Hypothalamic gene expression during voluntary hypophagia in the Sprague-Dawley rat on withdrawal of the palatable liquid diet, Ensure.

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Abstract

Sprague Dawley rats over-consume calories over a 10 week period and develop diet-induced obesity (c. 100g body weight differential vs controls) when fed a control pellet diet supplemented with chocolate Ensure liquid. Subsequent withdrawal of Ensure immediately reduces caloric intake by more than 50%, and results in weight loss, despite control pellet being available *ad libitum*. To assess the molecular underpinnings of this phenomenon, brains were processed for energy balance and food reward-related gene expression analysis at two time points, 24h and 4 days after the withdrawal of Ensure, when energy intake was suppressed. Gene expression levels in hypothalamic arcuate nucleus and forebrain nucleus accumbens were compared with rats pair-fed to the same energy intake, i.e. imposed negative energy balance, and to controls fed control pellet *ad libitum* throughout. Cumulative energy intake was approximately 50% lower across the 4 day post-Ensure period, giving rise to a small reduction in body weight although body adiposity and blood leptin remained elevated (c. 100% and 50%, respectively vs controls) in rats that had previously been fed Ensure. In contrast, pair-feeding reduced blood insulin and leptin by 33% and 55%, respectively. Hypothalamic expression of neuropeptide Y and agouti-related peptide was down-regulated at 24h in rats previously fed Ensure, indicative of the apparent counter-regulatory changes seen in diet-induced obesity, but was normalised between the 24h and 4 day time points. By contrast, the effect of cumulative negative energy balance in the pair-fed groups increased with time, up-regulating expression of the orexigenic neuropeptides. There was also a reduction of suppressor of cytokine signalling-3 gene expression in pair-fed groups where leptin levels were low. There were no changes in opioid, dopamine receptor or cannabinoid receptor expression in the nucleus accumbens. Feedback from diet-induced obesity appears to drive

voluntary hypophagia upon withdrawal of palatable diet, and to override signals from intake restriction that would otherwise set in train an anabolic drive.

Keywords: diet-induced obesity; reward; neuropeptide Y; agouti related peptide; SOCS3

1. **Introduction**

Polygenic susceptibility and palatable diet challenge are the basis of rodent models of diet-induced obesity (DIO), mimicking aspects of the human condition. Some of the most interesting features of these models appear immediately after dietary manipulation [1], and are obviously diet-dependent. Accordingly, such manipulations frequently result in periods of elevated or suppressed caloric intake of variable duration and intensity that also have implications for body weight defence. The Sprague Dawley (SD) rat model of DIO, elegantly characterised by Levin and co-workers in a series of seminal papers, e.g. [2], has a number of fascinating features, revealed by dietary manipulation. SD rats exhibit relatively limited upward or downward deflections in energy intake on switching between control diet and high energy (HE) pellet diet, and largely defend their HE-elevated body weight [2,3,4]. However, when SD rats are fed Ensure (EN) as a supplement to HE diet, initial high levels of energy intake decline but remain elevated relative to controls, apparently indefinitely [2,4], leading to additional accumulation of adipose tissue. Return to control diet, i.e. simultaneous withdrawal of both HE pellet diet and liquid EN, induces a prolonged voluntary hypocaloric intake episode and weight loss, although total normalisation of body weight may not be achieved [2,4]. Similar outcomes have been recorded with cafeteria feeding of palatable solid diets ([5,6] and references therein).

Lengthy voluntary excursion into negative energy balance as a result of reduced caloric intake on withdrawal of EN is observed in unselected populations of rats [1,4], as well as in selected sub-groups of animals that are more or less susceptible to DIO, where the response in SD rats was first described [2]. There appears to be

'recognition' of excessive body weight and adiposity that suppresses energy intake once the over-riding stimulus of a palatable diet has been removed. This is evidenced by apparent counter-regulatory changes in hypothalamic gene expression observed in DIO animals [4,7,8]. Thus genes which show a characteristic response to negative energy balance, such as following food restriction, exhibit the opposite response to DIO, e.g. suppression of orexigenic gene expression in obese hypercaloric rats.

The mechanistic basis of hypocaloric intake upon withdrawal of palatable food is poorly defined. We employed the simplified dietary manipulation of EN supplementation of control diet [9] in order to maximise the effect sizes of both hypercaloric intake with palatable diet and reduced voluntary intake upon its withdrawal. We compared rats in which EN access was terminated (EN-C) with those held on control diet throughout but pair-fed (C-PF) to the energy intake of EN-C rats over the post-EN period. To our knowledge this is the first assessment of hypothalamic and forebrain regulatory systems that have been implicated in negative energy balance, DIO and food reward during this acute dietary transition phase. We hypothesised that despite equicaloric intake, energy balance and/or reward systems would differentiate between voluntary hypocaloric intake and imposed restriction.

