group and cohort were unbalanced because the number of recording pads for the implanted transmitters (see below) was fixed at 64. Body mass and food intake were recorded for 3 days on a daily basis prior to the intermittent starvation regime. Animals were stochastically exposed to 24 h fasting or restriction on 9 occasions over a period of 42 days. Each starvation day was always followed by a non-starvation day. Probability of next day would be starvation was set at 0.21 and was decided using random integers. During the treatment, days 4, 7, 10, 14, 16, 20, 26, 29 and 39 were assigned as fasting/restriction days. Body mass and food intake were recorded for 3 days on a daily basis prior to the intermittent starvation regime. Animals were stochastically exposed to 24 h fasting or restriction on 9 occasions over a period of 42 days. Each starvation day was always followed by a non-starvation day. Probability of next day would be starvation was set at 0.21 and was decided using random integers. During the treatment, days 4, 7, 10, 14, 16, 20, 26, 29 and 39 were assigned as fasting/restriction days. On each treatment day, IF mice were completely deprived of food, while IR mice were given 50% of their average daily food intake measured over the baseline period at the start of the experiment. Mice were provided with ad libitum food between fasting/restriction days. Body mass and food intake were measured at 1500 h every day when food was given or removed. The first cohort of mice was killed on day 13 of the treatment, while the second cohort was killed on day 42.

2.2. Physical activity and body temperature

Prior to experimentation the mice were implanted with a telemetry transmitter to monitor body temperature (Tb) and physical...
activity (PA) (Model PDT-4000 E-Mitter, Mini-Mitter, Bend, OR, USA). The surgery was carried out under anesthesia induced by a mixed flow of isofluorane and oxygen. An incision of approximately 1.0 cm was made in the ventral skin, then a second smaller incision made through the peritoneal wall. The transmitter was inserted intraperitoneally, and the two layers were sutured separately (Ethicon Vicryl: W9982; W&J Dunlops Ltd., Dumfries, UK). The surgical procedure took around 15–20 min/mouse and mice were given 2 weeks with minimal handling to allow them to recover from the surgery. A receiver pad (ER-4000 Receiver, Mini-Mitter, Bend, OR, USA) under the cage where the mouse was housed received PA and Tb information from the transmitter which was collected every minute by a Windows PC-based data acquisition system (VitalView (TM): Mini-Mitter, Bend, OR, USA). PA and Tb recordings were averaged for each hour to show daily activity and Tb patterns. Daily means, and means during dark and light phases were calculated throughout the baseline and treatment periods.

2.3. Body fatness and plasma metabolites levels

Body fat mass (FM) was determined using dual energy X-ray absorptiometry (DXA; PIXImus2 Series Densimeters, GE Medical Systems Ultrasound and BMD, Bedford, UK). Mice were anesthetized during the scan. Data generated from the DXA software were corrected using an equation developed specifically for our machine [26]. DXA was performed to assess body fatness at baseline in both cohorts, and additionally on day 12 for cohort 1 and on days 24, 30, 37 for cohort 2. Mice were sacrificed after fasting for 4 h. Blood samples were collected by cardiac puncture for the determination of circulating hormone and metabolite levels (leptin, insulin, TG and NEFA). Fat depots (epidydimal fat, perirenal fat, mesenteric fat and subcutaneous fat) were dissected and weighed. All blood samples were placed in EDTA-treated vials and centrifuged at 4 °C at 10,000 rpm for 10 min. Blood plasma was stored at −80 °C until analysis.

Plasma leptin and insulin were measured with ELISA-based methods using commercially available kits (mouse leptin ELISA kit, Millipore Billerica, MA, USA; mouse insulin ELISA kit, Crystal Chem Inc, Downers Grove, IL, USA, respectively), while plasma NEFA and TG were measured using colorimetric assays (NEFA-HR (2), Wako Chemicals, Neuss, Germany; Triglyceride and Free Glycerol Kit, Sigma-Aldrich, Kent, UK, respectively).

2.4. Gene expression of neuropeptides

2.4.1. Tissue collection and preparation

Mice were killed by CO2 inhalation. Their brains were immediately removed, frozen in isopentane over dry ice and stored at −80 °C. Coronal 20 μm sections of the hypothalamic region were cut on a cryostat (Leica, CM3050S, Milton Keynes, UK) and thaw mounted on poly-L-lysine-coated slides. 20 slides (with approximately 6 sections on each slide) through the hypothalamus were collected per animal, and they were numbered as 1–10, 11–20. Two slides numbered as 1 and 11 (or 2 and 12, etc.) were used for one specific neuropeptide.

