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Mice that are resistant to diet-induced weight loss have greater food anticipatory activity and altered melanocortin-3 receptor (MC3R) and dopamine receptor 2 (D2) gene expression

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ABSTRACT

Diet-induced weight loss varies considerably between individuals, but the mechanisms driving these individual differences remain largely unknown. Here we investigated whether key neuropeptides involved in the regulation of energy balance or reward systems were differentially expressed in mice that were prone or resistant to caloric restriction (CR) induced weight loss. Mice (n = 30 males and n = 34 females) were fed 70% of their own baseline ad libitum intake for 25 days, after which their brains were collected and expression of various neuropeptides were investigated and compared between the 10 male and 10 female mice that showed the greatest (high weight loss, HWL) or lowest weight loss (LWL) (n = 40 in total). HWL mice showed a differential neuropeptide profile to LWL in both sexes, characterised by increased expression of neuropeptide Y (NPY), agouti-related peptide (AgRP), leptin receptor (ObRb), and melanocortin 3 receptor (MC3R) in the arcuate nucleus. No changes in the expression of fat mass and obesity related gene (FTO) or suppressor of cytokine signalling 3 (Socs3) were observed. Levels of dopamine D2 receptor were decreased in the nucleus accumbens in HWL compared to LWL mice. HWL mice showed a stronger increase in food anticipatory activity (FAA) in response to CR than LWL mice. These results indicate that the mice prone to diet-induced weight loss experienced greater hunger, potentially driving their elevated FAA.

Introduction

The most common self- and clinician administered intervention to achieve loss of excess body weight (fat) is caloric restriction (i.e., dieting, CR). CR involves adequate intake of micronutrients, but reduced energy intake, and in theory should lead to reductions in body and fat mass because individuals are forced into negative energy balance and must supply the shortfall between supply and demand from their body tissue reserves. Individual responses to this intervention vary enormously (Astrup et al., 1995; Mutch et al., 2007; Sorbris et al., 1982). For instance, in a dietary intervention consisting of 36 weeks provision of a 4.2 MJ/day low-fat high carbohydrate diet, weight loss varied from 4 to 22 kg between the subjects studied (Astrup et al., 1995). Whether an individual is prone or resistant to weight loss may be the result of differences in genetic and/or physiological factors between individuals (Astrup et al., 1995; Mutch et al., 2007; Sorbris et al., 1982). For instance, reasons for unsuccessful weight loss may include pre-existing differences in metabolic parameters between individuals, like energy expenditure, capacity for fat oxidation, insulin sensitivity and/or behavioural risk factors, such as low general activity or a tendency to gorge (Astrup et al., 1995; Blundell et al., 2005; Hambly et al., 2007a; Mutch et al., 2007; Pavlou et al., 1986). Another major factor involved in the variation in weight loss under CR is the amount of compensation that occurs in energy expenditure that can offset the caloric deficit (Hambly and Speakman, 2005). The variability in response to dieting that is observed in humans is also observed in mice (Gelegen et al., 2006; Vaanholt et al., 2012). Mice exposed to 30% CR varied in weight loss between 0.3 and 17.3 g of body mass and a large part (69%) of this variability in diet-induced weight loss could be predicted by differences in baseline food intake, resting metabolic rate, body mass and activity, together with compensatory changes in activity in response to CR (Vaanholt et al., 2012). Initial food intake and compensatory changes in activity were the strongest predictors in this model and together explained 57% of the variation in diet-induced weight loss.

Food intake and energy expenditure (e.g., levels of physical activity) are under neuroendocrine control (Magni et al., 2009; Sánchez-Lasheras et al., 2010). This homeostatic control of energy balance involves endocrine signals from the periphery, like leptin and insulin that provide information on the nutritional state and energy level of the animal, which

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are received and integrated in the brain stem and various nuclei in the hypothalamus. These stimulate the expression of various neuropeptides which then impinge on receptors elsewhere in the brain. These include orexigenic (e.g., neuropeptide Y, NPY; and agouti-related peptide, AgRP) and anorexigenic (e.g., proopiomelanocortin, POMC; and cocaine-, and amphetamine-regulating transcript, CART) neuropeptides in the arcuate nucleus (ARC) interacting with for example melanocortin receptors in the paraventricular nucleus (see for instance: Jeong et al., 2014; Koch and Horvath, 2014; Schwartz et al., 2000). In addition to these homeostatic mechanisms, reward-associated neurotransmitters have also been implicated in the control of energy balance and are thought to potentially override homeostatic control in some situations (Magni et al., 2009; Sánchez-Lasheras et al., 2010). An essential role for dopaminergic signalling in maintaining normal feeding behaviour has been shown; i.e., dopamine-deficient mice die of starvation at 3–4 weeks of age (Zhou and Palmiter, 1995). Dopamine D2 receptor availability is decreased in obese individuals in proportion to their body mass index (BMI) (Wang et al., 2001), mutant mice with an obese phenotype show reduced dopamine D1 receptor expression (Zhang et al., 2014), and treatment of leptin-deficient mice with dopamine D1/D2 receptor agonists normalizes the phenotype of hyperphagia and body weight gain (Bina and Cincotta, 2000). An important role for the dopaminergic system in regulating goal-directed behaviour, i.e., food anticipatory activity (FAA), and timing of these behaviours has also been suggested based on evidence that the total amount of activity prior to mealtime is reduced following acute treatment with D1 and D2 specific antagonists (Liu et al., 2012; Mistlberger and Mumby, 1992). In addition, it has been shown that activation of dopamine D2 receptors can shift the onset of FAA in rats maintained in LD (i.e., light–dark cycle) or LL (i.e., continuous light) (Smit et al., 2013), indicating that signalling at dopamine D2 receptors is involved in the phase control of food entrainable oscillators (FEOs) responsible for circadian food anticipatory rhythms. Interestingly, it has recently been shown that the melanocortin 3 receptor (MC3R) is expressed in up to a third of dopaminergic neurons of the ventral tegmental area (VTA, Lippert et al., 2014). Projections from the VTA to the nucleus accumbens (NAcc) in the mesolimbic area are well characterised regarding their role in drug addiction and the rewarding aspects of food (Sánchez-Lasheras et al., 2010). Because arcuate AgRP and NPY neurons are known to innervate and regulate VTA signalling, the MC3R in dopaminergic neurons provides a specific input for communication of nutritional state within the mesolimbic dopamine system. The link between MC3R and dopaminergic neurones may indicate that previous evidence showing important roles for both MC3R (Sutton et al., 2008) and the dopaminergic system (Smit et al., 2013) on FAA is part of the same regulatory system.

We hypothesised that differences in the expression of neuropeptides and their receptors involved in the homeostatic control of energy balance and reward systems form the basis of individual variation in diet-induced weight loss. The aim of the present study was to explore the differences in metabolic and physiological factors and expression of neuropeptides, and their receptors, in the brains of individual mice that were prone or resistant to CR-induced weight loss.

Methods & procedures

Animals and housing

Male and female (30 males and 34 females) MF1 mice, obtained from Charles River UK (Kent, UK) at 8 weeks of age, were housed individually in standard plastic cages ($23 \times 33 \times 18$ cm^3) with sawdust and paper shreds for bedding. Mice had ad libitum access to food (D12450B, 10% fat, Research Diets, USA) and water and were maintained in a temperature controlled room (21 ± 1 °C) under a 12:12-h light–dark cycle, with lights on at 6:00 h and a "dawn/dusk" period of 20 min at either end of the light period.

At 21–22 weeks of age all mice were implanted intraperitoneally with temperature transmitters (PDT-4000 E-Mitter, Mini Mitter Company Inc., USA) under general anaesthesia (mixture of isoflurane and oxygen). Mice were allowed at least 3 weeks to recover from the surgery before the start of the experiment. All procedures concerning animal care and treatment were approved by the ethical committee for the use of experimental animals of the University of Aberdeen, and licenced by the UK Home Office (Project license PPL60/3706).

