Brief communication

# High fat diet causes rebound weight gain

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## ABSTRACT

Obesity is at epidemic proportions but treatment options remain limited. Treatment of obesity by calorie restriction (CR) despite having initial success often fails due to rebound weight gain. One possibility is that this reflects an increased body weight (BW) set-point. Indeed, high fat diets (HFD) reduce adult neurogenesis altering hypothalamic neuroarchitecture. However, it is uncertain if these changes are associated with weight rebound or if long-term weight management is associated with reversing this.

Here we show that obese mice have an increased BW set-point and lowering this set-point is associated with rescuing hypothalamic remodelling. Treating obesity by CR using HFD causes weight loss, but not rescued remodelling resulting in rebound weight gain. However, treating obesity by CR using non-HFD causes weight loss, rescued remodelling and attenuates rebound weight gain. We propose that these phenomena may explain why successful short-term weight loss improves obesity in some people but not in others.

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## 1. INTRODUCTION

Obesity is at epidemic proportions in many developed nations, but treatment options remain limited. The homoeostatic body weight (BW) set-point theory is strongly supported by the counter-measures that are enabled to resist weight loss under calorie restriction (CR) [1], and the subsequent return to their original body weight after dieting is terminated, called rebound weight gain [2]. However, such robust homoeostatic regulation is difficult to reconcile with environmental influences on BW; feeding mice a high fat diet (HFD) induces obesity (DIO) which can be reversed by feeding regular chow [3]. One proposed solution to this paradox is that the BW set-point is altered by environmental factors via changes in the neuroarchitecture of the homoeostatic energy balance circuit [4] and indeed, the properties of homoeostatic energy balance are altered by HFD and restored by feeding regular chow [3]. Recently, remodelling of the hypothalamus has been identified in obese mice and humans [5] and we have discovered that dietary regulation of hypothalamic neurogenesis alters hypothalamic neuroarchitecture [6].

Treatment of obesity by reducing calorie intake, despite having a good success rate in promoting initial weight-loss, has a generally poor outcome for long-term weight control [7]. Indeed the large scale Diabetes Prevention Program [8] and its follow up the Look Ahead trial [9] demonstrate that the short-term success of lifestyle change in lowering obesity is offset in the long-term by rebound weight gain. The mechanisms underlying long-term rebound weight gain remain unclear although the homoeostatic BW set-point theory suggests that obese subjects have a higher BW set-point reflecting an underlying defect of the homoeostatic energy balance circuit. Those individuals who achieve long-term weight management after caloric restriction (CR) may have successfully lowered their set-point while rebound weight gain may result from a failure to lower the raised set-point during the period of restriction. However, it is uncertain if obese individuals do have an increased BW set-point and if successful long-term weight management is associated with reversing this.

Here we follow up on our recent study of dietary regulation of proliferative remodelling in the murine hypothalamus [6] and find that obese mice do have an increased BW set-point and that lowering this set-point is associated with rescuing proliferative remodelling. We show that hypothalamic remodelling and long-term BW control are distinct from short-term BW changes, and propose that these phenomena may explain why successful short-term weight loss improves obesity in some people but not in others.

## 2. RESULTS AND DISCUSSION

### 2.1. Both calorie restriction and HFD reduce hypothalamic proliferative remodelling

The hypothalamic energy balance circuit is remodelled by ongoing adult neurogenesis; in lean mice old neurons are replaced by newborn neurons [6]. However, neurogenesis is reduced in both diet induced obesity (DIO) and genetic obesity; in obese mice newborn neurons are not generated and old neurons are retained [6]. This change is not permanent, and inducing weight loss in DIO animals by CR rescues proliferative remodelling in the hypothalamic niche [6]. However, it is unclear if this rescue is complete, and the extent to which macronutrient composition of diet during CR affects the extent of rescue. To test the extent of proliferative remodelling during weight loss and the role of dietary macronutrients in this process, we fed 6 week old mice HFD for 10 weeks to induce obesity. We then treated these 16 week old DIO mice with diets rich in a single macronutrient high protein (HPD), high carbohydrate