2. Materials and Methods

2.1 Animals

Procedures were licensed under the UK Animals (Scientific Procedures) Act and received local ethical approval. SD rats (Charles River Laboratories, Kent, UK) were housed individually at 19-21°C on a 12:12h light-dark cycle and fed Purina 5001 (control diet; 3.34kcal/g: 23% protein, 12% fat, 65% carbohydrate by energy; PMI

Nutrition International, Nottingham, UK) *ad libitum*. Where detailed, control diet was supplemented with liquid chocolate Ensure (EN; 1.06kcal/ml: 14% protein, 22% fat, 64% carbohydrate; Abbott Nutrition, Abbott Laboratories Ltd., Kent, UK), also available *ad libitum*, and renewed daily. Dietary regimes were initiated at a body weight of c. 200g.

Study 1: Energy intake and body weight gain were recorded for 20 rats (EN-C) during baseline feeding on control diet (30 days), while control diet was supplemented with EN (70 days), and following the withdrawal of EN (28 days). Controls (C-C) were fed control diet throughout. The EN supplementation period of 10 weeks was selected to approximate to the duration of dietary manipulation used in Levin and Keesey's original study [2], and in our earlier study [4], in both of which EN was provided as a supplement to HE diet. This regime was intended to give rise to similar body weight differentials (c. 100g), and energy intake responses upon withdrawal, to those previously reported.

Study 2: Using a similar study design, rats were fed control diet *ad libitum* before being divided into 3 weight-matched groups, each of 30 rats, one of which had control diet supplemented with EN for 73 days (EN-C). At the end of this period, EN was withheld in the first half of the light phase; control diet continued to be available *ad libitum*. EN-C rats were killed by decapitation in two sub-groups after a further 24 hours or 4 days. The weight of control diet eaten during the previous 24 hours by individual EN-C rats, with EN withheld, was used to pair feed identified individuals in one of the control diet-fed groups (C-PF). The restricted ration was delivered in the second half of the light phase. Rats in the remaining control diet-fed group (C-C)

were fed *ad libitum* throughout and killed in two sub-groups 24 hours after C-PF animals. Serum and plasma were prepared from trunk blood. Brains were frozen for *in situ* hybridisation. The following tissues were dissected and weighed: subcutaneous, epididymal, retroperitoneal, omental and mesenteric white adipose tissue (WAT), interscapular brown adipose tissue (IBAT) and liver. IBAT was dissected free from any visible adhering WAT prior to weighing.

2.2 Blood parameters

Serum leptin concentrations were measured using a rat-specific radioimmunoassay kit (Linco; RL-83K, Biogenesis, Poole, Dorset, U.K.). Plasma insulin was measured using a rat-specific radioimmunoassay kit (Linco; RI-13K; Biogenesis). Plasma glucose was determined using the KONE analyser.

2.3 Gene expression

Expression of energy balance and feeding related genes was quantified in the hypothalamus or forebrain using *in situ* hybridisation, as described in detail elsewhere [3,4,7,8]. Riboprobes complementary to partial fragments of neuropeptide Y (NPY), agouti related peptide (AgRP), proopiomelanocortin (POMC), cocaine and amphetamine-regulated transcript (CART), leptin receptor (ObRb), dynorphin (DYN), enkephalin (ENK), suppressor of cytokine signalling-3 (SOCS3), melanocortin-3 receptor (MC3R), dopamine D_2/D_3 receptors (D_2R, D_3R), and cannabinoid type 1 receptor (CB_1R) were generated from cloned cDNAs. Brain sections (20 micron) were collected from the caudal extent of the hypothalamic arcuate nucleus (ARC) rostrally onto a set of 10 slides and from the forebrain caudally onto two sets of 10 slides. Sets of slides spanned the ARC from approximately –4.52 mm to –2.30mm, and the

forebrain, from 2.28mm to 0.36mm relative to Bregma [10]. Sections were fixed, acetylated (except for POMC, CART and NPY), and hybridised overnight at 58°C using ^{35}S-labelled antisense riboprobes (10^7 dpm/ml). Processed slides were apposed to Kodak Biomax MR film (Sigma, UK). Autoradiographic images were quantified using a computerised image analysis system, the Image-Pro Plus system (Media Cybernetics, Maryland, USA). Scanned images from sheet film were analysed at the level of individual nuclei or identified forebrain regions, measuring the intensity and area of the hybridisation signal. The integrated intensity of the hybridization signal was then computed using set parameters and standard curves generated from ^{14}C autoradiographic microscales (Amersham, UK). Image analysis was performed on 4 or 5 representative sections from each brain region, by an observer blind to the treatment groups.

2.4 Statistical analysis

Mean values are reported ± S.E.M. and statistical significance was set as $P<0.05$. Tissue weights, blood parameters and gene expression were analysed by two-way ANOVA using SigmaStat v3.1 (Jandel Corporation, Ergath, Germany), with dietary manipulation and time (since withdrawal of Ensure) as factors. For simplicity, ANOVA results use the terminology 'effect of diet' and 'effect of time'. Energy intake and body weight gain data were analysed by two-way RM ANOVA. Due to the staggered termination of the *in vivo* study in *Study 2* (EN-C, C-PF, C-C), energy intake and body weight gain were realigned over the final 4 days for each experimental group and compared separate from the pre-withdrawal data.