2.4.2. Riboprobe synthesis

Template DNA, complimentary to the mRNA sequence of interest, was gifted from Dr. Sharon Mitchell. Briefly fragments were amplified from mouse brain cDNA by polymerase chain reaction (PCR). Primers (Eurofins MWG Operon, London, UK), fragment size and Accession numbers are detailed in Table 1. PCR products were cloned into either the pCR-Script Amp SK (+) vector (Stratagene, CA, USA) or the pGMET Easy Vector (Promega, Southampton, UK) and transformed into competent cells. Plasmid DNA was isolated using the QiAprep Spin Mini Prep kit (Qiagen, Germany) and linearized with appropriate restriction enzymes (Promega, Southampton, UK). All sequences were verified by Eurofins Sequencing Services (London, UK). The linearized DNA was transcribed using T7, T3 or SP6 RNA polymerase promoters matched to a restriction site contained within the vector. Riboprobes were labeled with 35S-UTP (Perkin Elmer, UK) and unincorporated label removed by spinning through Chromospin columns (BD Biosciences, UK). Finally riboprobes were made up to a final concentration of 1 × 106 c.p.m ml−1 in hybridization buffer. Antisense and sense riboprobes were tested for specificity and background binding.

2.4.3. In situ hybridization

Labwar was baked at 200 °C and all solutions made up in 0.1% diethylpyrocarbonate (DEPC) to prevent RNase contamination. Sections were fixed in 4% paraformaldehyde (Sigma-Aldrich, UK)/0.1 M phosphate buffer (144 mM NaH2PO4 and 5.6 mM Na2HPO4, pH 7.4; PB) for 20 min on ice and washed in 2 × 0.1 M PB. To eliminate background signal and inactivate RNases, sections were first immersed in 0.1 mM triethanolamine (TEA) (Sigma-Aldrich, UK) for 2 min before acetylation in 0.1 mM TEA/0.25% acetic anhydride (Sigma-Aldrich, UK) for 10 min at room temperature. Sections were again washed in 0.1 M PB for 4 min, dehydrated through increased concentrations of ethanol (50%, 70%, 90% and 100%) and dried under vacuum. Labeled probe was pipetted onto cover slips, annealed to sections and sealed with DPX before an overnight hybridization at 58 °C. Post-hybridization, cover slips were removed by soaking slides in 4 X saline-sodium citrate buffer (150 mM sodium chloride and 15 mM sodium citrate, pH7; SSC). Non-specific hybridization was dissociated by increasing the temperature, up to 60 °C for 30 min. Unhybridized RNA was digested with RNase for 30 min. Sections were desalted over a series of SSC solutions: 2 X SSC, 1 X SSC, 0.5 X SSC and 0.1 X SSC and finally dehydrated through graded increased concentrations of 50%, 70%, 90% and 100% ethanol. The slides were air-dried and exposed to Kodak BioMax Film (Sigma, UK) for time appropriate to specific riboprobe. Autoradiographs of sections including microscale standards were scanned on Umax Power Look II (Umax Data System, Fremont, CA, USA), and gene expression was taken as the integrated optical density (IOD) in the area of interest using ImageJ software system (WinZip Computing Inc, USA). The slides were observed by a blinded observer.

2.5. Statistical analysis

All data were expressed as means ± SD. General linear modeling (GLM) with individual ID included as a random factor nested within the treatment to account for the repeated measures was used to compare changes of body mass, body fat mass, food intake, PA and Tb throughout the experiment. One-way analysis of variance (ANOVA) with post hoc Tukey tests was used to test the between-group differences of body mass, body fat mass, food intake, cumulative food

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Agouti-related peptide, (AgRP); cocain- and amphetamine-regulated transcript (CART); neuropeptide Y (NPY); pro-opiomelanocortin (POMC); signaling form of leptin receptor (obRb); suppressor of cytokine signaling 3 (SOCS 3).
intake, PA and T_{3} at different time points where group differences were significant over the whole time course. The two cohorts were analyzed separately. Analysis of covariance (ANCOVA) was performed to examine differences body fat mass using body mass as a covariate and ANCOVA was also used to test differences in plasma leptin levels using fat mass as a covariate. Group differences in plasma insulin, TG, NEFA levels and fat depots were examined with one-way ANOVA. Group differences in neuropeptides gene expression were examined by comparison of the total integrated optical density (IOD) for each gene using ANOVA. Gene expression data were presented as percentage of the mean for AL control. P values < 0.05 were considered statistically significant. All data were analyzed using SPSS 18.0 statistical package for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Body mass and fatness