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| Diet | %Energy Fat | %Energy Protein | %Energy Carbohydrate | Gross energy (kJ/g) | Manufacturer | Reference |
|------|-------------|-----------------|----------------------|---------------------|--------------|-----------|
| HFD | 45 | 20 | 35 | 20.1 | Research Diets | D12451 |
| HCD | 10 | 20 | 70 | 16.3 | Research Diets | D12450B |
| HPD | 13.2 | 42.6 | 44.3 | 15.9 | Teklad | TD90018 |
| KD | 93.4 | 4.7 | 1.8 | 30.1 | Bio-Serv | F3666 |
| Chow | 9.1 | 22.0 | 68.9 | 13.8 | Special diets services | 801722 |

Table 1: Composition of the diets used (data as provided by manufacturer).

(HCD), ketogenic (KD = high fat and low protein and carbohydrate) or maintained them on HFD for 4 weeks (see Table 1 for diet details). Diet treated mice where further sub-divided by feeding either ad-lib (AL) or CR at −30% of daily energy intake with a final group size of 7 mice per diet/feeding regime combination. The extent of proliferative remodelling in the arcuate nucleus (ARC)/median eminence (ME) niche was measured by BrdU incorporation during the last week of treatment (Figure 1).

As expected DIO mice maintained on ad-lib HFD did not lose weight while DIO mice treated with HFD-CR lost weight (Figure 1a). Treatment of DIO mice with non-HFD diets either ad-lib or under CR also caused weight loss (Figure 1a). When fed ad-lib, macronutrient composition had a significant effect on final BW (p < 0.001), but had no effect on final BW under CR (Figure 1b). For all diets, CR of DIO mice resulted in a final BW similar to chow fed controls treated with CR (Figure 1b). DIO mice fed HFD with 45% of energy derived from fat (Table 1) had a significantly reduced proliferative remodelling compared to chow fed controls (Figure 1c). This is in agreement with our previous finding in mice fed HFD with 58% of energy derived from fat [6] suggesting that reduced proliferative remodelling is a general feature of mice fed HFDs. Treating DIO mice with ad-lib HPD, HCD and KD led to a significant recovery of proliferative remodelling, although this recovery did not reach the level seen in ad-lib controls (Figure 1c). Likewise, treating DIO mice with HPD, HCD and KD under CR led to a significant recovery of proliferative remodelling (Figure 1c). This recovery of proliferative remodelling under CR was significantly less than seen in ad-lib fed mice (p < 0.001). The finding that CR inhibits proliferative remodelling compared to ad-lib feeding was confirmed in chow fed controls; CR significantly inhibited hypothalamic proliferative remodelling in non-obese mice (Figure 1c).

While CR had a significant inhibitory effect on recovery of proliferative remodelling there was no compounding effect of individual dietary macronutrients (p = 0.865, HCD, HPD and KD). However, DIO mice fed HFD-CR showed no recovery of proliferative remodelling despite weight loss, indicating a significant (p < 0.001) and specific effect of HFD on the rescue of proliferative remodelling (Figure 1c).