Experimental procedure

Baseline measurements started at the age of 25 weeks and were taken over a period of 20 days. During the baseline phase mice had ad libitum access to food and water (-20 to -1 days) and body mass and food intake were measured each day 1 h before lights off (between 16:00–17:00 h). Food intake of all mice was then restricted to 70% of their individual baseline intake for a period of 25–26 days (CR phase; days 0–26, on average mice received 3.1 ± 0.3 g). During the CR phase, mice were weighed every day before providing them with their daily food ration in their cage (between 16:00–17:00 h).

Body temperature and general activity

Mice in their home cages were placed onto transponder energizers (ER-4000 Receiver, Mini Mitter Company Inc., USA) for periods of 4 days at a time allowing us to non-invasively monitor body temperature and general activity at various stages during the protocol. The VitalView™ Data Acquisition System (Mini Mitter Company, Inc., USA) was used to collect the data in 1 minute intervals (for a detailed description see Harkin et al., 2002). For each phase, i.e., baseline, CR1 (days 0–2), CR2 (days 10–13), CR3 (days 22–25) mean body temperature and activity (number of counts) were calculated per day. FAA was determined by calculating the amount of activity in the 3 h prior to food provision (13:00–16:00) and was expressed as a % of total 24 h activity.

Basal metabolic rate

Basal metabolic rate (BMR) was determined in all animals during baseline (-4 ± 2 days) and after 22 ± 2 days of CR. All measurements took place during the light phase between 8:00 and 16:00. Animals were fasted for 2.5 h prior to the baseline measurement to ensure mice were post-absorptive and to enable comparison between measurements during baseline and CR phase.

BMR was measured in an open-flow respiratory system (as described by Speakman and McQueenie, 1996). In short, fresh air was pumped (Charles Austin Pumps) through a sealed Perspex chamber (volume 885 ml) within an incubator (INL-401N-010, Gallenkamp) set at 30 °C (within the thermal neutral zone for these mice) (Speakman and Keijer, 2012). Mass-flow controllers (MKS Instruments UK Ltd., Cheshire, UK) provided 500–700 ml O_2 min^{-1} which was monitored using an Alexander Wright DM3A flow meter. Air leaving the animal chamber was dried using silica gel and 150 ml min^{-1} was passed through a gas analyser (Servomex Xentra). CO_2 was not absorbed prior to gas analysis as this maximizes the accuracy of energy expenditure measures (Koteja, 1996). Gas concentrations were measured continuously, and averaged values were stored every 30 s for 180 min. BMR was quantified as the oxygen consumption over the lowest 20 consecutive values (10 min interval) and corrected for temperature and pressure, using the appropriate equation (Hill, 1972). The data (in ml O_2 min^{-1}) were converted to energy equivalents using an oxycalorific value of 21.117 J ml^{-1} O_2, derived from the equation of Weir (1949) for a respiratory quotient (RQ) of 1 (Speakman, 2000). Mean body mass was calculated from mass before and after each run.

Gorging behaviour

Gorging behaviour was assessed during baseline and during the CR phase. During baseline, gorging tendency was measured in each mouse immediately after the respirometry measurement on day -4 ± 2 when mice had been fasted for approximately 6 h (i.e., 2.5 h fast + 3.5 h in respirometer). Animals were returned to their home cage and a known amount of food was provided. After 2 h the amount of food missing from the hopper in grams was calculated.

During days 10–15 of the CR phase gorging tendencies were determined by weighing the amount of food left 1 h and 2 h after food provision. Most animals ate all their allotted food within 2 h, so in the final analysis the amount of food eaten within the first hour after food provision, expressed as a % of the total amount of food available, was used.

Body composition

Fat mass and fat free mass (FFM) of mice was determined 3 times during the experimental protocol using dual energy X-ray absorptometry (DXA; PIXImus2 Series Densitometers with software version 1.46.007, GE Medical Systems Ultrasound and BMD, Bedford, UK); at day −2 (baseline), day 10 and day 25 of the CR phase. Mice were anaesthetised using a face mask which provided a mixture of isoflorane and oxygen for the duration of the scan (~3 min). The software enabled a region of interest (ROI) to be created to exclude the head with the mask from analysis (Hambly and Speakman, 2005). Data were corrected with a calibration formula specific to our machine that has been generated by the linear regression of fat content determined by DXA analysis, with the fat content as determined by soxhlet chemical extraction (see Johnston et al, 2005 for detailed description of the procedure).

After 25–26 days on CR (morning of days 26–27 between 10:00 and 13:00) all mice were euthanized and dissected into 20 body parts as described previously in Johnson et al., 2001. A blood sample was collected via cardiac puncture and plasma was stored at −80 °C until analysis of leptin levels (ELISA kit, Millipore, Hertfordshire, UK). Brains were frozen in an isopentane solution (Sigma, UK) on dry ice and stored at −80 °C until sectioning on the cryostat (Leica CM1950, Leica Biosystems, Milton Keynes, UK). Collected tissues were dried to constant weight in a drying oven at 60 °C.

Sectioning and in situ hybridisation

Frozen brains were mounted onto a specimen disc and coronal 20 μm sections of the hypothalamic region were cut using a cryostat (Leica, CM3050S, Milton Keynes, UK) and thaw mounted on poly-L-lysine-coated slides. 30 slides (with 5–7 sections on each slide) through the nucleus accumbens (NAcc) and hypothalamus were collected per animal, and they were numbered as 1–10, 11–20, and 21–30. For expression of dopamine receptor D2 in NAcc one slide was used and two slides numbered as 11 and 21 (or 12 and 22, etc) were used for each neuropeptide in the hypothalamus. The NAcc, and ARC and ventromedial area (VMH) of the hypothalamus were located using the atlas of the mouse brain (Franklin and Paxinos, 1997).

Riboprobe synthesis

Template DNAs, complimentary to the mRNA sequences of interest, were created by Dr. Sharon Mitchell. Fragments were amplified from mouse brain cDNA by polymerase chain reaction (PCR). 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 Miniprep kit (Qiagen, Germany) and linearised with appropriate restriction enzymes (Promega, Southampton, UK). All sequences were verified by Eurofins Sequencing Services (London, UK). The linearised DNA was transcribed using T7, T3 or SP6 RNA polymerase promoters matched to a restriction site contained within the vector. Riboprobes were labelled with ^{35}S-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 1×10^6 c.p.m ml^{-1} in hybridisation buffer. Antisense and sense riboprobes were tested for specificity and background binding.

In situ hybridisation

To prevent RNase contamination, labware was baked at 200 °C and all solutions made up in 0.1% diethylpyrocarbonate (DEPC). Sections were fixed in 4% paraformaldehyde/0.1 M phosphate buffer (PB) for 20 min on ice and washed in 2 × 0.1 M PB for 5 min each. To eliminate background signal, sections were first immersed in 0.1 mM triethanolamine (TEA) for 2 min before acetylation in 0.1 mM TEA/0.25% acetic anhydride for 10 min at room temperature. Sections were washed twice in 0.1 M PB for 5 min each time, dehydrated through increased concentrations of ethanol (50%, 70%, 90% and 100%) and dried under vacuum. The labelled probe was then pipetted onto baked cover slips, annealed to sections and sealed with DPX before an overnight hybridisation at 58 °C. Post-hybridisation, cover slips were removed by soaking slides in 4× saline–sodium citrate buffer (150 mM sodium chloride and 15 mM sodium citrate, pH 7; SSC). Non-specific hybridisation was dissociated by increasing the temperature, up to 60 °C for 30 min and unhybridised RNA was digested with RNase (Promega, Southampton, UK) for 30 min. Sections were desalted over a series of SSC solutions: 2 × SSC, 1 × SSC, 0.5 × SSC and 0.1 × 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-Aldrich, UK) for a length of time appropriate to each 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).

Data analysis

All data were tested for normality using the Kolmogorov–Smirnov test in SPSS (version 22) and when necessary data were log-transformed to obtain a normal distribution.