3.1.1. Cohort 1

Prior to the start of intermittent starvation regime, animals had similar body mass on day 0 (IF: 24.46 ± 1.15 g; IR: 24.28 ± 1.24 g; AL: 24.29 ± 1.27 g; ANOVA: F_{2,29} = 0.08, P = 0.926; Fig. 1A). The intermittently fasted (IF) mice significantly decreased body mass on the first day (4) and second day (7) of fasting (ANOVA: day 4, F_{2,29} = 7.00, P = 0.003; day 7, F_{2,29} = 4.71, P = 0.017; Fig. 1A). The IF mice did not exhibit a significant decrease in body mass on the third day of fasting (day 10) (ANOVA: F_{2,29} = 1.96, P = 0.159). After 3 days of fasting and 7 days of free feeding, on days 11–13, IF mice maintained a significantly higher body mass in comparison to both AL control and the intermittently restricted (IR) mice (ANOVA: day 11, F_{2,29} = 4.18, P = 0.025; day 12, F_{2,29} = 3.89, P = 0.032; day 13, F_{2,29} = 3.78, P = 0.035; Fig. 1A).

3.1.2. Cohort 2

There were no significant differences in body mass between groups before the treatment started (ANOVA: F_{2,29} = 0.1, P = 0.989; Fig. 1B). On day 0, the average body mass of IF, IR and AL groups were 23.11 ± 1.19 g, 23.12 ± 1.21 g and 23.19 ± 1.21 g, respectively. The responses of the second cohort over the first 13 days of treatment were almost identical to those observed in cohort 1. After the first starvation day, IF mice showed a significantly lowered body mass compared to AL control (Post hoc Tukey test: P = 0.001) and IR mice (Post hoc Tukey test: P = 0.004), whereas no differences were detected between IR mice and AL control (Post hoc Tukey test: P = 0.05) (ANOVA: F_{2,29} = 11.22, P < 0.001; Fig. 1B). The body mass of IF mice returned to the AL control level after one-day of free feeding (P = 0.005). The second starvation day resulted in a similar response in IF mice exemplified by a significant reduction in body mass (ANOVA: F_{2,29} = 11.45, P < 0.001), which was followed by an immediate increase after refeeding (ANOVA: F_{2,29} = 1.31, P = 0.285). Moreover, after the second fasting event the IF mice showed a trend for body mass to increase above that of the AL control animals (0.69 g higher) although this difference was not statistically significant (ANOVA: F_{2,29} = 2.39, P = 0.109). Different from the first two starvation days, no significant body mass loss relative to the masses of the other groups was not observed in IF mice after the third fasting day and no between groups difference in body mass was detected (ANOVA: F_{2,29} = 2.70, P = 0.08). Free feeding following the third starvation day caused a pronounced weight increase in the IF mice above AL control levels which lasted for 3 continuous free feeding days (ANOVA: F_{2,29} = 4.57, P = 0.019; F_{2,29} = 4.10, P = 0.027; F_{2,29} = 4.20, P = 0.025; respectively. Post hoc Tukey test: IF vs AF, P = 0.05; IR vs AF, P = 0.05). As the treatment progressed, this response of elevated body mass weakened. Significant mass losses in IF mice occurred on fasting days 4 to 9 (ANOVA: P < 0.05) while body mass on the free feeding days did not increase significantly above the levels of AL control or IR mice (ANOVA, P > 0.05). Over the 42 days of treatment, there was a significant effect of intermittent starvation on body mass within groups but not between groups (GLM repeated measures: day of treatment, F_{2,29} = 63.29, P < 0.001; day of treatment × group, F_{2,29} = 12.80, P < 0.001; group, F_{2,29} = 0.48, P = 0.622; Fig. 1B).

All mice in cohort 1 and cohort 2 had similar fatness before the treatment started (IF: 3.52 ± 0.50 g; IR: 3.44 ± 0.57 g; AL: 3.64 ± 0.70 g; ANCOVA: F_{2,29} = 0.18, P = 0.836; Fig. 1C). After 3 starvation days and 9 free feeding days on day 12, there was a significant difference in body fat mass between three groups (ANOVA: F_{2,29} = 4.24, P = 0.025; Fig. 1C). IF mice had higher body fat than AL control (Post hoc Tukey test: P = 0.020) while no difference was found between IR mice and AL control (P = 0.068) or between IF and IR (P = 0.816) (Fig. 1C). On day 24 (after 6 starvation days and 18 free feeding days), the fat mass of IF mice were significantly higher than AL control (ANOVA: F_{2,29} = 5.02, P = 0.014; Post hoc Tukey test: IF vs AL, P = 0.01; Fig. 1C). No differences were found between the IR group and the AL control or between the IR and IF groups (Post hoc Tukey test: P = 0.162; P = 0.384, respectively). However, the differences in body fatness observed at days 11–13 had disappeared by day 30 (after 8 starvation days and 22 free feeding days) and day 37 (after 8 starvation days and 29 free feeding days) (ANOVA: F_{2,29} = 1.66, P = 0.209; F_{2,29} = 1.56, P = 0.227, respectively; Fig. 1C).

