Contribution of the hypothalamus and gut to weight gain susceptibility and resistance in mice

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Abstract

Obesity susceptibility in humans and in rodent strains varies in response to the consumption of high-energy density (HED) diets. However, the exact mechanism(s) involved in this susceptibility remain(s) unresolved. The aim of the present study was to gain greater insight into this susceptibility by using C57BL/6J (B6) mice that were separated into obesity-prone (diet-induced obese (DIO)) and obesity-resistant (diet-induced resistant (DR)) groups following an HED diet for 6 weeks. Physiological, biochemical and gene expression assessments of energy balance were performed in the DIO and DR mice on an HED diet and chow-fed mice. The increased weight gain of the DIO mice as compared to the DR mice was associated with increased energy intake and higher plasma leptin and adiponectin levels but not with reduced physical activity or resting energy expenditure. Hypothalamic *Pomc* gene expression was elevated, but there were no changes in *Npy* or *Agrp* expression. Adipose tissue leptin and adiponectin gene expression were significantly reduced in the DIO group as compared to the DR group. Interestingly, ileum expression of G protein-coupled receptor (*Gpr*) 40 (*Gpr40*) was significantly increased, whereas *Gpr120*, *Gpr119*, *Gpr41*, and glucagon-like peptide 1 (*Glp1*) were reduced. Contrastingly, the lower weight gain of the DR group was associated with elevated adipose tissue leptin and adiponectin gene expression, but there were no differences in plasma hormone or hypothalamic gene expression levels as compared to chow-fed mice. Therefore, the present data demonstrate that susceptibility and resistance to diet-induced weight gain in B6 mice appears to be predominantly driven by peripheral rather than hypothalamic modifications, and changes in gut-specific receptors are a potentially important contributor to this variation.

Key Words

- ▶ diet-induced obesity
- ▶ high-energy density diet
- ▶ body weight regulation

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Introduction

Loss-of-function mutations in single genes have been linked to obesity in human subjects; however, such mutations only represent rare cases and cannot account for the overwhelming majority of obesity cases (O'Rahilly 2009). Genome-wide association studies have identified more than 40 loci related to human obesity, and the fat mass

and obesity-associated (*FTO*) gene is the most replicated and shows the highest statistical significance (Marti & Ordovas 2011). However, the combined effect of all of these loci only account for 2–3% of the inherited contribution to obesity risk (Marti & Ordovas 2011), which highlights the multifactorial and complex nature of the disease. It is therefore

generally accepted that 'common obesity' is driven by the sensitivity of an individual's genetic predisposition to the harmful effects of environmental influences, such as increased energy intake (particularly of high-energy density (HED) diets) and sedentary lifestyle.

It is clear from the continued rise in global obesity that we do not completely understand the mechanism(s) involved in its development, particularly seeing as the capacity for weight gain is not the same across our population. Inter-individual variability, particularly in response to an HED diet, can lead to excessive weight gain in those that are susceptible to these environments, whereas others can resist these effects and become only mildly overweight or remain lean (Levine *et al.* 1999, Ravussin & Bouchard 2000, Blundell *et al.* 2005). Although this phenomenon is self-evident, it has been clinically demonstrated in a key study where lean, healthy individuals were overfed 1000 kcal of energy daily for 8 weeks. This led to a large range in weight gain, which was subsequently attributed to the activation of an element of energy expenditure, non-exercise activated thermogenesis (NEAT); the individuals that gained more weight exerted less NEAT and those that remained lean exerted increased NEAT (Levine *et al.* 1999). However, this is only one study that showed a potential mechanism for this diversity; it is still unclear what biochemical/genetic mechanisms contribute to this phenomenon.

Rodent models of obesity are an ideal tool for studying human obesity, not only because they present with the same physiological responses in terms of appetite, energy expenditure and fat deposition profiles but also because they exhibit the same pattern of weight gain divergence in response to HED diets (Chang *et al.* 1990, Pagliassotti *et al.* 1994, Levin *et al.* 1997, Levin & Dunn-Meynell 2000, 2002, Burcelin *et al.* 2002). Outbred strains, such as Sprague-Dawley (SD) and Wistar rats, have reported several physiological explanations for obesity susceptibility and resistance, including changes in energy intake, whole-body fat oxidation and pancreatic sympathetic tone (Levin *et al.* 1987, Chang *et al.* 1990), whereas increases in circulating leptin, insulin and cholecystokinin and reductions in ghrelin have been reported to contribute to the separation in SD rats (Li *et al.* 2011). Similarly, the inbred C57BL/6J (B6) mouse model can be divided into susceptible and resistant populations, with some authors reporting that changes in the appetite stimulators neuropeptide Y (*Npy*) and agouti related peptide (*Agrp*) and in the appetite-suppressing melanocortin pathway (Huang *et al.* 2003a,b) were the predominant contributors, whereas other authors showed changes only in the

suppressor of cytokine signalling 3 (*Socs3*) but not in *Npy* or *Agrp* (Enriori *et al.* 2007). Although there has been some effort to explore the mechanisms that underpin this body weight variance in the outbred models, the current literature has only provided a little insight into these potential mechanisms, and they remain poorly understood. We hypothesise that the cause of diet-induced obesity (or resistance) is mediated by changes in the hypothalamic neuropeptides that govern appetite/satiety.

In the present study, we sought to gain a better understanding of these mechanisms by utilising the B6 mouse model and performing a more detailed analysis of the physiological and genetic factors (hypothalamic and gut) that govern body weight following 6 weeks on an HED diet.