Mice of each sex were divided into three groups; 1) the 10 mice that lost least body mass on CR (low weight loss group, LWL), 2) the 10 mice that lost most body mass on CR (high weight loss group, HWL) and 3) intermediates (n = 10 in males and n = 14 in females; see also Fig. 1). Mean weight loss was calculated as the difference between mean body mass over days −6 to −1 and days 20 to 25. The mean weight lost was 2.3 ± 3.4% and 14.4 ± 3.0% in LWL and HWL females respectively and 3.0 ± 3.8% and 17.3 ± 3.2% in LWL and HWL males respectively. General linear models (GLMs) with sex (females vs. males), group (HWL vs. LWL) and sex × group (S × G) as fixed factors were used to investigate differences between HWL and LWL groups at baseline. Where appropriate, body mass (or fat mass) was added as a covariate in these tests. In addition, compensatory responses in variables were investigated using repeated measures GLM, where sex and group were added as between-subjects factors and time (i.e., baseline vs. CR phase) was added as within-subjects factor. For variables that were related to body mass, residuals were used in these analyses. Effect sizes for all GLMs were computed in SPSS (partial eta squared, η_P^2).

Linear regressions were performed for some variables, in this case data for all mice (n = 64) were used unless stated otherwise (i.e., HWL, LWL and intermediates). All tests were two-tailed and significance was set at $p \leq 0.05$.

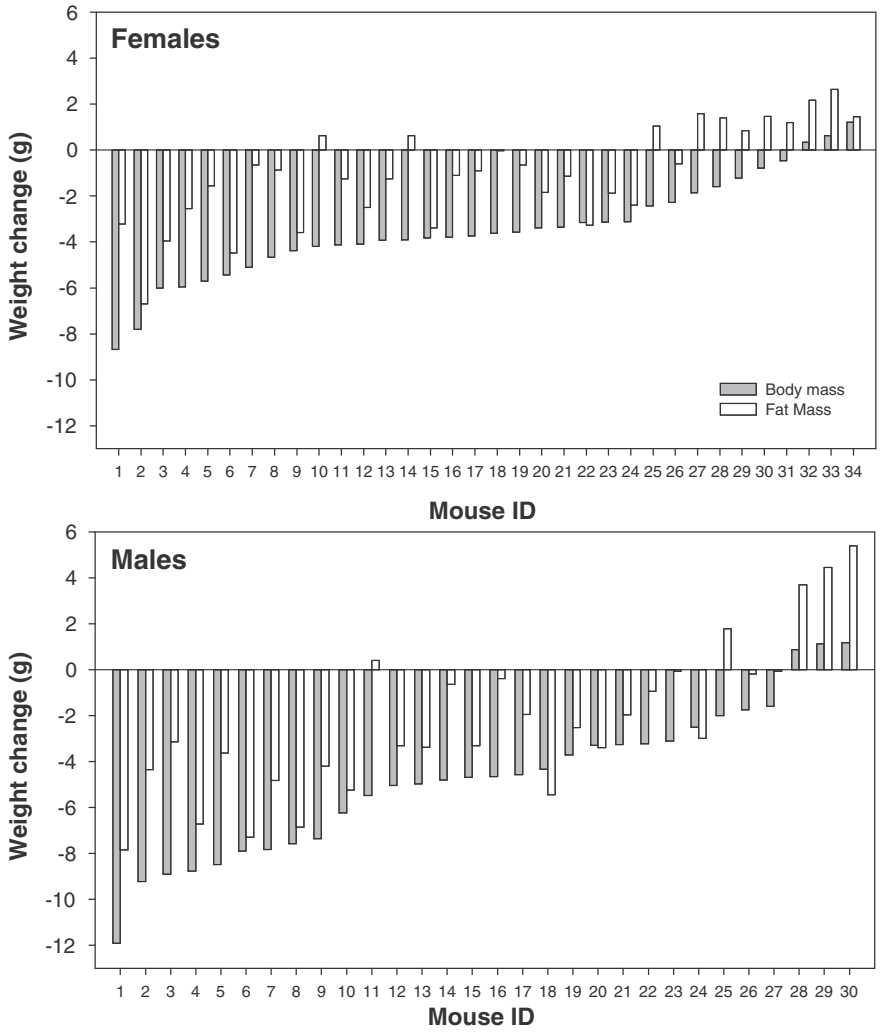

Fig. 1. Absolute body mass loss (grey bars) and fat mass loss (white bars) in female (top panel) and male (bottom panel) MF1 mice after 25 days of 30% caloric restriction. The mice are ordered from the mouse that lost most body mass to the mouse that lost least body mass.

Results

Baseline variables

No significant differences were found between HWL and LWL mice in initial body mass, fat mass, FFM, BMR, general activity or mean body temperature (see Table 1). At baseline, RQ (LWL: 0.83 ± 0.01, HWL: 0.79 ± 0.01; GLM: group: $F_{1,36} = 8.4$, $p = 0.06$, $\eta^2_P = 0.18$, sex: $F_{1,36} = 0.1$, $p = 0.86$, $\eta^2_P = 0.002$, G × S: $F_{1,36} = 0.3$, $p = 0.58$, $\eta^2_P = 0.006$) and food intake (LWL = 4.60 ± 0.47 g day^{-1}, HWL = 4.34 ± 0.44 g day^{-1}; GLM with body mass as covariate: group: $F_{1,35} = 7.3$, $p = 0.01$, $\eta^2_P = 0.17$, sex: $F_{1,35} = 8.8$, $p = 0.005$, $\eta^2_P = 0.20$, G × S: $F_{1,35} = 0.5$, $p = 0.48$, $\eta^2_P = 0.02$) were reduced in HWL mice compared to LWL in both sexes.

Variability in weight loss

There was a large variation in the amount of body mass lost on 30% CR in both male and female mice (Fig. 1). Weight loss ranged from −25% to +3% (i.e., weight gain) of baseline body mass, equivalent to −12 g to +1 g in absolute mass. Diet-induced weight loss was significantly elevated in the HWL group compared to the LWL and this difference was most pronounced in the males (GLM with initial body mass as covariate: group: $F_{1,35} = 144.7$, $p < 0.001$, $\eta^2_P = 0.81$, sex: $F_{1,35} = 2.7$, $p = 0.111$, $\eta^2_P = 0.07$ and G × S: $F_{1,35} = 2.9$, $p = 0.034$. $\eta^2_P = 0.12$). On average diet-induced weight loss in LWL and HWL mice was −0.8 ± 1.3 g and −5.8 ± 1.5 g respectively in females and −1.4 ± 1.8 g and −8.4 ± 1.5 g in males.

Interestingly, when we examined the body composition of the mice, all made adjustments to their body composition, but individual mice used different strategies; i.e., some mice reduced both fat mass and FFM, whereas other mice increased their fat mass at the expense of FFM (see Fig. 1). When we compared the HWL group and LWL group, we found a significant difference in the change in fat mass (Table 1: GLM with initial body mass as covariate: group: $F_{1,35} = 30.9$, $p < 0.001$, $\eta^2_P = 0.47$, sex: $F_{1,35} = 0.3$, $p = 0.61$, $\eta^2_P = 0.008$, G × S = $F_{1,35} = 0.01$, $p = 0.96$, $\eta^2_P = 0.001$, see Table 1), but the change in FFM was similar between the groups for both sexes (GLM: group: $F_{1,35} = 0.09$, $p = 0.76$; sex: $F_{1,35} = 0.4$, $p = 0.54$; G × S: $F_{1,35} = 0.1$, $p = 0.80$).

Compensatory mechanisms

Body composition

Total dry mass was reduced in HWL groups compared to LWL in both males and females (Supplementary Table 1: GLM: group: $F_{1,36} = 17.1$, $p < 0.001$, $\eta^2_P = 0.32$, sex: $F_{1,36} = 17.3$, $p < 0.001$, $\eta^2_P = 0.33$, G × S: $F_{1,36} = 1.2$, $p = 0.29$, $\eta^2_P = 0.03$). No differences in dry organ masses of LWL and HWL mice were found after 25 days of CR (see Supplementary Table 1, GLM with total dry mass as covariate), except

Table 1
Body composition, metabolic rate, activity and body temperature in LWL and HWL mice at baseline and on caloric restriction (CR).