3.2. Food intake

3.2.1. Cohort 1

Before intermittent starvation treatment started, all mice had a similar amount of food intake (IF: 2.64 ± 0.31 g; IR: 2.67 ± 0.51 g; 2.59 ± 0.33 g; ANOVA: F_{2,29} = 0.12, P = 0.899; Fig. 2A). The IF mice in cohort 1 displayed hyperphagia on the first two free feeding days after the fasting days (ANOVA: days 5–6, days 8–9, day 11: P < 0.001; day 12, P = 0.012; Fig. 2A). By the end of this treatment (day 13), cumulative food intake differed significantly between the three groups (ANOVA: F_{2,29} = 5.73, P = 0.008; Fig. 2B). Overall, IR mice consumed 2.79 g (9%) less food (P = 0.012) than AL controls while the amount of cumulative food intake consumed by IF mice was similar to that of AL control (P = 0.902) over the course of the treatment (Fig. 2B).

3.2.2. Cohort 2

Under the free feeding baseline condition, there was no difference in food intake between groups (ANOVA: P = 0.05). On the first free feeding day following the first starvation (day 5), IF and IR mice consumed significantly more food than the AF control (ANOVA: F_{2,29} = 12.41, P < 0.001; Post hoc Tukey test: P < 0.001, P = 0.029, respectively; Fig. 2C). On the second free feeding day (day 6), the food intake of IF mice was still higher than AL control (ANOVA: F_{2,29} = 11.917, P < 0.001; Post hoc Tukey test: P = 0.002; Fig. 2C) while the food intake of the IR mice returned to the similar level of AL control (Post hoc Tukey test: P = 0.858). Compared to AL controls, no increase in food intake following the treatment days was found in IR mice from the second restriction day onwards (Post hoc Tukey test: P = 0.05 in all cases). In contrast, IF mice showed a hyperphagic response to starvation on the first two free feeding days that followed both the second and third restrictions (days 8–9 and days 11–12) (ANOVA: day 8, F_{2,29} = 12.58, P < 0.001; day 9, F_{2,29} = 6.42, P = 0.005; day 11, F_{2,29} = 33.83, P < 0.001; day 12, F_{2,29} = 5.11, P = 0.013; Post hoc Tukey test: IF vs AL, P < 0.05 for all cases; Fig. 2C). The magnitude of hyperphagia was less on the second refeeding day than that on the first refeeding day. The patterns in cohort 2 matched those of cohort 1 over the first 13 days of treatment almost exactly. From days 14–38, hyperphagia following fasting in the IF group was present only on the first free feeding days after starvation (ANOVA: day 15, F_{2,29} = 11.21, P < 0.001; day 17, F_{2,29} = 16.86, P < 0.001; day 21, F_{2,29} = 18.44, P < 0.001; day 27, F_{2,29} = 4.56, P = 0.019; day 30, F_{2,29} = 10.51, P < 0.001; Post hoc Tukey test: IF vs AF, P < 0.05 for all
cases; Fig. 2C). Unexpectedly, on the first free feeding day after the ninth starvation (day 40), IF and IR mice consumed significantly less food than AL control (ANOVA: F2,29=29.19, P < 0.001; Post hoc Tukey test: IF vs AL, P < 0.001; IR vs AL, P = 0.01; Fig. 2C). There was no significant difference between three groups in cumulative food intake over 42 days of the treatment (ANOVA: F2,29=2.56, P=0.095; Fig. 2D).