To further investigate the effect of CR and HFD on proliferative remodelling, alongside ad-lib fed DIO and control mice we pair-fed mice HCD or HFD from 6 to 20 weeks of age, with hypothalamic remodelling measured by BrdU incorporation at 20 weeks as above. Each pair-fed mouse was fed a single daily meal containing the age matched energy intake of ad-lib fed controls (Control AL in Figure 1) i.e. 0%CR (Supplementary Figure 1). This experiment revealed that HFD consumption under pair-feeding (0%CR) did not result in either weight gain or weight loss while hypothalamic proliferative remodelling was reduced to the level seen in both HFD ad-lib fed DIO mice and DIO mice treated with HFD 30%CR. This data confirms that this pattern of proliferative remodelling reflects HFD consumption and not current BW. Remarkably, HCD pair-fed mice (0%CR) had a similar final BW to HCD ad-lib controls but the moderately reduced level proliferative
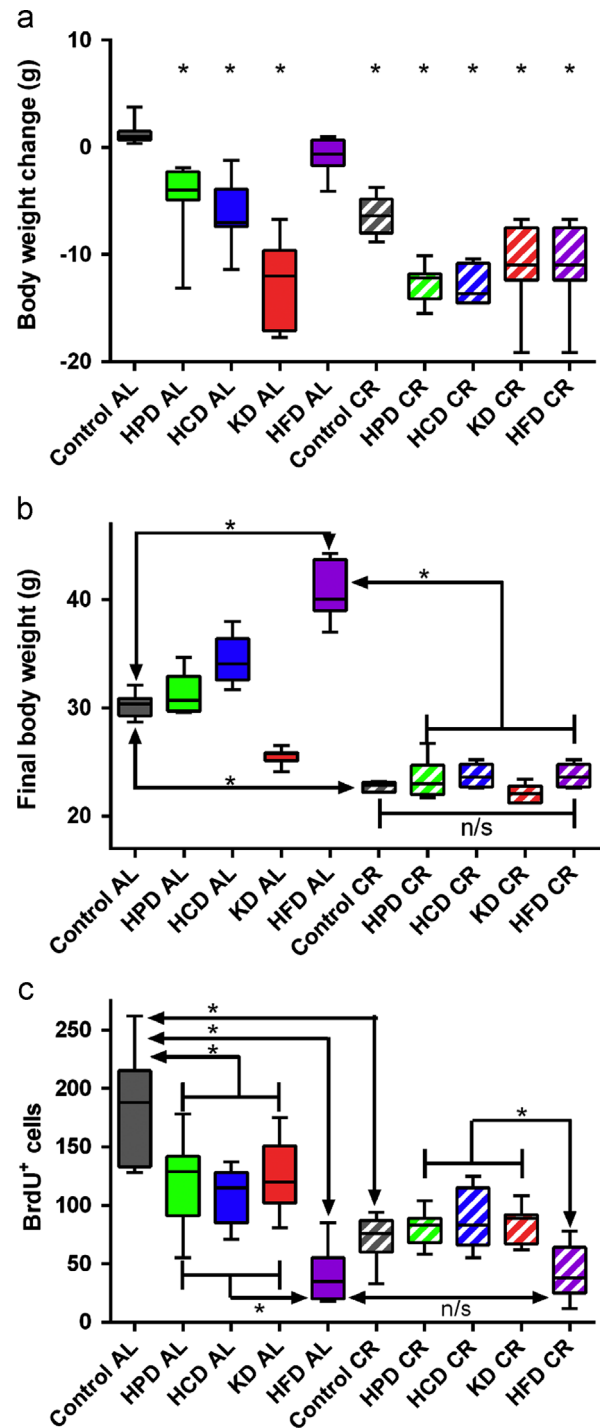


Figure 1: Calorie restriction of obese mice using HFD fails to rescue hypothalamic proliferative remodelling. 16 week old obese mice were treated using 4 diets for 4 weeks, either ad-lib (AL) or at 70% of normal daily calorie intake of lean controls (CR). (a) Treatment of obese mice caused significant BW loss in all groups except mice fed HFD AL. (b) Treating obese mice by CR resulted in identical final BW irrespective of macronutrient composition, while AL treatment resulted in differential final BW dependent on macronutrient composition. (c) Although CR with HFD lowered BW, it did not rescue hypothalamic proliferative remodelling. Non-HFD diets rescued proliferative remodelling irrespective of dietary macronutrient composition under both CR and AL. However, CR inhibited the rescue of proliferative remodelling compared to AL treatment (p < 0.001). CR also reduced proliferative remodelling in lean controls. The rescue of hypothalamic proliferative remodelling during treatment of obesity was incomplete in all groups and did not reach the level seen in control mice not previously exposed to HFD. (*p < 0.05, graphs show box plots, N = 7 mice per group).

remodelling seen in control mice treated with HCD 30%CR (Supplementary Figure 1). These data suggest that the moderate reduction in hypothalamic proliferative remodelling seen in CR treated

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mice is not due to reduced energy intake but a response to the feeding regime (i.e. a single daily meal during the day).