Variables	Females				Males			
	Baseline		CR		Baseline		CR	
	LWL	HWL	LWL	HWL	LWL	HWL	LWL	HWL
Body mass (g)	38.1 ± 1.2	40.0 ± 1.8	37.2 ± 1.3	34.2 ± 1.6**	48.6 ± 1.9	48.7 ± 1.2	47.2 ± 2.0**	40.3 ± 1.2**
Food intake (g day^{-1})	4.6 ± 0.1	4.3 ± 0.1	3.2 ± 0.1**	3.0 ± 0.1**	4.6 ± 0.2	4.4 ± 0.2	3.3 ± 0.1**	3.0 ± 0.1**
Fat mass (g)	8.2 ± 0.9	9.9 ± 1.3	9.5 ± 1.0**	6.8 ± 0.8**	14.4 ± 1.5	14.2 ± 0.9	15.3 ± 1.8	8.8 ± 0.8**
Fat free mass (g)	31.3 ± 0.9	31.2 ± 1.0	29.1 ± 0.9**	28.9 ± 0.9**	35.3 ± 0.8	35.1 ± 1.0	32.9 ± 0.7**	32.4 ± 0.9**
BMR (kJ day^{-1})	27.4 ± 1.3	27.4 ± 0.9	23.8 ± 1.4	23.2 ± 1.7**	28.8 ± 1.3	29.1 ± 1.3	25.1 ± 1.6**	23.2 ± 1.1**
RQ	0.84 ± 0.02	0.78 ± 0.02*	0.79 ± 0.03	0.74 ± 0.03	0.83 ± 0.01	0.79 ± 0.01	0.76 ± 0.03**	0.79 ± 0.04
Activity (counts day^{-1})	11,802 ± 736	10,320 ± 494	10,589 ± 685**	10,329 ± 1059	8486 ± 400	7750 ± 369	7561 ± 304**	9383 ± 1017
Body temperature (°C)	37.3 ± 0.1	37.1 ± 0.1	36.4 ± 0.2**	35.8 ± 0.2***	36.6 ± 0.1	36.7 ± 0.1	36.0 ± 0.1**	35.8 ± 0.1**

Data were analysed using GLM in SPSS. Body mass was added as a covariate for analysis of food intake, fat mass, fat free mass and BMR (see text for results). Shown are mean ± sem.
* Indicates differences between LWL or HWL mice at baseline or CR ($p < 0.05$, post-hoc t-tests).
** Indicates significant differences between baseline and CR within LWL or HWL mice ($p < 0.05$, post-hoc paired t-test).

for skin mass in females, and BAT in males, that were both significantly reduced in the HWL mice (post-hoc t-tests, $p < 0.05$). Total fat mass, calculated based on the weights of the fat pads collected during dissection (i.e., subcutaneous, gonadal, mesenteric, BAT and abdominal fat) correlated strongly with the fat mass measured by DXA one day previously ($r = 0.93$, $p < 0.001$) and was lower in HWL mice compared to LWL mice in both sexes (GLM: group: $F_{1,36} = 17.5$, $p < 0.001$, $\eta_p^2 = 0.32$, sex: $F_{1,36} = 7.9$, $p = 0.008$, $\eta_p^2 = 0.18$, G × S: $F_{1,36} = 0.8$, $p = 0.38$, $\eta_p^2 = 0.02$), but this difference was not significant when corrected for total dry mass (GLM: group: $F_{1,35} = 0.6$, $p = 0.45$, $\eta_p^2 = 0.02$, sex: $F_{1,35} = 4.5$, $p = 0.041$, $\eta_p^2 = 0.11$, G × S: $F_{1,35} = 0.1$, $p = 0.78$, $\eta_p^2 = 0.002$).

Basal metabolic rate

Animals showed several compensatory changes in their metabolic and behavioural parameters in response to CR. These are summarised in Table 1 for HWL and LWL mice (i.e., difference between baseline and CR values). Mice showed a reduction in BMR by 15% (mean of all groups ± sem, −4.2 ± 0.9 kJ day^{-1}), which was a greater reduction than expected based on changes in body mass or FFM alone (i.e., residuals were significantly lower on CR, RM GLM: time: $F_{1,36} = 13.5$, $p = 0.001$, $\eta_p^2 = 0.27$, group: $F_{1,36} = 0.1$, $p = 0.98$, $\eta_p^2 = 0.001$, sex: $F_{1,36} = 6.1$, $p = 0.018$, $\eta_p^2 = 0.15$; see Supplementary Fig. 1 showing relationship between body mass and BMR at baseline and CR).

Compensation in BMR (absolute change or using residuals from relationship between BMR at baseline and CR) did not differ significantly between LWL and HWL mice (GLM: group: $F_{1,36} = 0.6$, $p = 0.45$, $\eta_p^2 = 0.02$, sex: $F_{1,36} = 0.3$, $p = 0.60$, $\eta_p^2 = 0.008$, G × S: $F_{1,36} = 0.2$, $p = 0.67$, $\eta_p^2 = 0.005$). As expected, mice lowered their RQ when on CR compared to baseline, indicating that they were using more fat as their primary energy source (baseline: RQ = 0.83 ± 0.05, CR: RQ = 0.77 ± 0.05, Table 1, RM GLM: within subjects effect: time: $F_{1,36} = 6.0$, $p = 0.020$, $\eta_p^2 = 0.14$). However, no differences were found between LWL and HWL mice (RM GLM, between-subject effects: group: $F_{1,36} = 1.7$, $p = 0.20$, $\eta_p^2 = 0.05$, sex: $F_{1,36} = 0.03$, $p = 0.86$, G × S: $F_{1,36} = 1.4$, $p = 0.25$, $\eta_p^2 = 0.04$).

General activity, food anticipatory behaviour and gorging tendencies

Gross motor activity (counts day^{-1}) was significantly higher in females than in males, but did not change significantly in response to CR, and did not differ significantly between HWL and LWL mice (Table 1, RM GLM: time: $F_{1,36} = 0.5$, $p = 0.50$, $\eta_p^2 = 0.01$, group: $F_{1,36} = 0.2$, $p = 0.69$, $\eta_p^2 = 0.04$, sex: $F_{1,36} = 19.3$, $p < 0.001$, $\eta_p^2 = 0.35$, G × S: $F_{1,36} = 1.24$, $p = 0.27$, $\eta_p^2 = 0.03$). However, an interaction effect between time and group ($F_{1,36} = 4.6$, $p = 0.038$, $\eta_p^2 = 0.11$) indicated that HWL and LWL mice responded differently to CR. As shown in Table 1, on average LWL mice showed a decrease in activity in response to CR (−1069 counts day^{-1}), whereas HWL mice showed an increase in activity (+830 counts day^{-1}). Note though, that within the HWL mice only the males showed a pronounced increase (+1633 counts day^{-1}, Table 1), whereas females did not (+9 counts day^{-1}), but within the LWL mice both sexes showed a decrease (males: −925 counts day^{-1}, females: −1213 counts day^{-1}). The increase in activity in HWL mice may be partly explained by an increase in FAA. Fig. 2 shows the 24 h pattern of activity during baseline and during days 22–25 of CR and whereas at baseline there was a peak in activity at 16:00 (i.e., during weighing) this peak appeared earlier (i.e., at 15:00) and was ~3 fold greater during CR. This peak in activity occurred before any disturbance to the animals and indicates that animals became active prior to provision of food, which is known as FAA (expressed as the number of activity counts during the 3 h prior to food provision in absolute number or relative to 24 h activity) (Mistlberger, 1994).

The amount of FAA that mice displayed increased over the duration of the CR (data not shown). Mice that displayed more FAA lost significantly more weight during CR, and this was apparent in both males and females (linear regression, $p < 0.01$, Fig. 3). FAA differed significantly between HWL and LWL mice after 25 days of CR at 16.1 and 26.7% of total activity, respectively (GLM: group: $F_{1,36} = 16.1$, $p < 0.001$, $\eta_p^2 = 0.31$, sex: $F_{1,36} = 1.7$, $p = 0.20$, $\eta_p^2 = 0.05$ G × S: $F_{1,36} = 1.5$, $p = 0.23$, $\eta_p^2 = 0.04$).