3.3. Physical activity

Fig. 3A illustrates physical activity over a free feeding day prior to the experimental treatment starting. Mice from all groups showed a similar circadian pattern in physical activity with a low level of activity during the light phase (punctuated by a brief rise when disturbed to be fed at 1500 h) and a high level of activity during the dark phase. On the first fasting/restriction day (day 4), there was no significant difference in 24 h physical activity between the three groups (GLM repeated measures: day 4, F2,29=0.28, P=0.758; Fig. 3B). When the treatment proceeded to the second fasting/restriction day, 24 h physical activity levels tended to differ between the three groups although the difference did not reach significance (GLM repeated measures: day 7, F2,29=3.08, P=0.062; Fig. 3C). From the third fasting/restriction day (day 10) and onwards, there was a significant effect of fasting/restriction on 24 h physical activity (GLM repeated measures: day 7, F2,29=9.42, P<0.001; Post hoc Tukey test: IF vs AL, P=0.001, IR vs AL, P=0.104; IF vs IR, P=0.086, Fig. 3D; data on days 14, 16, 20, 26, 29 and 39 not shown). In addition to changes in 24 h activity, there was no difference in daily average activity between the three groups on the first two fasting/restriction days (ANOVA: P>0.05 for both cases; Fig. 3E). However, IF mice showed a significant decrease in daily average physical activity on the third to ninth fasting/starvation days (ANOVA, P<0.05, Post hoc Tukey test, IF vs AL, P<0.05) and IR mice significant decreased daily average activity on the fifth to ninth fasting/starvation...
days (ANOVA, P < 0.05, Post hoc Tukey test, IR vs AL, P < 0.05) (Fig. 3E). Furthermore, day of treatment representing the number of events had a significant effect on physical activity over the 13 fasting/restriction days (GLM repeated measures: group, F2,29 = 7.27, P = 0.003; group × day of treatment, F2,16 = 3.31, P < 0.001; Fig. 3E).

The first day of refeeding (day 5) following the first fasting/restriction (day 4) 24 h physical activity levels differed significantly between groups (GLM repeated measures: F2,29 = 24.62, P < 0.001). IF and IR mice displayed lower activity levels than AL control (Post hoc Tukey: IF vs AL, P < 0.001; IR vs AL, P = 0.001; IF vs IR, P = 0.025; Fig. 3F). Similar responses persisted on the second day of refeeding on day 6 (GLM repeated measures: F2,29 = 7.20, P = 0.003; Post hoc Tukey: IF vs AL, P = 0.002; IR vs AL, P = 0.312; IF vs IR, P = 0.076) even though the magnitude of responses became weaker. On the third day of refeeding (day 13) after the third fasting/restriction event (day 10), the difference in physical activity between groups totally disappeared (GLM repeated measures: F2,29 = 0.80, P = 0.462; Fig. 3G). Additionally, IF mice and IR mice displayed a decrease in daily physical activity on the first refeeding days following all fasting/restriction events (ANOVA: P < 0.05) except the fourth one on day 14 (ANOVA: P = 0.081) and on the second refeeding days after the first-third, fifth, seventh and ninth fasting/restriction events (ANOVA: P < 0.05) (Fig. 3H). Moreover, day of treatment (representing number of events) had a significant effect on physical activity during all free feeding and refeeding days (GLM repeated measures: group, F2,29 = 3.74, P = 0.037; group × day of treatment, P = 0.001). Overall, total activity levels of IF mice were significantly lowered by 17% in comparison with AL control over 42 days of treatment compared with AL controls (ANOVA, F2,29 = 4.79, P = 0.017; Post hoc Tukey test, IF vs AL, P = 0.017) whereas no differences were found between IR mice and AL control (P = 0.82) (Fig. 3I). Consistent with changes in total daily activity, there were significant differences between three groups in activity during the light phase (ANOVA: F2,29 = 11.13, P < 0.001). However, activity levels were unchanged during the dark phase across the three groups (ANOVA: F2,29 = 2.22, P = 0.128). In cohort 2, intermittent fasting and restriction had exactly the same impact on physical activity as during the first 13 days of treatment in cohort 1.
3.4. Body temperature

All mice showed a similar circadian pattern of body temperature when fed ad libitum (Fig. 4A). On day 4, 24 h fasting and food restriction both led to pronounced hypothermia (GLM repeated measures: $F_{2,29} = 32.52$, $P < 0.001$; Post hoc Tukey test: IF vs AL, $P < 0.001$; IR vs AL, $P = 0.002$; Fig. 4B). IF mice had a significantly lower body temperature compared with IR mice (Post hoc Tukey test: IF vs IR, $P < 0.001$). The effect of fasting/restriction on daily average temperature was observed on each fasting/restriction day (ANOVA: $P < 0.001$ in all 9 cases; Fig. 4C). Compared with control fed ad libitum, average body temperature in IF and IR mice on fasting/restriction days was decreased by 2.48 °C and 0.90 °C, respectively.