## 2.2. Both overweight and underweight mice show reduced hypothalamic proliferative remodelling

This complex regulation of hypothalamic proliferative remodelling excludes a linear relationship between BW and hypothalamic remodelling i.e. ad-lib HFD fed mice and HFD-CR treated mice have identical levels of hypothalamic proliferative remodelling despite the former being obese while the later underweight. Moreover, chow fed controls and HPD-AL treated DIO mice have similar BWs but different levels of proliferative remodelling (compare Figure 1a and b).

The relationship between body weight and proliferative remodelling was determined by regression across all previously obese mice which showed no linear relationship (p = 0.478) but a second order polynomial relationship (p < 0.001) with hypothalamic proliferative remodelling highest in normal weight mice and lower in both overweight and underweight mice (Figure 2). We [6] and others [11] have recently suggested that obesity is associated with a loss of new neurons in the adult hypothalamus while others have argued that obesity is associated with increased new neurons in the adult hypothalamus [10]. However, the finding that reduced proliferation is associated with both overweight and underweight mice strongly suggests that neither position is correct and that the relationship between BW and hypothalamic remodelling is qualitative rather than quantitative.

This discovery of a non-linear relationship supports a complex mechanism underlying the role of hypothalamic proliferative remodelling in regulation of BW i.e. a lower rate of hypothalamic remodelling is not always associated with obesity. Rather the data suggests that changes in hypothalamic proliferative remodelling reflect adaptive remodelling of the energy balance circuit in response to diet which in turn leads to an altered BW set-point i.e. HFD induced proliferative remodelling alters hypothalamic neuroarchitecture increasing the BW set-point in obesity [6]. This view is supported by the finding that blocking hypothalamic proliferative remodelling by irradiation prior to exposure to HFD attenuates weight gain [10] i.e. proliferative remodelling is required for weight gain; while IKKβ-mediated inhibition of hypothalamic neurogenesis induces obesity [11]. The discovery that reducing hypothalamic neurogenesis both prevents and causes obesity supports the view that the relationship between BW and hypothalamic
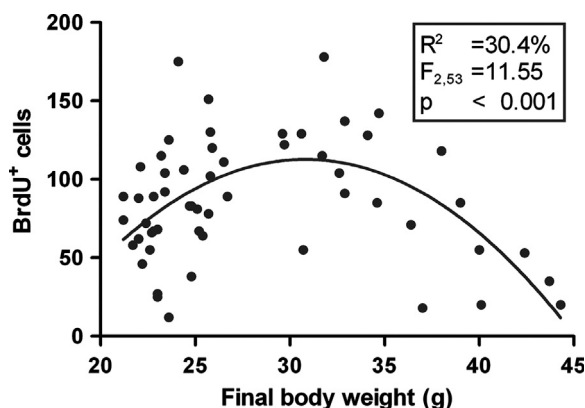
remodelling is qualitative, with BW determined by the delicate balance between orexigenic and anorexigenic neurons mediated by subtle alteration of neuroarchitecture. This is supported by our finding that subtle alteration of the energy balance circuit neuroarchitecture by transplantation of small numbers of neurons results in long-term BW change [12].

## 2.3. HFD raises the BW set-point and prevents successful treatment of obesity by calorie restriction

As ad-lib HFD alters hypothalamic proliferative remodelling [6], a process which is required for weight gain in DIO [10], and DIO mice subsequently fed the same HFD under CR retain this pattern despite weight loss, we next directly measured the BW set-point in mice exposed to HFD. As before mice were fed HFD from 6 weeks of age for 10 weeks to induce obesity. At 16 weeks of age, DIO was treated by feeding mice the 4 diets (HCD, HPD, KD and HFD) for 4 weeks under CR with a final group size of 5–6 mice. At 20 weeks of age mice were released from CR onto ad-lib chow with rebound BW and post-release hyperphagia measured (Figure 3).