Gorging behaviour was scored once during baseline and from days 10 to 15 of CR. Because the measures taken at these time points were obtained using different methods, measurements at baseline and CR were analysed separately (Supplementary Fig. 2). Gorging tendencies were increased in HWL mice compared to LWL mice at both baseline (Supplementary Fig. 2A, HWL: 0.37 ± 0.52 g, LWL: 0.09 ± 0.16 g, GLM: group: $F_{1,36} = 5.3$, $p = 0.027$, $\eta_p^2 = 0.13$, sex: $F_{1,36} = 0.4$, $p = 0.53$, $\eta_p^2 = 0.01$, G × S: $F_{1,36} = 0.5$, $p = 0.52$, $\eta_p^2 = 0.01$) and during CR (Supplementary Fig. 2B, HWL: 99.5 ± 2.4%, LWL: 85.4 ± 18.6%, GLM: group: $F_{1,36} = 12.0$, $p = 0.001$, $\eta_p^2 = 0.25$, sex: $F_{1,36} = 2.6$, $p = 0.12$, $\eta_p^2 = 0.07$, G × S: $F_{1,36} = 1.8$, $p = 0.19$, $\eta_p^2 = 0.05$). A positive relationship between weight loss and gorging tendency was observed in both sexes (linear regression: $R^2 = 0.22$, $p = 0.002$). In addition, the amount of gorging behaviour that animals displayed was positively related to FAA during CR ($r = 0.47$, $p = 0.002$). Mice that showed increased FAA also ate their food in a shorter time and had greater diet-induced weight loss.

Body temperature and torpor incidence

Body temperature decreased significantly during CR in both groups, but did not change by the same amount in HWL and LWL mice as indicated by a significant interaction between time and group (see Fig. 2 for 24 h pattern during both phases: RM GLM: within-subject effects: time: $F_{1,36} = 175.4$, $p < 0.001$, $\eta_p^2 = 0.83$, T × S = $F_{1,36} = 5.6$, $p = 0.023$, $\eta_p^2 = 0.14$, T × G = $F_{1,36} = 8.6$, $p = 0.006$, $\eta_p^2 = 0.19$, between-subject effects: group: $F_{1,36} = 9.8$, $p = 0.004$, $\eta_p^2 = 0.21$, sex: $F_{1,36} = 20.2$, $p < 0.001$, $\eta_p^2 = 0.35$, G × S: $F_{1,36} = 6.8$, $p = 0.013$, $\eta_p^2 = 0.16$). Female and male mice responded differently: female HWL mice

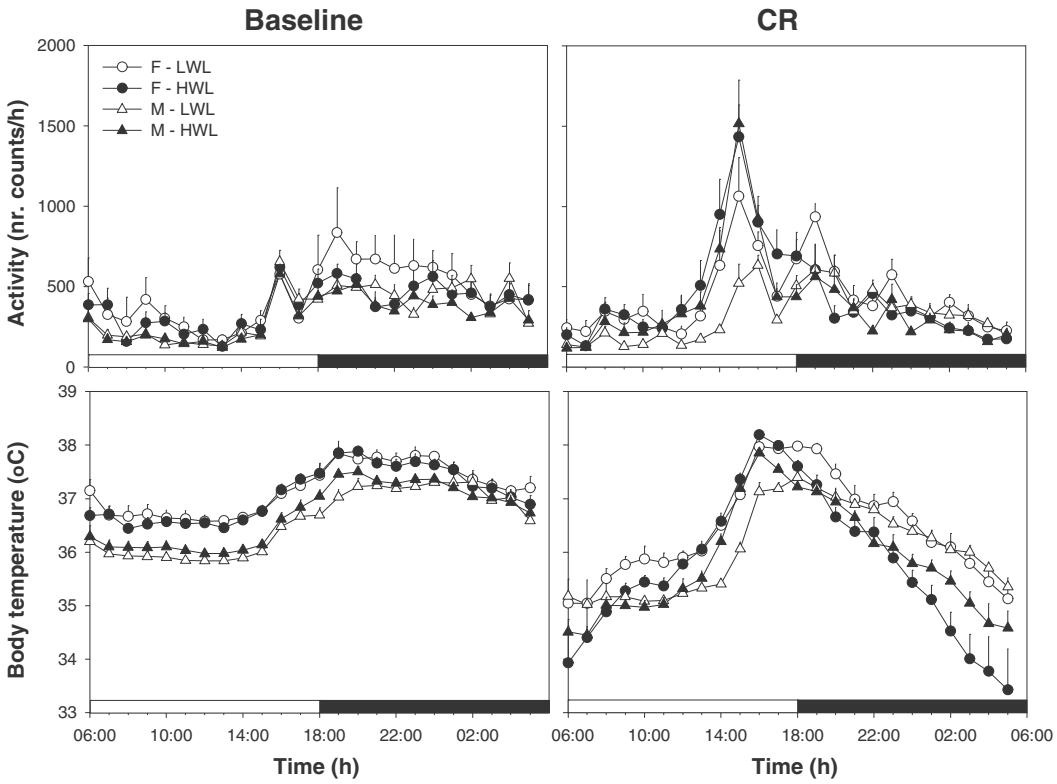

Fig. 2. Twenty-four hour rhythms of general activity and body temperature at baseline and after 25 days of caloric restriction (CR). Black bars indicate the dark (i.e., active) phase of the animals. During baseline there is a peak in activity at 16:00 (time of weighing), whereas during CR this peak occurs at around 15:00 (i.e., food anticipatory activity). Shown are mean and sem.

lowered their body temperatures more than LWL and the opposite occurred in male mice (i.e., significant group × sex effect).

Approximately 25% (15 out of 64, 14 females & 1 male) of mice showed periods of reduced body temperatures that included periods below 31 °C, known as torpor, on at least 1 day during the measurement period. On average torpor bouts lasted 336 ± 68 min (mean ± SD) with a minimum temperature averaging 29.1 ± 1.2 °C. The lowest body temperature recorded was 26.5 °C. Torpor can result in large daily savings of energy expenditure and is a known strategy in calorically restricted mice (Rikke et al., 2003). Whether a mouse entered torpor was not related to their weight loss on CR i.e., there was no significant correlation between weight loss and minimum body temperature, and mice that showed torpor were represented equally in all weight loss groups in females 5 HWL, 4 LWL and 5 intermediates males: 1 HWL. The fact that 41% of females used this strategy and only 3% of males, can be explained by sex differences in absolute body mass; females are significantly lighter. Consequently, in a linear regression, most of the variation in minimum body temperature was explained by initial body mass ($R^2 = 0.28$, $F_{1,62} = 25.2$, $p < 0.001$; see Supplementary Fig. 3) with no independent sex effect ($p = 0.394$).

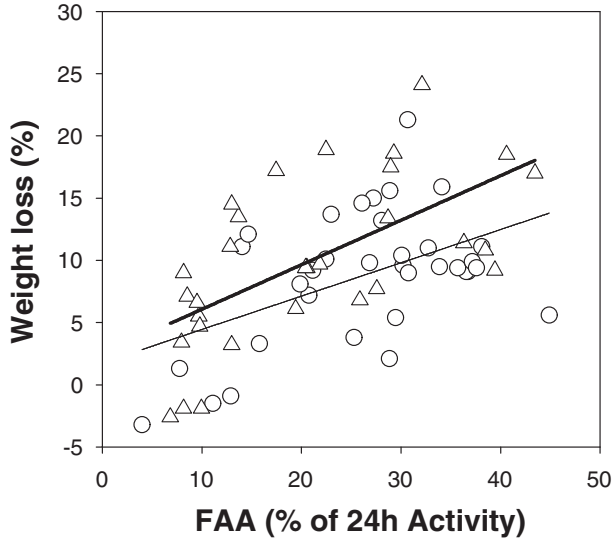

Fig. 3. Relationship between food anticipatory activity (FAA, in % of total 24 h activity) and diet-induced weight loss after 25 days of 30% caloric restriction in male (triangles, n = 30) and female (circles, n = 34) mice. Linear regression lines are shown for both sexes separately (regression analysis: males (bold line): $R^2 = 0.38$, $p < 0.001$, and females: $R^2 = 0.24$, $p = 0.004$).