Body temperature was returned to the AL control levels after 1 day of refeeding (GLM repeated measures: $F_{2,29} = 1.21$, $P = 0.313$; Fig. 4D). There were no significant differences in daily average temperature across the three groups on all refeeding days (ANOVA: $P > 0.05$ in all cases; Fig. 4E). In cohort 2, the effects of intermittent fasting and restriction on body temperature were exactly the same as in cohort 1 for the first 13 days of treatment.

3.5. Circulating hormone and metabolite levels

Circulating levels of leptin were positively correlated with body fat mass on both day 13 and day 42 of the treatment, (Linear regression: day 13, $F_{1,30} = 7.34$, $P = 0.011$, adjusted $R^2 = 0.17$; Fig. 5A; day 42, $F_{1,30} = 12.13$, $P = 0.001$, adjusted $R^2 = 0.288$; Fig. 5B). On day 13, there was no significant difference in circulating leptin levels across the three groups (ANCOVA using fat mass as a covariate: $F_{2,29} = 0.139$, $P = 0.87$; Fig. 5C). However on day 42 IF mice had a significantly
elevated plasma leptin levels compared with the AL control and IR mice (ANCOVA using fat mass as a covariate: $F_{2,28}=4.92$, $P=0.015$; ANOVA post hoc Tukey: IF vs AL, $P=0.043$, IF vs IR, $P=0.002$; Fig. 5C). Circulating insulin were unchanged on days 13 and 42 (ANOVA, $F_{2,29}=1.60$, $P=0.220$; $F_{2,29}=1.65$, $P=0.209$; Fig. 5D). Intermittent fasting and food restriction had no effect on TG or NEFA levels (ANOVA: $P>0.05$ for both TG and NEFA on days 13 and 42; Fig. 5E,F).

3.6. Hypothalamic neuropeptides gene expression

On day 13 (cohort 1) after 3 starvation days and 10 free feeding days, levels of NPY gene expression in arcuate nucleus was significantly elevated by 49% in IF mice compared with AL control (ANOVA: $F_{2,29}=3.39$, $P=0.047$; Post hoc Tukey test: IF vs AL, $P=0.039$; Fig. 6A). No differences were detected between IR mice and AL control at this time point (Post hoc Tukey test: IR vs AL, $P=0.257$; Fig. 6A). However, the difference in NPY had disappeared after 42 days of treatment (cohort 2) (ANOVA: $F_{2,29}=0.22$, $P=0.805$; Fig. 6A). There were no effects of intermittent fasting/restriction on the levels of expression of POMC, CART, AgRP, Ob-Rb and SOCS 3 (ANOVA: $P>0.05$ for all on days 13 and 42; Fig. 6B-F).

4. Discussion

4.1. Effects of intermittent starvation on body mass and fatness in mice

We examined the changes in body mass and fatness in response to elevated starvation risk. Our finding that after 13 days of treatment there was a significant increase in both body mass and fat mass in the group that received intermittent complete food removal supports the suggestion that starvation risk defines the lower intervention level, according to the dual intervention points model [20]. However, mice that were stochastically exposed to only 50% food restriction showed no difference in body mass compared to controls that were fed ad libitum. This lack of response does not support the suggestion that starvation risk increases the lower intervention point. Moreover, when intermittent starvation was prolonged for a greater duration, the effects on body mass and adiposity were reduced and not significantly greater than the AL controls. The absence of significant elevation in body mass in response to the longer-term intermittent fasting is also at odds with the prediction from the dual intervention point model. It seemed that the immediate response of the mice to increased starvation risk was indeed to elevate fatness, but that as time went on different strategies were employed. One possibility is that a
short term intermittent starvation might provoke a more efficient compensation to defend the negative energy balance, thus generating over compensation in body mass.

Our result from long-term intermittent starvation is consistent with the finding that Swiss mice and striped hamsters (*C. barabensis*) also showed no change in body mass after exposure to stochastic food supplies for 4 weeks [23]. In their study, however, Swiss mice and striped hamsters showed no immediate response of body mass gain that attenuated over time. However, in this latter study the occurrence of fasting days was far more frequent than in the present study. Nevertheless, similar findings have been seen in humans. Healthy women subjects undergoing a restricted diet on 4 days each week exhibited no substantial weight change after 4 weeks [27]. However, in other studies where ADF regimens were administered for 12 weeks, body weight was found to decrease [28,29], but, when ADF regimens were applied for 16 weeks, no effect on body weight was observed [30,31]. Such findings imply that animals were unable to consume twice their daily food intake on the refeeding day for longer periods, which resulted in body weight loss.