3. Results

3.1 *In vivo* measures

Study 1: Supplementation with EN (EN-C) resulted in an immediate and sustained increase in total energy intake (Fig. 1A). Intake was maximal during the initial 2 weeks following supplementation before declining to a plateau that represented an excess energy intake of approximately 20% compared to C-C. Withdrawal of EN resulted in an immediate reduction in energy intake to 36% of C-C intake or 31% of the final supplemented level, before intake recovered slowly over a period of 3-4 weeks. Energy intake was elevated in EN-C rats (n=20) between day 32 and day 101 (P generally <0.001, but NS on days 51, 64-66, 74, 78, 85, 86, 97 and 102; Fig. 1A – significant difference indicated by filled bar), but was depressed relative to C-C between day 103 and day 116 (P generally <0.001 – shaded bar), but not from day 120 onwards, as energy intakes converged. Over the 10 weeks of supplementation, EN induced a body weight differential of 110g (Fig. 1B). Body weight gain differed between the EN-C and C-C groups from day 59 to day 111 (Fig. 1B – filled bar; including P<0.001 from day 79 to day 106), but not from day 112 onwards. The recovery in energy intake in the EN-C group combined with the maintenance of normal intake and weight gain in the C-C group, resulted in approximately 62% of body weight differential between the two groups being lost by the end of the study.

Based on these findings, sampling points of 24h and 4-days following EN withdrawal were selected for further study. Samples at the 24h time point correspond to maximal (acute) restriction, i.e. >60% vs C-C, whereas the 4-day time point corresponds to the last day of at least 50% energy restriction vs C-C. Although energy intake remains supressed beyond this period, daily energy intake gradually increases and dilutes the average restriction level.

Study 2: Total energy intake of the EN-C group (n=15) during EN supplementation was 18% higher than in the control groups (Fig.2A), with 84% of ingested calories taken as EN. As in *Study 1*, there was an immediate reduction in calorie intake when EN was withheld, from around 100kcal to 37kcal during the first 24h. Over the following 3 days, caloric intake increased only slowly, rising to approximately 50kcal by day 4 (Fig. 2A). Energy intake was elevated in EN-C rats compared to C-C from day 41 to day 114 ($P<0.05$, but NS on days 63, 70, 78, 90 and 97; Fig. 2A – filled bar). Over the final 4 days of the study, daily energy intake was depressed in both EN-C and C-PF rats compared to C-C rats (all $P<0.001$; Fig. 2A – shaded bar). Ensure supplementation gave rise to a body weight differential of c. 100g immediately prior to the withdrawal of EN from the EN-C group (Fig. 2B). Voluntary hypophagia in EN-C rats gave rise to body weight losses of 7.7g (1.4%) and 19.6g (3.5%) after 24h and 4 days, respectively (Fig. 2B). By comparison, imposed restriction to the same caloric intake in the C-PF group resulted in losses of 15.4g (3.4%) and 34.7g (7.5%) after 24h and 4 days, respectively. Body weight gain differed between the EN-C and C-C groups (EN-C>C-C) from day 54 to day 120 (mostly $P<0.001$; Fig. 2B – filled bar), and remained higher in EN-C compared to both C-C and C-PF following the withdrawal of Ensure.

3.2 Post mortem tissue weights

There were effects of diet (all $P<0.001$), but not time, for each dissected tissue (Table 1; n=15). WAT weights were higher in EN-C groups compared to C-C and C-PF groups at both time points (all $P<0.001$), but there were no differences between the latter two groups. IBAT weights were higher in EN-C than in C-PF at 24h and 4 days

(P<0.001 and P<0.05, respectively). Liver weight was reduced in C-PF groups compared to C-C and EN-C, at both time points (all P<0.001).

3.3 Blood hormones and metabolites

There were effects of diet on all blood parameters (all P<0.01; n=15), but no effects of time (Table 2). Blood glucose was lower overall in C-PF rats (P<0.001 vs EN-C; P<0.05 vs C-C), with low concentrations at 24h being normalised by day 4. The rank order of insulin concentrations was C-C>EN-C>C-PF, with C-C levels being higher than EN-C (P<0.05) and C-PF (P<0.001). Leptin levels also differed with all diet treatments, reflecting level of adiposity and energy balance state (EN-C>C-C>C-PF); statistical outcomes were very similar at 24h and 4 days.

3.4 Gene expression

There were treatment effects on expression of three of the ARC genes (Fig. 3; n=15). The expression profiles for NPY and AgRP were broadly similar, with effects of time for both orexigenic genes (day 4>24h; P<0.05 and P<0.01, respectively), and an effect of diet for AgRP (P<0.05). For NPY (Fig. 3A), expression levels were elevated at day 4 (vs 24h) in both EN-C and C-PF groups (P<0.05), whereas for AgRP (Fig. 3B), expression levels were higher overall in C-PF rats compared with EN-C rats (P<0.05), and also increased with time in C-PF groups (P<0.05). There were no effects on expression of the anorexigenic gene, POMC (data not shown), although there was a trend towards an effect of diet on CART gene expression (Fig. 3C; P=0.057), where mRNA levels in the EN-C groups were elevated relative to the C-C and C-PF groups. There was an effect of diet on SOCS3 gene expression (Fig. 3D; P<0.001), due to suppression of mRNA levels with pair-feeding (C-PF vs C-C, C-PF vs EN-C, both

P<0.001), an effect which was also evident at both 24h and 4 days (all P<0.001 or P<0.01). There was also a difference between the C-C and EN-C groups at day 4 (EN-C>C-C, P<0.05). There were no effects on ObRb or MC3R expression in the ARC, or on expression of D_2R, D_3R, DYN, ENK or CB_1R genes in the nucleus accumbens.