Leptin

Leptin levels were decreased in HWL mice compared to LWL mice in both males (0.98 ± 0.22 vs. 1.54 ± 0.22, respectively) and females (0.81 ± 0.13 vs. 2.37 ± 0.47 respectively, GLM: sex: $F_{1,36} = 0.1$, $p = 0.76$, $\eta_p^2 = 0.003$, group: $F_{1,36} = 14.5$, $p < 0.001$, $\eta_p^2 = 0.29$, G × S: $F_{1,36} = 1.5$, $p = 0.225$, $\eta_p^2 = 0.04$), but this difference was not significant when values were corrected for fat mass (sex: $F_{1,36} = 13.2$, $p < 0.001$, $\eta_p^2 = 0.27$, group: $F_{1,36} = 0.3$, $p = 0.59$, $\eta_p^2 = 0.009$, G × S: $F_{1,36} = 0.6$, $p = 0.45$, $\eta_p^2 = 0.02$, Fig. 4).

Expression of neuropeptides

Expression of various neuropeptides involved in the regulation of energy balance was investigated in HWL and LWL mice after 25 days of CR and results are shown in Fig. 5. Expression of POMC and CART in the ARC was slightly reduced in HWL mice compared to LWL mice, but these differences did not reach statistical significance (Fig. 5, top graph; Table 2). Expression of NPY, AgRP, ObRb and MC3R in the ARC were significantly increased in HWL mice compared to LWL in both sexes as indicated by the significant group effect without an interaction

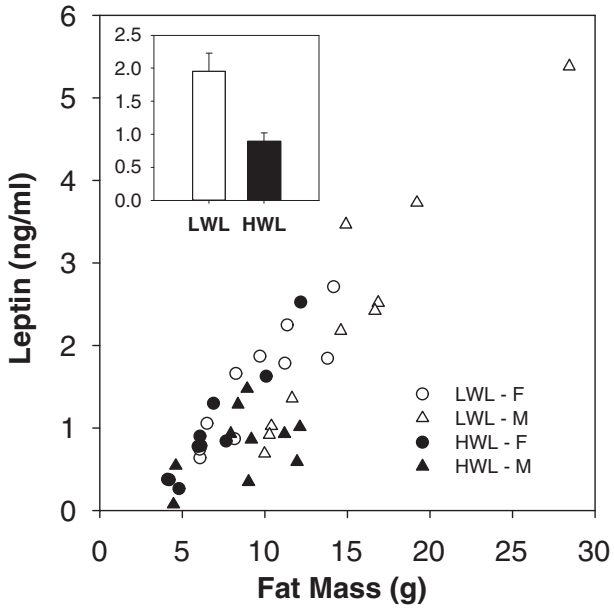

Fig. 4. Leptin levels after 25 days of caloric restriction in relation to fat mass in LWL and HWL male (M) and female (F) mice. The insert shows the mean ± sem. Leptin levels not corrected for fat mass in LWL (white bar) compared to HWL (black bar) mice (n = 20 in each group, includes both males and females).

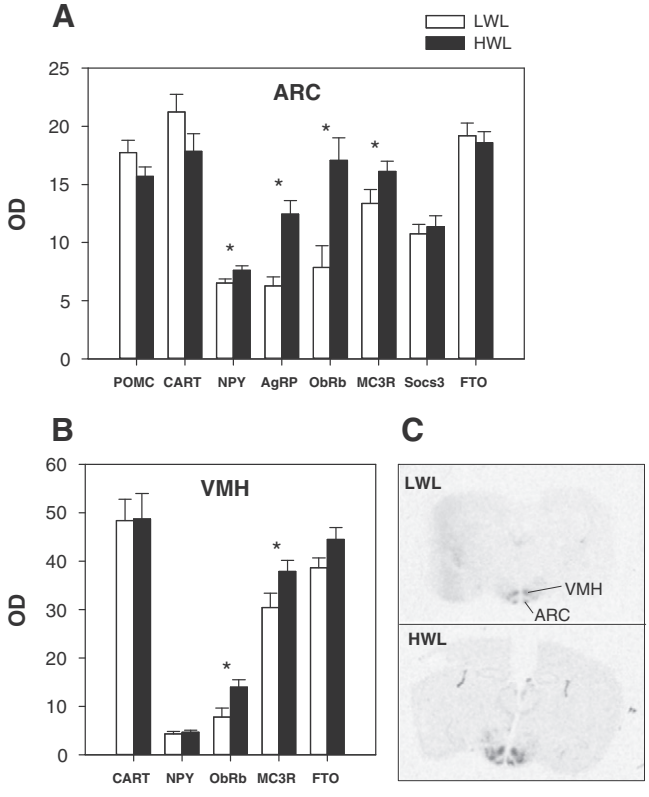

Fig. 5. Expression of various neuropeptides and receptors involved in the regulation of energy balance in male and female mice that show low (LWL) or high (HWL) weight loss in response to 30% caloric restriction in the arcuate nucleus (ARC, A) and ventromedial nuclei of the hypothalamus (VMH, B; n = 20 per group, includes males and females). C shows representative brain slides for LWL and HWL mice where melanocortin 3 receptor (MC3R) expression has been detected by in situ hybridization. Location of ARC and VMH are indicated. POMC = Proopiomelanocortin, CART = Cocaine and amphetamine regulated transcript, AgRP = Agouti-related peptide, NPY = Neuropeptide Y, ObRb = Leptin receptor, FTO = Fat mass and obesity-associated protein, Socs3 = Suppressor of cytokine signalling 3. * indicates that GLM showed a significant difference ($p < 0.05$) in the expression of neuropeptides between HWL and LWL mice.

effect (Table 2). FTO expression was slightly increased in HWL mice compared to LWL mice, but this difference did not reach statistical significance (see Table 2, p = 0.059). No changes in the expression of Socs3 were observed.

In the VMH an increase in MC3R and ObRb expression was found in HWL compared to LWL mice but no differences in the expression of FTO, CART and NPY were found (Fig. 5, bottom graph; Table 2). Fig. 6A & B show that expression of dopamine receptor D2 in the NAcc core (cNAcc) and shell (sNAcc), and the striatum was highly decreased in HWL mice compared to LWL mice (Table 2).

Significant sex effects were observed for various neuropeptides and receptors, e.g., expression of CART and MC3R was increased and expression of NPY, ObRb and Socs3 reduced in ARC of males compared to females (Table 2). A significant interaction between sex and group was not observed for any of the variables, indicating that male and female mice in the LWL vs. HWL groups showed similar responses in expression.

Discussion

Thirty percent CR resulted in a wide range of body mass responses in MF1 mice, as has been shown previously (Vaanholt et al, 2012). Also in agreement with the previous study (Vaanholt et al, 2012), mice that showed HWL had lower food intake at baseline and showed a different compensatory response in physical activity to CR, i.e., HWL increased activity whereas LWL mice decreased. Mammals that are provided with food intermittently (e.g., at the same time every 24 h) have been shown to entrain to the time of food provision and show anticipatory activity in the hours prior to food provision (Holmes and Mistlberger, 2000; Kobayashi et al., 2004; Sutton et al., 2008). This FAA was also observed in our mice with increasing intensity during the course of CR, and was primarily responsible for the differences observed in compensatory changes in daily activity, particularly in males. Although all mice showed FAA, the extent to which they showed this behaviour differed greatly between individuals. FAA was related to weight loss on CR and was more pronounced in male HWL mice (see also Gallardo et al., 2014a, who found no significant relationship with weight loss and FAA in response to 20% and 40% CR which may have been due to low sample size (n = 8–10), and a short study by Gelegen et al., 2008 which showed no relationship after 5 days of timed (2 h per day) CR). The extra amount of energy spent on FAA puts the animals in a greater energy deficit, and raises the question of how this is regulated. A study in MC3R knockout (KO) mice has shown that these KO mice show very little FAA in response to restricted feeding compared to wild types (Sutton et al., 2008), pointing to an important role for MC3R in regulating the expression of rhythms that anticipate nutrient availability. MC3Rs are part of the melanocortin signalling pathway in the hypothalamus that is important in the regulation of energy homeostasis. MC3R KO mice have increased body fat, reduced lean mass (Butler et al., 2000) and MC3R mutations have been shown to be a predisposing factor for obesity in humans (Lee et al., 2007). The melanocortin pathway is mainly regulated by leptin and may thus also play an important role in individual variability in weight loss under CR. Indeed, this is the first study to show differential expression of MC3R in hypothalamus of mice that are resistant (LWL) vs. prone (HWL) to diet-induced weight loss. In line with the results on KO mice (Begriche et al., 2012; Sutton et al., 2008; Chen et al., 2000), HWL mice were lean, showed high FAA and also had high levels of MC3R in both the ARC and VMH. Note however that studies using neuron-specific Cre transgenic mice have shown that reactivating MC3R in the VMH did not rescue FAA, indicating that actions of MC3R in neurons residing outside the VMH are required for the expression of FAA (Begriche et al., 2011).