There clearly is variability in the capacity of animals to compensate for a fast day on a fed day [32] particularly if fasting days are as frequently encountered as the fed days, and this may compromise their capacity to elevate body fatness. Furthermore, there could be longitudinal changes in this capacity to compensate dependent on the duration of treatment.

### 4.2. Physiological and behavioral responses to intermittent starvation

#### 4.2.1. Food intake

In the present study, mice experiencing intermittent fasting displayed pronounced hyperphagia on the first one or two days of refeeding days after the fasting day, which largely accounted for the weight restoration/elevation. It seemed necessary to have at least 2 days between the fasting days to build up a fat reserve and hence the difference between the current study and the previous studies by Zhao and colleagues may rest primarily in the frequency of fasting. If this frequency is too high there may be limited capacity to compensate on the fed days. However, the magnitude of hyperphagia was
associated with the severity of food deprivation since IF mice consumed more food than IR mice on the refeeding days. So the failure of IR mice to deposit a fat reserve was clearly not because they did not have the capacity to eat sufficient food on the days when food was available. Post fasting or restriction food compensation has been observed in a number of previous studies e.g. [33–35]. For instance, rats showed a dramatic post fast hyperphagia and consequently a rapid recovery of body weight lost during starvation [36,37]. Similarly, an initial hyperphagic response following refeeding was observed in C57BL/6 mice previously on 40% food restriction [33]. Hambly et al. (2007) further found that the magnitude of the hyperphagic response at refeeding was independent of the prior length of caloric restriction [34]. In contrast, golden hamsters (Mesocricetus auratus) of both sexes showed little or no post fast food compensation after 24 h of food deprivation and their daily food intake was no greater than daily intake during baseline testing [38]. Despite a hyperphagic response on refeeding days, IF mice consumed a similar total amount of food over the 13 days of intermittent fasting treatment, while IR mice consumed significantly less food compared to AL controls. However, with an even longer duration of treatment, the difference in accumulated food intake between IR mice and the AL control disappeared and mice from all the three groups consumed similar total amount of food over 42 days of intermittent fasting/restriction treatment. It has been shown that the amount of food consumed by rodents maintained on an ADF regimen varies depending on the strain and, in some cases (e.g., C57BL/6 mice), the animals gorge during the non-fasting time period to an extent that overall food intake is essentially equivalent to that of animals fed ad libitum [32]. Wan et al.

**Fig. 6.** Effects of intermittent fasting/food restriction on gene expression of neuropeptides in the hypothalamus in female C57BK/6j mice. A. Neuropeptide Y. B. Pro-opiomelanocortin (POMC). C. Cocaine- and amphetamine-regulated transcript (CART). D. Agouti-related peptide, (AgRP). E. Signaling form of leptin receptor (Ob-Rb). F. Suppressor of cytokine signaling 3 (SOCS 3). Filled bars represent intermittently fasted mice (IF), Slashed bars represent intermittently food restricted mice (IR), open bars represent control fed ad libitum (AL). Different letters over bars on the same treatment day indicate significant difference.
(2003) reported that rats on ADF regimen consumed 30% less food over time compared with rats fed ad libitum [39]. The intermittent starvation regimen provides stochastic food availability together with food abundance allowing compensation. Our results suggested that the responses to refueling varied with the extent of starvation. If mice were able to compensate for 24 h complete food deprivation on refueling days and maintained cumulative food intake at the same level as AL control. However the responses to refueling in IR mice changed over time. During the first 13 days of treatment, the decrease in cumulative food intake with unchanged body mass in IR mice implies a decrease in energy expenditure. On day 42, cumulative food consumption in IR mice was normalized to AL control level indicating other adaptations e.g. decreased energy expenditure might also be restored. When intermittently 50% food restricted, mice might employed different responses dependent on the duration of treatment.