4. Discussion

We assessed hypothalamic and forebrain gene expression correlates of restricted intake and weight loss when palatable diet was withheld following the establishment of DIO on that diet (EN-C), and compared responses to those of pair-fed rats fed on control diet throughout (C-PF). The phenomenon of hypocaloric intake following withdrawal of palatable diet has been reported in a number of different models, most of them involving solid, cafeteria-type diets [e.g. 5,6,11,12], but in the case of the EN model in the SD rat, the use of a palatable liquid has served to highlight the fact that not all palatable diets induce these 'withdrawal-type' effects – body weight is defended and food intake only minimally disturbed by withdrawal of HE pellet diet [2,3,4]. Although the withdrawal phase and attendant hypocaloric intake has been investigated in the context of anxiety or stress responses [6, 13] or addiction-like responses [12], our focus in the current study was on acute regulation of genes related to energy balance, leptin signalling and food reward, and the longitudinal development of responses over the period of maximal intake suppression following diet transition. The hypothalamic changes observed were subtle, but co-ordinated. EN-C rats exhibited regulatory changes in energy balance genes that appeared designed to counter DIO, but these had dissipated by day 4 following withdrawal, with cumulative hypocaloric intake minimising hypothalamic perturbation (vs C-C) by this time point, with the exception of SOCS3 gene expression. By contrast, C-PF

rats had a hypothalamic profile characteristic of negative energy balance, with complementary opposite changes in orexigenic (NPY, AgRP) and anorexigenic (CART) peptides, and low levels of SOCS3 gene expression.

For the EN-C group, the experimental protocol represented a simplified dietary manipulation compared to those frequently employed with the SD rat DIO model [2,4,14] - a regime limited to provision and subsequent withdrawal of EN. The *in vivo* outcomes were typical of similar manipulations described previously [2,4,9,14]. Long-term supplementation with EN, and elevated caloric intake (Figs 1A and 2A), gave rise to substantial additional body weight gain (Figs 1B and 2B), and increased adiposity (Table 1). The proportion of total caloric intake taken as EN was also in line with previous reports [4,7,14]. Withdrawal of EN resulted in a sustained reduction in energy intake [4,14], and reduction in body weight, although relative overweight persisted for many weeks. The hypocaloric intake response to withdrawal of cafeteria-type diets has been shown to be quite variable in magnitude and duration, with energy intake remaining depressed for between 1 and 3 weeks [5,6,11,12]. The data generated from *Study 1* and *Study 2* highlight the robust nature of the Ensure-SD rat model. Whereas gene expression responses in the C-PF group were due solely to ongoing food restriction, both previous dietary history and current energetic status (voluntary restriction) would have contributed to responses in the EN-C group. Energy intakes were matched in the EN-C and C-PF groups from the point of withdrawal of EN, but the relative reduction in energy intake was greater in the EN-C groups. Nevertheless, weight loss outcomes were more severe in the C-PF group, presumably reflecting differences in body composition. Another contribution to this differential response to the restricted but matched caloric intake could be that the C-

PF group received their ration within a particular time window (second half of light phase), rather than consuming it in an *ad libitum* pattern, as in the other two groups. This makes it likely that there was no food available in the second half of the dark phase, possibly accentuating the effect of the overall negative energy balance on C-PF outcome measures at the time of tissue sampling.

The genes selected for analysis in the EN-withdrawal/PF phase include energy balance genes such as NPY, AgRP and CART, which are regulated by food deprivation and food restriction [15,16,17,18], whereby orexigenic genes (e.g. NPY, AgRP) are up-regulated and anorexigenic genes (e.g. POMC, CART) are down-regulated. This creates a hypothalamic drive that favours the restoration of energy balance through reduced energy expenditure and hyperphagia once a normal food supply is restored. Some of these genes also exhibit apparent counter-regulatory changes in the positive energy balance state of DIO (e.g. NPY, AgRP, leptin receptor, MC3R [7,8], CART [4,19], melanocortin system [20]). Additional genes relevant to leptin signalling (SOCS3), and dopaminergic, opioid and cannabinoid systems in forebrain food reward centres [21,22] were also included. Analysis of hypothalamic gene expression suggested coordinated regulation of the orexigenic peptides, NPY and AgRP, opposite trends for the anorexigenic peptide, CART, and changes in the suppressor of cytokine signalling, SOCS3 (Fig. 3). Between 24h and 4 days there was only a gradual increase in energy intake, accompanied by continuing weight loss, and overall increases in expression of both NPY and AgRP. These differences appeared to reflect at least two processes, the normalisation of suppressed expression in EN-C rats, and up-regulation in C-PF rats as imposed cumulative energy deficit increased. It is notable that the gene expression changes in the current study are quite modest in