An important role for MC3R in regulating the mesolimbic dopamine system has recently been shown in female mice (Lippert et al., 2014). During deprivation, food and water are naturally rewarding and motivating substances that activate dopamine neurons and thereby

Table 2
Expression of various neuropeptides and receptors in male and female LWL and HWL mice.

Variables					General linear model								
	Females		Males		Sex			Group			Sex × group		
	LWL	HWL	LWL	HWL	$F_{1,36}$	p	η^2_P	$F_{1,36}$	p	η^2_P	$F_{1,36}$	p	η^2_P
Arcuate nucleus													
POMC	19.3 ± 1.1	15.0 ± 0.9	16.1 ± 1.7	16.4 ± 1.3	0.4	0.509	0.01	2.5	0.125	0.06	3.2	0.081	0.08
CART	20.1 ± 1.3	14.8 ± 1.3	22.4 ± 2.7	20.9 ± 2.4	4.3	**0.045**	0.11	2.8	0.106	0.07	0.8	0.367	0.02
NPY	7.3 ± 0.5	8.2 ± 0.6	5.9 ± 0.9	7.1 ± 1.4	8.5	**0.006**	0.20	5.8	**0.021**	0.14	0.2	0.643	0.01
AgRp	8.7 ± 1.0	12.4 ± 1.3	3.9 ± 0.6	12.4 ± 2.0	3.2	0.084	0.08	21.3	<**0.001**	0.37	3.2	0.082	0.08
ObRb	12.7 ± 3.0	19.7 ± 3.1	3.1 ± 0.7	14.4 ± 2.3	9.2	**0.005**	0.20	14.0	**0.001**	0.28	0.8	0.381	0.02
MC3R	10.3 ± 1.1	13.4 ± 1.0	16.4 ± 1.6	18.8 ± 0.9	23.8	<**0.001**	0.40	5.4	**0.026**	0.13	0.1	0.726	0.003
Socs3	12.1 ± 1.3	14.2 ± 1.1	9.4 ± 0.8	8.7 ± 1.0	15.0	<**0.001**	0.30	0.5	0.506	0.01	1.8	0.192	0.05
FTO	19.4 ± 1.8	19.7 ± 1.1	19.0 ± 1.4	17.4 ± 1.5	0.8	0.364	0.02	0.2	0.686	0.005	0.4	0.516	0.01
VMH													
CART	43.6 ± 3.1	34.8 ± 4.2	53.2 ± 8.2	62.5 ± 7.6	9.1	**0.005**	0.20	0.0	0.963	0.001	2.2	0.151	0.06
NPY	4.3 ± 0.4	4.3 ± 0.3	4.3 ± 0.9	5.0 ± 0.8	0.2	0.624	0.007	0.2	0.628	0.007	0.2	0.671	0.005
ObRb	10.6 ± 3.4	15.5 ± 8.5	5.0 ± 1.1	12.4 ± 1.5	3.4	0.073	0.09	6.8	**0.013**	0.16	0.3	0.601	0.008
MC3R	22.6 ± 3.3	30.2 ± 1.9	38.3 ± 3.4	45.4 ± 2.3	30.6	<**0.001**	0.46	7.0	**0.012**	0.16	0.0	0.927	0.001
FTO	35.4 ± 2.8	39.6 ± 2.0	41.8 ± 2.8	49.4 ± 4.1	7.3	**0.010**	0.17	3.8	0.059	0.10	0.3	0.580	0.009
D2 R — sNAcc	248.5 ± 67.0	93.2 ± 11.8	259.1 ± 44.7	166 ± 52.8	1.0	0.316	0.03	6.5	**0.015**	0.15	0.3	0.569	0.009
D2 R — cNAcc	292.25 ± 80.3	96.0 ± 19.5	320.0 ± 57.2	196.0 ± 75.2	0.7	0.396	0.02	6.6	**0.015**	0.15	0.4	0.526	0.01
D2 R — Striatum	1513.6 ± 428.0	468.08 ± 121.0	1266.9 ± 210.7	769.8 ± 269.2	0.0	0.927	0.001	7.6	**0.009**	0.17	1.0	0.332	0.03

Shown are mean ± sem OD measured in male and female mice that show high (HWL) or low (LWL) weight loss in response to 30% caloric restriction (CR) for various factors involved in the regulation of energy balance in the arcuate nucleus (ARC) and ventromedial nuclei of the hypothalamus (VMH), and expression of dopamine receptor D2 (D2 R) in nucleus accumbens shell and core (sNAcc and cNAcc respectively) and striatum. POMC = Proopiomelanocortin, CART = Cocaine and amphetamine regulated transcript, AgRP = Agouti-related peptide, NPY = Neuropeptide Y, ObRb = Leptin receptor, MC3R = Melanocortin receptor 3, FTO = Fat mass and obesity-associated protein, Socs3 = Suppressor of cytokine signalling 3. Data were analysed using GLM with sex, group and sex * group as fixed factors. Significant differences between groups are indicated by bold p-values ($p < 0.05$) and effect sizes for these effects computed by SPSS are shown (partial eta squared, η^2_P).

facilitate goal-directed behaviour, including FAA, which results in the acquisition of food and water (Liu et al., 2012; Mistlberger and Mumby, 1992). Projections from the VTA to the NAcc in the mesolimbic area are well characterised regarding their role in drug addiction and the rewarding aspects of food (Sánchez-Lasheras et al., 2010) and recent studies suggest that information on nutrient status is passed from the ARC in the hypothalamus to MC3R in the VTA, thereby providing a link between hypothalamic nutrient sensing and dopaminergic reward systems (Lippert et al., 2014; Sánchez-Lasheras et al., 2010). HWL mice had reduced levels of D2 expression, but showed increased FAA. This is in agreement with a study showing reduced expression of D2 in C57BL/6J mice that displayed FAA, compared to A/J mice that did not display FAA in response to CR (Gelegen et al., 2008), but disagrees with results suggesting reduced FAA in response to D2 antagonists (Liu et al., 2012; Mistlberger and Mumby, 1992) and a study showing no effect on FAA in D2 KO mice (Gallardo et al., 2014b). It may be that the lack of food found in response to increased FAA resulted in down-regulation of the reward system and thus lower expression of dopamine

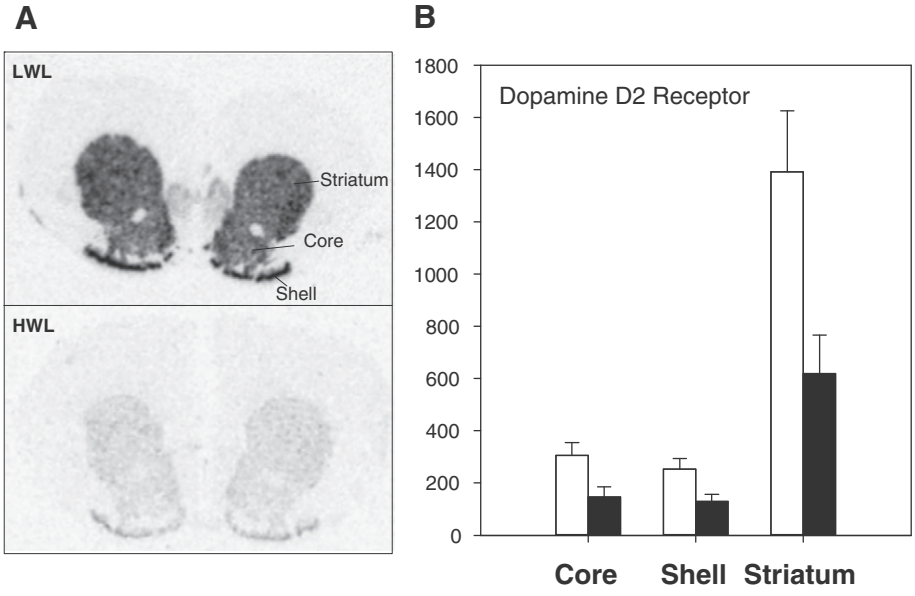

Fig. 6. Expression of dopamine D2 receptor in the nucleus accumbens (core & shell) and striatum. A shows representative brain slides for a low weight loss (LWL) and high weight loss (HWL) mouse where dopamine D2 receptor expression has been detected by in situ hybridization. Location of nucleus accumbens core and shell and the striatum are indicated. B shows the mean expression of dopamine D2 receptor in the nucleus accumbens core and shell and the striatum in mice that showed low (white bars) or high (black bars) diet-induced weight loss (n = 20 per group, includes males and females).

receptor. This is in line with the fact that chronic food restriction and maintenance of low body weight increase the self-administration of abused drugs (Carroll, 1997). In our study, animals were culled between 10:00 and 13:00, prior to the food anticipatory peak in activity, and we do not yet know if/how expression of dopamine D2 receptor was affected shortly after the occurrence of FAA. Moreover, studies in KO mice have indicated an important role for dopamine D1 receptor, but not D2 receptor in regulating FAA (Gallardo et al., 2014b).