CR successfully induced weight loss in all DIO mice with all mice reaching a similar BW and adiposity to CR treated controls (Figure 3a
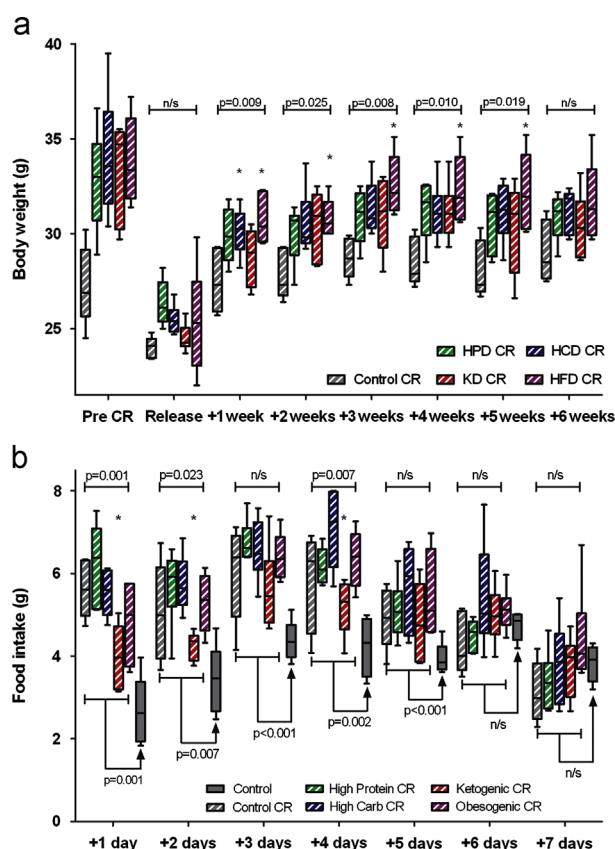


**Figure 2:** Both overweight and underweight mice show reduced hypothalamic proliferative remodelling. 16 week old obese mice were treated using 4 diets for 4 weeks, either ad-lib (AL) or at 70% of normal daily calorie intake (CR). Regression analysis of hypothalamic proliferative remodelling and BW across all mice showed no linear relationship (p = 0.478) but a second order polynomial relationship (p < 0.001) with hypothalamic proliferative remodelling highest in normal weight mice and lower in both overweight and underweight mice.



**Figure 3:** Treatment of obesity by calorie restriction using HFD results in long-term rebound weight gain. 16 week old obese mice were treated using 4 diets for 4 weeks at 70% of normal daily calorie intake and released onto ad-lib chow at 20 weeks. (a) All mice treated by CR lost BW. At the point of release from CR onto ad-lib chow the BW of all groups was similar and not affected by CR diet macronutrient composition or previous obesity. Obese mice treated with HFD-CR rebounded to a higher BW compared to controls and obese mice treated with CR using non-HFD diets indicating an increased BW set-point. Although there was a trend for obese mice treated with non-HFD diets to have a higher BW than control mice this only reached statistical significance at a single time point in the case of HCD. (b) All CR treated mice displayed post-restriction hyperphagia compared to control mice fed chow ad-lib. The increase in rebound BW in obese mice treated with HFD-CR was not due to increased post-restriction hyperphagia in these mice. Likewise, while post-restriction hyperphagia was reduced in obese mice treated with KD-CR this did not result in reduced rebound BW in KD-CR treated mice. (*p < 0.05, graphs show box plots, N=5–6 mice per group).

and data not shown). Hence, when released from CR mice differed only in previous macronutrient exposure and not BW, adiposity or current diet. We predicted that DIO animals treated with HFD-CR would rebound to a higher BW than those on the other 3 diets, who would in turn rebound higher than mice that were placed under CR but had not previously been DIO.