magnitude compared with some of those reported with complete food deprivation or long term food restriction [15,16,17,18], although they are more in line with reported responses to DIO and positive energy balance [4,7,8,19,20], the difficulty in comparing studies and quantitative methodologies notwithstanding. Since palatability-induced over-consumption of calories and variable short-medium term caloric restriction are arguably more representative of common human experiences, and as drivers of our own regulatory processes, more knowledge is required of the relevance of such subtle changes. There was no effect of time on either adipose tissue weights (Table 1) or leptin levels (Table 2), either between the EN-C groups, or between the C-PF groups. Significantly, similar preservation of adipose tissue was observed when designated 'DIO-resistant' SD rats made obese on a HE+EN diet were transferred back to control diet for 2 weeks; rats maintained their elevated adipose tissue mass and leptin levels despite a 60% reduction in energy intake and 7% weight loss [2]. Reduced caloric intake, and hypoglycaemia in the C-PF group, over the initial 24h of restriction did not have a major effect on expression of genes other than SOCS3. Initial depression of expression of orexigenic genes in EN-C DIO animals is consistent with a pre-existing feedback from excess adipose tissue, i.e. counter-regulatory in action [4,7,8], although is interesting that this effect was dissipated by day 4, even though energy intake remained low, and body adiposity and leptin levels unchanged. Thus the trajectories of changes in NPY and AgRP gene expression were similar in EN-C and C-PF groups ingesting the same calories from control diet but coming from different dietary backgrounds, and with contrasting body composition.

Marked differences were seen between the EN-C and C-PF groups with respect to SOCS3 gene expression, which was suppressed in the latter group at both time points,

along with insulin and leptin (Table 2). This is consistent with results from fasting studies in rats [23], implicating hypoleptinaemia rather than low levels of energy intake, and suggesting that SOCS3 gene expression is a more acute marker of food restriction than NPY and AgRP. SOCS3 was elevated in hyperleptinaemic EN-C rats after 4 days, in line with other data from DIO rodents [e.g. 24], and suggestive of leptin resistance [25,26,27], although the absence of equivalent change at 24h, when leptin levels were also elevated, was unexpected. As with the neuropeptides, expression levels of SOCS3 presumably represent the integration of responses to high background leptin levels that reflect body adiposity and the depressive effect of low voluntary food intake.

The hypothalamic energy balance system does appear to differentiate between voluntary and imposed caloric restriction, with ongoing voluntary restriction in EN-C animals pushing hypothalamic indices that reflect DIO back toward a normal baseline, whereas the same level of imposed restriction in C-PF animals cumulatively drives gene expression towards a compensatory anabolic profile. However, whereas the hypothalamic gene expression profile (coupled with hyperleptinaemia) 24h after withdrawal of EN seems likely to be responsible for driving down food intake, this relationship appears to have broken down by day 4. This would not be predicted from the outcome of *Study 1*, in which energy intake took several more weeks to recover, with body weight continuing to decline, albeit at a slower rate, across this period, and ultimately settling above the C-C level. By contrast, the trajectories of gene expression in the C-PF groups between 24h and day 4 reflected mounting negative energy balance, with SOCS3 mRNA and blood leptin levels remaining depressed. The longevity of counter-regulatory hypothalamic changes in DIO animals following

return to a control diet has not been studied systematically, although analysis in animals that had been returned to control diet for 3 weeks revealed an increase in NPY and a decrease in CART [4], relative to HE- or HE+EN-fed rats, indicative of normalisation of such DIO-related changes. Analysis of hypothalamic neuropeptide gene expression in a cafeteria DIO model provides further evidence of up-regulation of NPY gene expression upon return to control diet [6]. Sixteen weeks on a cafeteria diet resulted in a body weight differential in excess of 100g, driven by a doubling of daily energy intake. Withdrawal of cafeteria diet reduced energy intake to a level half that of controls, and which increased only gradually over 9 days of control diet feeding. Ventral hypothalamus NPY gene expression was significantly higher 9 days after withdrawal than in rats that continued to be fed the cafeteria diet [6]. As in the SD rat model of DIO [4], this outcome could reflect the normalisation of expression levels that had been suppressed by developing DIO.

In addition to apparent 'recognition' of excess body weight and adiposity, another possible influence on feeding behaviour upon transfer back to the less palatable control diet is successive negative contrast (SNC; [5]). This effect sees animals transferred from a more- to a less-preferred reward perform worse (i.e. in this case, consume less calories) than animals that have only ever received the less preferred reward. SNC, as it relates to feeding, is observed whether the food reward is presented in solid or liquid form [28]. The relative importance of adiposity recognition and SNC has been assessed by feeding rats a restricted ration of a cafeteria-type diet to prevent development of DIO, prior to transfer back to chow [5]. This manipulation suggested that the hypocaloric episode ascribed to SNC was less pronounced and of shorter duration (5-7 days) than the counter-regulatory effect of elevated body weight (3-4

weeks) observed in DIO rats. Whereas calories in liquid form may contribute to the level of over-consumption through reduced satiety and less accurate compensation [29], this may not be directly relevant upon withdrawal where the forces in play (body composition and SNC) are likely to be independent of the physical form of the historically consumed calories. Although the current study was not designed to examine the phenomenon of SNC directly, the time scale of normalisation of gene expression observed is closer to that presumed for SNC [5], although this seems an unlikely relationship. The hedonic, or reward, system is a more likely candidate for any SNC effect [30], although our examination of dopamine receptor, cannabinoid receptor and opioid genes in the nucleus accumbens at both 24h and 4 days, found no evidence of perturbations in expression of these systems, despite studies showing that striatal opioid gene expression may be regulated following EN feeding [31], and ventral tegumental area up-regulation of dopamine receptor expression on transfer to cafeteria diet [6].