4.2.2. Physical activity

In contradiction to previous studies demonstrating an elevation in physical activity level which occurred in several species such as lemurs (Lemur catta) [40], Siberian hamsters (Phodopus sungorus) [41] and mice [42] in response to food restriction, we observed a decrease in physical activity level in mice when fasted or 50% food restricted for 24 h, suggesting a response to decrease energy expenditure and conserve energy. This is consistent with the finding that mice exposed to 20% food restriction showed decreased activity which contributed to 75.5% of altered energy expenditure to compensate for reduced energy intake [43]. Similarly in female monkeys (Macaca mulatta), a rapid decrease in physical activity was observed to counteract diet-induced weight loss [44]. Since the energy cost of physical activity is an important component of energy expenditure, this physiological response to reduce energy expenditure is potentially an integral factor contributing to protection against excessive weight loss during caloric restriction and potentially predisposes to weight re-gain in post-obese individuals [45]. Further analyses of activity data showed that the decrease was mainly due to activity during the light phase coincident with the occurrence of hypothermia. However, IF and IR mice had similar levels of activity during the dark phase compared to the controls fed ad libitum. Increased activity during food restriction has been previously interpreted to reflect an increase in foraging behavior, which may enhance the chances of survival in a free living animal [46]. This inconsistency could be explained by the short duration of starvation (24 h), which failed to induce food foraging behavior in mice. Additionally, Gutman et al. (2007) observed that desert gold spiny mice (Acomys russatus) employed two strategies for coping with food shortage, some significantly reduced activity level concentrating their activity around feeding time lowering energy expenditure while others significantly increased activity level searching for food and thus losing body mass rapidly [47]. It is currently unclear whether and how the response in activity is correlated with the severity or duration of starvation. On refueling days after starvation, our mice still maintained a lowered level of physical activity, which also contributed to the recovery and the increased body mass of the IF mice. Although it has been suggested that hypoleptinemia can influence starvation induced hyperactivity [48,49], in our study the decreased physical activity during intermittent starvation was accompanied by either unaltered (13 days) or elevated (42 days) leptin levels, indicating that lowered leptin was unlikely to be the stimulus of the activity changes.

4.2.3. Body temperature

Mice are documented as an attractive model for studying the response of body temperature to dietary restriction because body temperature of mice is sensitive to reduced food intake [50]. Indeed mice from our study demonstrated a decrease in body temperature in response to 24 h of complete fasting or 50% food restriction. Notably, hypothermia was only present when food was deprived or restricted and refueling immediately restored body temperature to control levels. Caloric restriction is associated with lower mean body temperature in most homeotherms that have been examined including mice, rats, monkeys and humans [49]. The use of torpor has been described as one of the key strategies for coping with food shortage in rodents [51,52]. In the present study, to what extent the reduced body temperature contributed to the saving of energy and thus the prevention of weight loss remained unclear, particularly when the reduction in body temperature was restored rapidly following refueling.

4.3. Effects of intermittent fasting/restriction on neuroendocrine status

Leptin is primarily known for its role in regulating food intake and energy homeostasis [53]. At the end of 12 days of intermittent fasting/restriction, no changes in circulating leptin and insulin levels were detected. This was unexpected given both the increased body fatness and food intake in the IF mice. Moreover, after a long-term intermittent starvation, IF mice exhibited elevated leptin levels while their body fatness and food intake were restored to the same level as AL controls. It is possible that leptin sensitivity might have been impaired; however, the absence of changes in gene expression of Ob-Rb and SOCS 3 suggested this was not the case.

With respect to other neuropeptides involved in eating behavior, NPY gene expression was increased after a short-term intermittent starvation. NPY and AgRP neurons of the hypothalamic arcuate nucleus are thought to promote feeding and consistent with an orexigenic role, fasting induced a 4-fold increase in the basal action potential frequency of NPY/AgRP neurons [54]. It seems likely that the elevation in NPY expression stimulated the increased food intake in the IF mice. Similarly, an increase in NPY expression and a parallel decrease in POMC expression were observed in the hypothalamic arcuate nucleus of rats killed just before rebound hyperphagia following a restricted feeding/repletion protocol [55].

5. Conclusions

C57BL/6J mice displayed an increased body mass and fatness in response to 13 days of intermittent fasting, which were both restored to baseline levels when the treatment was prolonged to 42 days. Over the first 13 days of treatment the data were consistent with the dual intervention point model as the mice showed both increased body mass and adiposity, however the effect disappeared over the more protracted period of 42 days. Whether the data are considered consistent with the dual intervention point model therefore depends critically on the time course of the experiment. Over 13 days the response was consistent but over 42 days it was not. In the short term, an increase in food intake, decreased physical activity as well as a reduction in body temperature contributed to the elevated body mass and fatness. In contrast, the body mass of mice treated with intermittent fasting for 42 days were not significantly elevated. This finding was inconsistent with the dual intervention point model. Intermittent starvation resulted in unaltered cumulative food intake and meanwhile a decreased physical activity level, suggesting an absence of overcompensation in energy intake but a decrease in energy expenditure in coping with stochastic food.

Overall, mice exhibited different behavioral and physiological responses to intermittent starvation which were critically dependent on the duration of treatment.

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