As expected, all mice showed significant rebound weight gain after release from CR (Figure 3a, $p < 0.001$). However, rebound BW was significantly different across treatment groups with obese mice treated with HFD-CR rebounding to a higher BW than controls (Figure 3a). Although DIO mice treated with non-HFD showed a trend towards higher rebound BW than controls, this only reached statistical significance at a one point in the case of HCD (Figure 3a). Hence the failure to achieve long-term weight control following CR only occurred in obese mice fed HFD during CR, coinciding with the maintenance of altered hypothalamic remodelling.

The increase in rebound BW in DIO mice treated with HFD-CR was not permanent but resolved after 6 weeks of ad-lib chow re-feeding. Given the similarly in macronutrient content between chow and HCD (see Table 1) this may reflect the loss of the altered HFD pattern of hypothalamic remodelling due to consumption of a high carbohydrate, low fat diet.

This increase in BW long-term in HFD-CR treated mice was not due to an acute increase in post-CR hyperphagia; HFD-CR treated DIO mice did not show increased hyperphagia compared to CR treated controls (Figure 3b). However, macronutrient composition during CR did have an effect on post-release hyperphagia. Mice treated with KD-CR had significantly reduced post-CR hyperphagia compared to both controls and DIO mice treated with other diets (Figure 3b). However, this did not result in decreased rebound weight gain (Figure 3a).

### 2.4. The success of treating obesity in the short-term does not predict long-term success

Together these and previous data [4,6,7,10] suggest that alteration of hypothalamic neuroarchitecture via proliferative remodelling alters the BW set-point and this mediates the long-term success of obesity treatment. This suggests that measuring the efficacy of obesity treatment immediately following CR will not predict long-term success. To test this, we analysed the predictive power of BW and adiposity (%body fat) at the end of CR treatment of obesity as well as post-CR hyperphagia (24 h food intake, 3rd day after release from CR) in predicting long-term BW (BW 4 weeks after release from CR) in the CR treated DIO mice as a single group (Figure 4).

Neither BW nor adiposity at the end of CR predicted long-term BW. Likewise, individual hyperphagic response following release from CR did not predict long-term BW. Taken together the acute success of obesity treatment as measured by BW, adiposity and food intake at the end of CR treatment predicted none of the variation in BW one month after cessation of treatment (Figure 4 and data not shown). These data support the view that treating obesity with CR does not by itself cure obesity despite treating the overt symptom of increased BW. Rather both the amount of calories consumed during restriction and the macronutrient composition of the diet are both important factors influencing successful long-term BW control.

While these data confirm that HFD increases the BW set-point such that following weight loss DIO mice rebound to a higher BW than lean controls, and supports a role for proliferative remodelling of the hypothalamic energy balance circuit in this process, the precise mechanism by which proliferative remodelling causes altered hypothalamic function remains unclear. Although our data focuses on overall rate of proliferative remodelling suggesting that the ratio of new to older neurons may be important, alteration of the balance between NPY to POMC neuronal populations or the alteration of neuronal subtypes within NPY and POMC populations may also play a role in altered hypothalamic function.