5. Conclusions

The defence of body weight and regulation of body adiposity, whether through set point or some other model, remain areas of active debate [32]. The relationships between diet composition and palatability, the development and severity of DIO, and the persistence of elevated body weight once dietary transitions are reversed, are poorly understood. However, it is apparent that some diets induce body weight gain that will be defended completely when dietary palatability is reduced, e.g. on return to control diet, whereas others give rise to additional body weight that is only partially defended. Here we have obtained foundation data on the hypothalamic correlates of acute hypocaloric intake in one of these models, on withdrawal of EN in the SD rat.

This manipulation is known to result in a failure to defend elevated DIO body weight in unselected populations [4], in selected DIO-resistant rats [2], and in selectively-bred DIO-sensitive rats [14]. The current study suggests that 24h after withdrawal of EN, hypothalamic gene expression primarily reflects 'inappropriate' body composition, i.e. recognition of excess body adiposity, and removal of the overriding stimulus of the palatable diet allows suppression of food intake. However, by day 4, although caloric intake is still suppressed, downward body weight trajectory is apparently sufficiently well established for the compensatory hypothalamic profile to dissipate. Pair-feeding control rats to the level of energy intake voluntarily consumed by animals coming off of EN results in neuropeptide gene expression changes with a similar trajectory, but which reflect cumulative negative energy balance, with the minimal effect at 24h being augmented by 4 days. These findings, and additional major differences in effect on SOCS3 gene expression, support the contention that energy balance systems differentiate between voluntary and imposed negative energy balance where energy intakes are identical, but dietary history, body weight and body composition are contrasting. The subtle nature of some of the changes in gene expression with the food withdrawal manipulation underlines the need for more investigation of the regulation of hypothalamic and food reward systems beyond substantial challenges such as complete food deprivation.

6. Acknowledgements

This work was funded by the European Commission, Quality of Life and Management of Living Resources, Key action 1 'Food, nutrition and health' programme (QLK1-2000-00515), and the Scottish Government. The authors thank Dr. Vernon Rayner for technical assistance.

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8. Figure legends

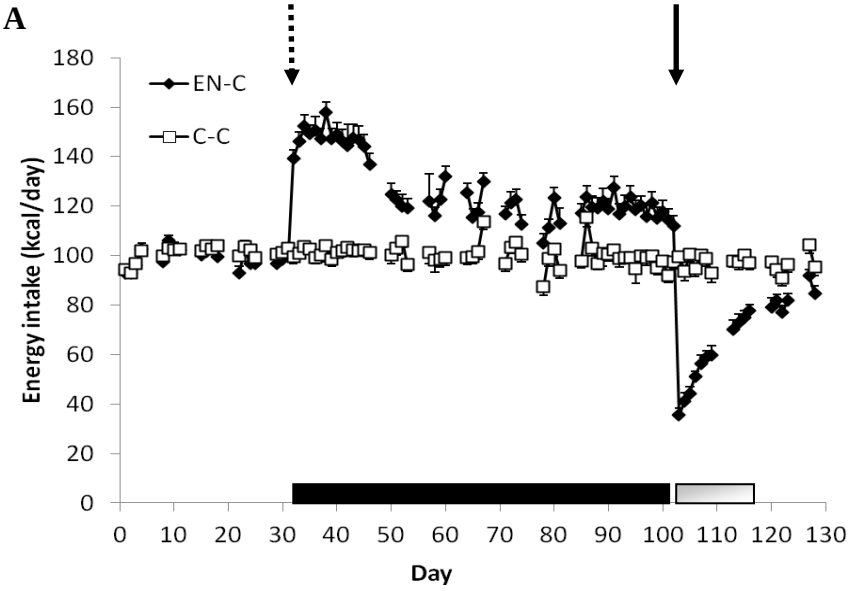
Figure 1. A. Energy intake (kcal/day) and B. body weight gain (g) of rats in *Study 1*. Rats were either fed control diet only throughout (C-C), or were fed control diet for 30 days followed by control diet supplemented with Ensure (EN) for 70 days, after which EN was withheld (EN-C), and control diet only was available for a further 28 days. Data are mean±SEM (n=20/group). Points of diet transition are indicated by vertical arrows (broken arrow, introduction of EN supplement; solid arrow, withdrawal of EN). The filled and shaded horizontal bars on A indicate phases of hypercaloric and hypocaloric energy intake, respectively, in EN-C rats. The filled horizontal bar on B indicates the period of significant body weight differential between groups.