A significant correlation between the amount of FAA and gorging tendencies was found in male mice. Gorging is defined as the high consumption of food in a short time period and can generally be triggered by CR (Hambly et al., 2007a; Hambly and Speakman, 2015). In our mice, particularly in the males, gorging tendencies were already apparent during baseline after a 6 h fast and the amount of food consumed during the 2 h after food provision was predictive of subsequent weight loss on CR. In males gorging behaviour on CR was also highly correlated with weight loss which is in agreement with previous studies showing greater weight loss in mice that gorged during restriction than in non-gorgers (Hambly and Speakman, 2015; Hambly et al., 2007a). Also in agreement with the current study, FAA, measured using behavioural observations rather than implanted transponders as performed here, was also shown to be higher in gorgers than in non-gorgers (Hambly and Speakman, 2015). Mice that were looking for food prior to provision thus also ate their food in a shorter time and this may indicate that these mice experienced greater hunger. The pattern of expression of the main orexigenic and anorectic neuropeptides involved in regulation of energy balance in HWL versus LWL supports this interpretation. HWL mice showed an increase in orexigenic neuropeptides (NPY and AgRP), consistent with an experience of greater hunger (Hambly et al., 2007b).

Leptin, which circulates in the periphery in relation to fat mass, provides information on the animals' nutritional status and signals to key regulatory centres in the hypothalamus. Leptin's effects on controlling food intake are largely mediated via the melanocortin pathway (for recent reviews see Koch and Horvath, 2014; Jeong et al., 2014; Münzberg and Morrison, 2015). Binding of leptin to its receptors (ObRb) in the ARC inhibits the action of neurons co-expressing the orexigenic NPY and AgRP and activates the action of neurons co-expressing POMC and CART. Both subsets of neurons make numerous connections with other hypothalamic areas including the lateral hypothalamus, VMH and paraventricular nucleus. POMC undergoes post-translational cleavage into several biologically active products of which α-melanocortin stimulating hormone (α-MSH) competes with AgRP for binding to the melanocortin receptors (MC3R and MC4R, which are expressed in the hypothalamus), resulting in up- or downregulation of food intake (Jeong et al., 2014; Koch and Horvath, 2014; Schwartz et al., 2000).

Reduced levels of leptin, as were observed in HWL mice compared to LWL mice, should thus result in stimulation of the orexigenic NPY/AgRP neurons and suppression of anorectic POMC/CART neurons. This is in agreement with our results showing higher expression of NPY/AgRP neurons and lower expression of POMC/CART neurons in HWL compared to LWL mice. Changes in the expression of these neurones stimulates food intake, and in the absence of food as in the current study, may result in increased foraging behaviour, which is reflected as an increase in FAA that was observed in the HWL mice. Indeed, results from activation and ablation studies indicate a direct role of NPY/AgRP neurons in the stimulating food-seeking behaviours; i.e., neonatal ablation of AgRP neurons reduced FAA on CR (Tan et al., 2014) and activation of AgRP neurons produced an increase in activity when food was absent (Krashes et al., 2011). In line with this, MC3R KO mice that display reduced FAA have reduced AgRP and NPY expression in the ARC (Girardet et al., 2014).

Leptin exerts its action by binding to its receptor (ObRb) which is highly expressed on neurons in the ARC, VMH and other areas of the brain. Activation of ObRb initiates a cascade of signal transduction pathways, most importantly, the JAK/STAT (Janus kinases/Signal transducers and activators of transcription) pathway. The JAK/STAT pathway is subject to negative feedback regulation by the suppressor of cytokine signalling (SOCS) family of proteins; in particular, leptin induces the expression of Socs3, which has been shown to inhibit the phosphorylation and activation of JAK2 and thus decreases the effect of leptin on reducing food intake. Indeed, over-activation of Socs3 is proposed to have an important role in leptin resistance that is observed in obesity (Bjorbaek et al., 1999). A reduction in Socs3 activity enhances the weight reducing effects of peripherally administered leptin (Kievit et al., 2006; Mori et al., 2004). HWL mice in this study showed an increase in ObRb expression without a change in Socs3 expression compared to LWL mice, which may suggest that leptin signalling was not the primary driver of neuropeptide responses.

Besides stimulating food intake, changes in the expression of NPY/AgRP neurons and their receptors have also been suggested to reduce energy expenditure (Koch and Horvath, 2014). Here we observed that BMR in mice exposed to CR was reduced more than expected based on changes in body mass alone, which may reflect a physiological response to the changes in expression of orexigenic and anorectic neuropeptides. However, we did not detect any significant differences in BMR between LWL and HWL mice (when correcting for body mass), despite them showing differential expression of various neurones and receptors (i.e., NPY/AgRP and ObRb and MC3R).

Studies comparing the expression of hypothalamic neuropeptides between ad libitum fed controls and CR mice show a similar pattern to that observed here when comparing LWL and HWL mice; i.e., they typically show an increased expression of NPY/AgRP and reduced expression of POMC/CART (Hahn et al., 1998; Hambly et al., 2007b; Schwartz et al., 2000). However, the magnitude of these responses did differ; i.e., comparing controls to CR mice there was a 2–4 fold increase in NPY/AgRP expression (Hambly et al., 2007b), compared to a 1.2–2 fold increase between LWL and HWL mice, and a 40–60% decrease compared to a 15% (not significant) decrease in expression of POMC/CART, in control vs. CR and HWL vs. LWL mice, respectively. Obviously, LWL and HWL both experienced alterations in their neuropeptide patterns in response to CR, but the fact that the expression of orexigenic and anorexigenic neuropeptides differed, does indicate that these differences may underpin the individual variability that is observed in weight loss between these groups via action on food intake and activity (see Vaanholt et al., 2012). Since it is only possible to kill the mice once, we do not know whether the expression of these neuropeptides was already different under baseline conditions or whether it is the response in neuropeptide expression to CR that differs.

FTO expression in ARC or VMH did not differ between HWL and LWL mice. FTO has been associated with increased BMI in humans and remains the strongest association of any common genetic polymorphism with human obesity (Frayling et al., 2007; Speakman et al., 2008). The biological mechanism underlying this link remains obscure however (for reviews see Larder et al., 2011; Speakman, 2015) and our results indicate that it is not involved at the transcriptional level in regulating responses in diet-induced weight loss.

In conclusion, outbred mice exposed to CR show a great variability in diet-induced weight loss. Animals that were prone to high levels of diet-induced weight loss showed increased FAA and a differential neuropeptide profile to mice that showed low levels of weight loss. The HWL individuals were characterised by increased expression of NPY/AgRP, the long form of the leptin receptor (ObRb), and MC3R expression, indicating that these mice experienced greater hunger when placed on CR. In addition, they show reduced levels of dopamine D2 receptor confirming a potentially important role for reward systems in responses to CR. It remains to be investigated whether this differential expression of neuropeptides between HWL and LWL mice occurs only in response to CR, or whether differential expression of neuropeptides is already present under baseline conditions and thus drive the differences between individuals that are resistant or prone to diet-induced weight loss.

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