It is of particular interest that the specific effect of moderately high fat diet (HFD) to alter hypothalamic proliferative remodelling is not shared by ultra high fat ketogenic diet (KD). While this could be due to the difference in the source of dietary fat, in both diets fat is predominantly of animal origin (lard and/or butterfat). This may point to a specific
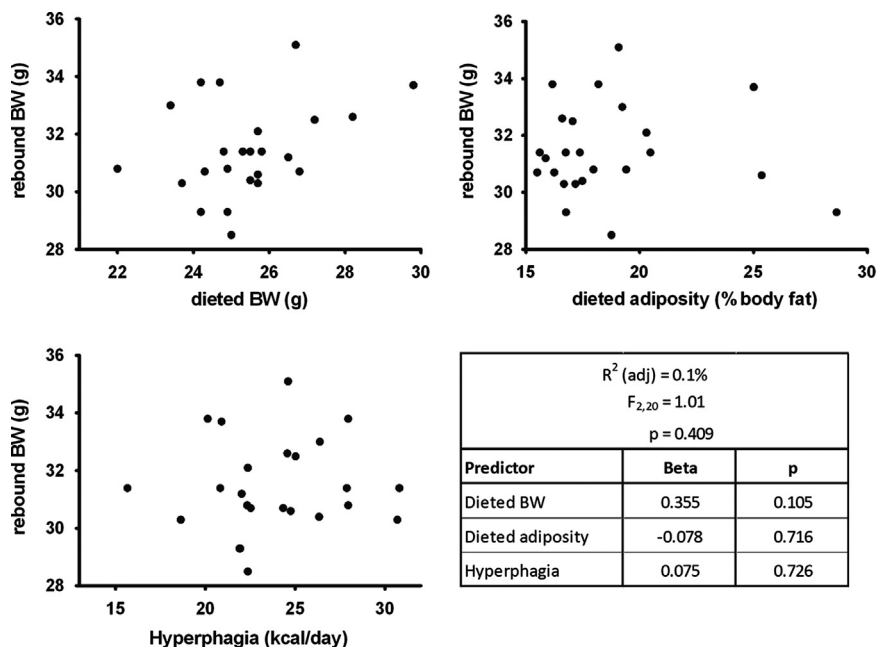


| | | |
|---|---|---|
| $R^2$ (adj) = 0.1% | | |
| $F_{2,20} = 1.01$ | | |
| $p = 0.409$ | | |
| **Predictor** | **Beta** | **p** |
| Dieted BW | 0.355 | 0.105 |
| Dieted adiposity | -0.078 | 0.716 |
| Hyperphagia | 0.075 | 0.726 |

**Figure 4:** Short-term efficacy of obesity treatment by CR does not predict long-term efficacy. Individual variation in BW, adiposity and hyperphagic response (release + 3 days) following cessation of CR treatment of obesity do not predict BW 4 weeks later either individually or together. This indicates that the success of obesity treatment measured at the end of treatment does not predict the long-term efficacy in preventing rebound weight gain.

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requirement for the presence of both fat and carbohydrate in the diet, although a protective effect of ketosis masking the effect of dietary fat in the case of KD cannot be excluded. Likewise, while both HCD and HFD share a 1:1 sucrose to starch ratio, HFD which is lower in sucrose (17% by energy content) reduces hypothalamic remodelling and causes rebound weight gain while HCD which is higher in sucrose (35% by energy content) does not. Together these data suggest that hypothalamic remodelling is regulated complex mechanisms involving response to multiple dietary macronutrients.

While the mechanism by which HFD specifically alters hypothalamic remodelling remains unclear, the discovery that the IKKβ/NF-κB pathway regulates hypothalamic neurogensis [11] and identification of inflammatory changes in the hypothalamus of obese mice and humans [5] suggests that local inflammatory processes may play a critical role. This central role for proliferative remodelling of hypothalamic neurocircuitry in response to HFD does not preclude roles for other mechanisms in mediating homoeostatic plasticity in a variable environment.

Overall a growing pool of evidence supports the view that changes in the BW set-point in response to environmental signals is a critical factor in the pathogenesis of obesity and provides a model for integrating the considerable physiological evidence for a BW set-point with the equally considerable evidence for environmental causes underlying the obesity epidemic [4]. Our results provide a mechanistic framework for understanding how environmental factors such as macronutrient composition of diet can be integrated into the homoeostatic hypothalamus leading to long-term changes in the BW set-point, providing a bridge between internal homoeostatic control of body weight and the external environmental driven obesity epidemic. We confirm that short-term weight loss does not cure obesity, and describe a mechanistic process apparently underlying the failure of some individuals to achieve long-term weight loss despite successful short-term weight loss. Manipulation of hypothalamic remodelling provides an attractive and novel target in the quest for a successful long-term cure of obese individuals with refractory obesity.