Figure 2. A. Energy intake (kcal/day) and B. body weight gain (g) of rats in *Study 2*. Rats were either fed control diet only throughout (C-C), or had control diet supplemented with EN for 73 days, after which EN was withheld and control diet only was available *ad libitum* for a further 24h or 4 days (EN-C). A third group of rats was also fed control diet only *ad libitum*, but individuals were then pair-fed (C-PF) to the weight of control diet eaten during the previous 24 hours by individual EN-C rats. Data shown are for the C-C, EN-C and C-PF groups at the 4 day time point, and are mean±SEM (n=15/group). Points of diet transition are indicated by vertical arrows (broken arrow, introduction of EN supplement; solid arrow, withdrawal of EN). The filled and shaded horizontal bars on A indicate phases of hypercaloric and hypocaloric energy intake, respectively, in EN-C rats. The filled horizontal bar on B indicates the period of significantly elevated body weight in the EN-C group.

Figure 3. Gene expression for A. NPY, B. AgRP, C. CART and D. SOCS3 in the hypothalamic arcuate nucleus (ARC) of rats from *Study 2* subjected to the dietary manipulations detailed in the legend to Figure 2. Data are mean±S.E.M (n=15/group), and are expressed as a percentage of C-C 24hrs. Black lines depict significant differences between groups, $P<0.05$; * $P<0.01$ or <0.001 vs other groups at same time point. Autoradiographs are representative images showing gene expression for A. NPY, comparing C-PF rats at 24h and 4-day time points, B. AgRP, using adjacent sections to those hybridised for NPY, C. CART, comparing C-C and EN-C at the 24h time point, and D. SOCS3, comparing C-C and C-PF at the 24h time point.

FIGURE 1

A


EN-C
C-C
Energy intake (kcal/day)
Day

B

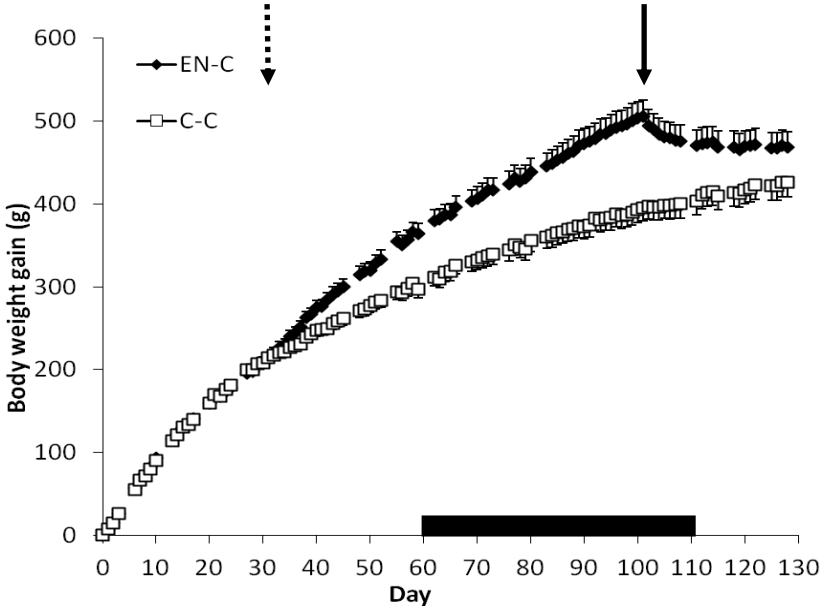
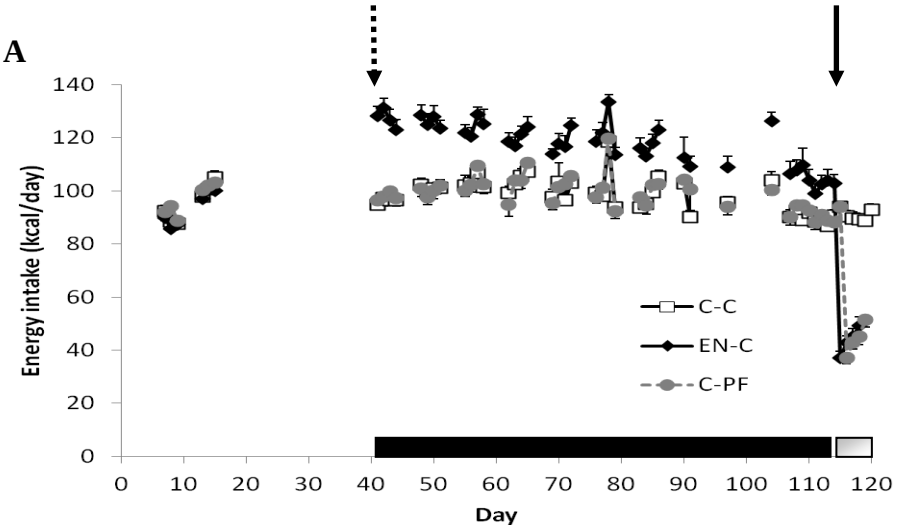

EN-C
C-C
Body weight gain (g)
Day

FIGURE 2

A


Energy intake (kcal/day)
140
120
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100 110 120
Day
C-C
EN-C
C-PF

B

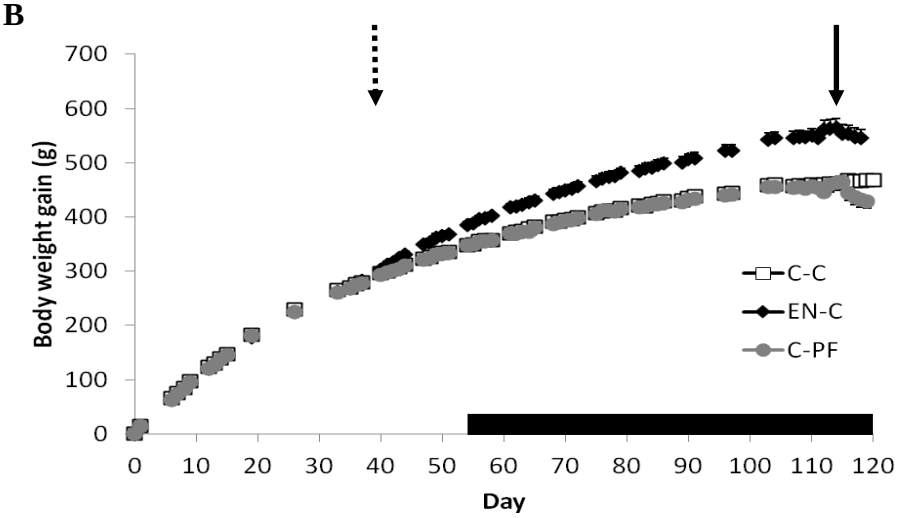
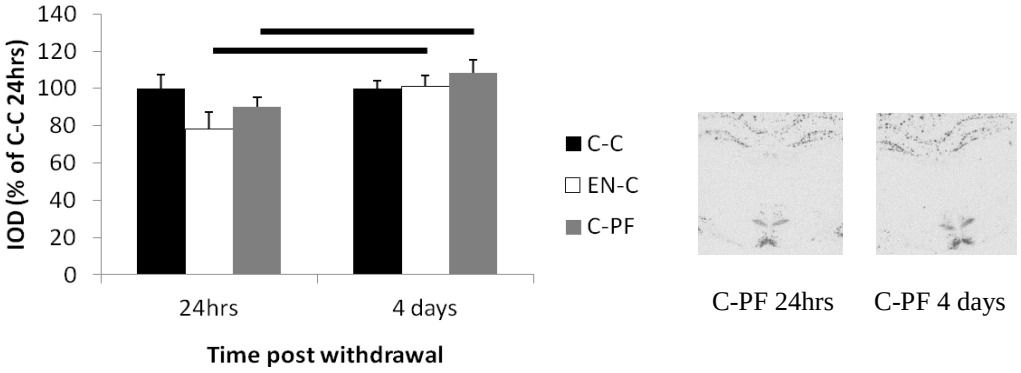
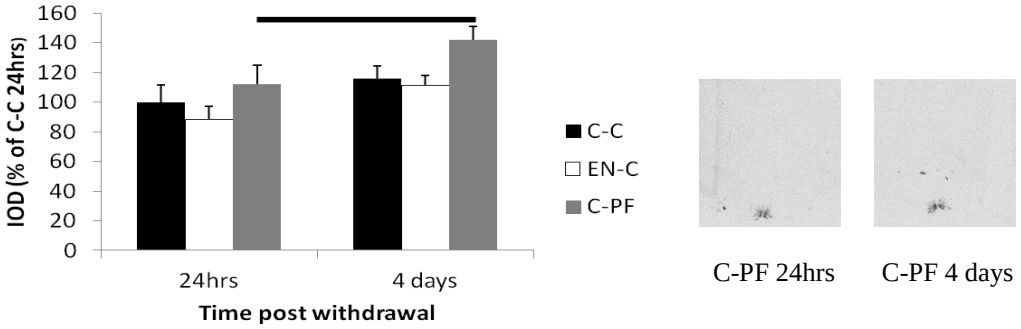

Body weight gain (g)
700
600
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90 100 110 120
Day
C-C
EN-C
C-PF

FIGURE 3

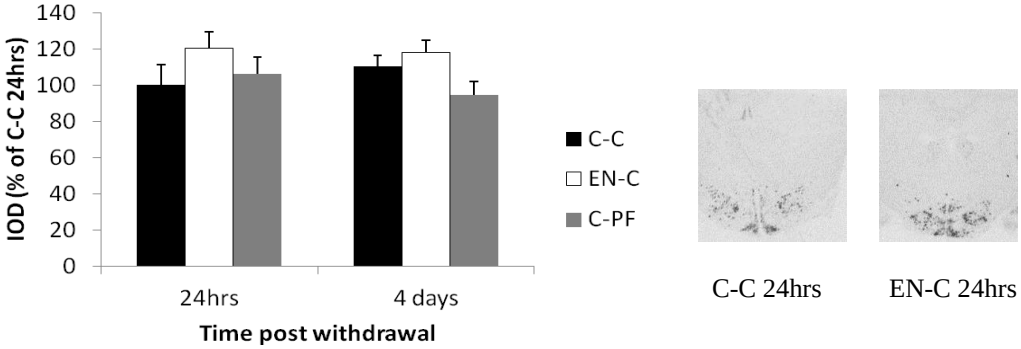
A NPY



B AgRP



C CART



D SOCS3