# 3. METHODS

## 3.1. Mice

Male C57BL/6 mice were obtained from the Charles River Laboratories at 4 weeks of age. Animals were housed under a 12/12 light/dark cycle. Mice were maintained at an ambient temperature of 21 °C with a relative humidity of $55 \pm 10\%$. Mice were group housed 3 per wire-top cage until 16 weeks of age and then singly housed. Food intake was determined by weighing food remaining in the hopper. All procedures were authorised by the College of Life Sciences and Medicine Ethics Review Board at the University of Aberdeen and by the UK Home office (PPL 60/3706).

## 3.2. Dietary manipulations

Mice were obtained from the Charles River Laboratories at 4 weeks of age and fed and chow (SDS Nutrition) from 4 to 6 weeks of age ad-lib. At 6 weeks of age DIO mice were fed HFD to induce obesity, while lean control mice were fed HCD diet. For details of diets see Table 1. At 16 weeks of age DIO mice were fed one of the four test diets either ad-lib or at 70% of the daily energy intake of lean control mice as a single daily pellet (30%CR). Likewise, at 16 weeks of age lean control mice were either maintained on HCD ad-lib (Control AL) or fed HCD at 70% of the daily energy intake of lean control mice as a single daily pellet (Control CR). Control daily food intake was the daily average between 8 and 16 weeks for all control mice (subsequently Control AL and Control CR in Figure 1) and was used for

all experiments (daily average = 49.15 kJ/day, 30%CR = 34.41 kJ/day). For re-feeding experiments mice were fed chow ad-lib from 20 weeks of age. For pair-feeding experiments mice were fed either HCD or HFD at 100% of the daily energy intake of lean control mice as a single daily pellet, from 6 weeks to 20 weeks of age. Control daily food intake was the daily average for all control mice (subsequently Control AL and Control CR in Figure 1) and was matched for week 6 (daily average = 39.86 kJ/day), week 7 (daily average = 45.38 kJ/day), and then the daily average between 8 and 16 weeks was used subsequently (daily average = 49.15 kJ/day).

## 3.3. Body composition

Body composition was determined by calibrated [13] dual energy X-ray absorptometry (PIXImus2) at 15 weeks and 20 weeks of age.

## 3.4. BrdU labelling

In brief (see [6]), mice under ketamine and xylazine anaesthesia (45 mg/kg and 5 mg/kg) were implanted with a subcutaneous osmotic minipump (2001, 1 μl/h, 7days, Alzet) at 19 weeks of age. Each minipump was filled with bromodeoxyuridine (BrdU, Sigma) in saline 20 μg/μl. 7 days later (at the end of BrdU infusion without a chase period) mice were killed by cardiac perfusion with 10% formalin.

## 3.5. Histology and cell counting

Following cardiac perfusion, brains were removed and post-fixed for 24 h in 10% formalin before cryoprotection in 30% sucrose (wt/vol). Brains were then embedded in OCT compound (CellPath), stored at −80 °C and cut into 12 μM coronal sections using a cryostat (10 series/brain). The total number of BrdU labelled cells in 3 central hypothalamic sections containing the ARC were counted manually.

## 3.6. Immunohistochemistry

Slides were treated with 2 N HCl for 2 h followed by $6 \times$ washing with PBS before incubation in primary BrdU antibody (1:200,Abcam) diluted in PBS+0.1%TritonX100+1%BSA overnight at 4 °C. Sections were washed $3 \times$ with PBS+0.1%TritonX100 before incubation in secondary Cy3-anti-rat antibody (1:400,Abcam) diluted in PBS+0.1%TritonX100+1%BSA for 2 h at room temperature. Sections were washed $3 \times$ with PBS+0.1%TritonX100 before imaging.

## 3.7. Imaging

Stained sections were examined using an Axiovert 200M microscope (Zeiss). Images were processed using Photoshop 7.0 (Adobe). Figures were prepared using Prism 5 (Graphpad).

## 3.8. Statistical analysis

Statistical analysis was carried out using Minitab 16 (Minitab). Z-tests were used to determine significance of weight loss. One way or two way ANOVA was used to determined differences between treatment groups followed by post-hoc Tukey's comparisons to identify specific differences. Regression analysis was used to determine the relationship between variables. $p < 0.05$ was considered significant.

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