Materials and methods

Mice and diets

Four-week-old female C57BL/6J (B6) mice were purchased from the Walter and Eliza Hall Animal Facility (Parkville, Vic., Australia) and individually housed at the Bio-Resources Facility, Austin Health (Heidelberg, Vic., Australia), where they were subjected to a controlled 12 h light:12 h darkness lighting cycle (darkness started at ~1900 h) and a room temperature of 22 °C. After 1 week of acclimatisation, the animals were weight-matched into groups (to control for potential maternal influences). One-third were fed a standard rodent chow diet (9% fat (w/w) (25% energy), 22% protein (w/w) (26% energy) and 69% carbohydrate (w/w) (49% energy); 13.2 kJ/kg total energy, Ridley AgriProducts, Pakenham, Vic., Australia), and the remaining mice were fed a modified version of the sweetened condensed milk diet used by Levin (Levin *et al.* 1997, Levin & Dunn-Meynell 2000) (HED diet; 15% fat (w/w) (31% energy), 19% protein (w/w) (17% energy) and 57% carbohydrate (w/w) (51% energy); 18.6 kJ/g total energy, Speciality Feeds, SF08-044, Glen Forrest, WA, Australia). Mice were allowed to feed *ad libitum* during the experimental period. Sample collections were standardised and performed at the same time of the day (0900–1100 h) as described in the following sections for all of the groups. Experimental procedures were carried out in accordance with the protocols approved by the Austin Health Animal Ethics Committee (AEC no. A2010/04028).

Feeding protocol and energy balance measurements

Body weight and food intake were determined weekly (Wong *et al.* 2011). After 4 weeks of feeding, mice were

stratified based on body weight gain into diet-induced obese (DIO; mice that gained equal to or greater than the average of the HED-fed group) and diet-induced resistant (DR; mice that maintained a similar weight gain pattern as that of the chow-fed only B6 mice) groups. Mice that fell between these groups ($n=11$, or $\sim 20\%$ of the mice on the high-fat (HF) diet) were not used, which was in line with other studies that have used similarly stratified B6 groups (Huang *et al.* 2003a,b, Enriori *et al.* 2007). Mice were tracked for a further 2 weeks on their respective diets. During the 6th week of feeding, voluntary physical activity (individually housed running wheels, which have been shown to correlate well with infrared photoelectric measurements of spontaneous motor activity (Thorburn *et al.* 2000)), resting energy expenditure (REE) and respiratory quotient (RQ; indirect calorimetry; 10 min interval sampling over 75 min during the resting period (daytime)) were measured (Fam *et al.* 2007, Mangiafico *et al.* 2011, Wong *et al.* 2011, Visinoni *et al.* 2012). At the conclusion, mice were anaesthetised in the non-fasted state (0900 h) (Ahren & Scheurink 1998, Fam *et al.* 2007) with an i.p. injection of sodium pentobarbitone, and blood was collected via cardiac puncture (0900–1100 h). Brain and gut ilea were rapidly removed and frozen in liquid nitrogen. Fat depots (subcutaneous, infra-renal, gonadal white fat (WAT) and brown fat (BAT)) were excised, weighed and frozen in liquid nitrogen. All tissues were stored at -80°C .

Plasma hormone concentrations

Samples were immediately added to EDTA-coated tubes that contained 10 μl of protease inhibitors per 1 ml of blood (100X stock containing Pefabloc (Roche, cat. no. 11429868001), DPPIV inhibitor (Millipore, cat. no. DPP4-010; Bayswater, Victoria, Australia) and protease inhibitor cocktail (Sigma, cat. no. P2714)). Plasma was collected and stored at 4°C for the measurement of leptin, adiponectin (total) and ghrelin (acylated) (one assay using the Mouse Gut Hormone MILLiplex kit, Millipore), insulin (mouse-specific ELISA; 80-INSMS-E01, ALPCO Diagnostics, Salem, NH, USA) and glucose (Analox Glucose Analyser; Helena Laboratories Pty Ltd, Melbourne, Vic., Australia).

Hypothalamic dissection and RNA/cDNA preparation

Whole hypothalami were dissected, and the sections that contained the arcuate nuclei (ARC) were excised, as previously reported (Steinberg *et al.* 2006, Fam *et al.* 2007, Wong *et al.* 2011, Visinoni *et al.* 2012). Sections that

contained the paraventricular nuclei (PVN) were also collected to act as a site-specific control for gene expression. RNA and cDNA were prepared from hypothalamic sections and dissected tissues (Fam *et al.* 2007, 2012, Wong *et al.* 2011, Visinoni *et al.* 2012).

Gene expression profiling

Gene-specific Taqman Gene Expression Assays (Applied Biosystems, Scoresby, Victoria, Australia) were used to analyse mRNA expression levels of the genes listed in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article, according to the manufacturer's protocol. A PerkinElmer ABI Prism 7700 Sequence Detector and Sequence Detection Systems Software version 1.9.1 (Applied Biosystems) was used for detection, and the results were analysed with the $2(-\Delta\Delta C_T)$ method, which is based on relative quantification of the treated group to the untreated group (Wong *et al.* 2011).

Microarray analysis

Microarray analysis was performed only in ARC samples from chow-fed, DIO and DR mice by the Australian Genome Research Facility (AGRF) with the Illumina Mouse WG-6 v2.0 BeadArray. This array uses 45 281 probe sets that measure the gene expression of 26 766 well-established coding transcripts, 6856 provisional annotated coding transcripts and 56 well-established non-coding transcripts, all of which come from the Mouse RefSeq Rel 22 content. In addition, the chips are supplemented with probes that target the mouse exonic evidence-based oligonucleotide set (MEEBO) and the exemplar protein coding sequences (RIKEN FANTOM24-6). Between 1 and 2 μg of total RNA from ARC samples of the chow-fed, DIO and DR mice were extracted. DNase was treated and cleaned post-extraction using the RNeasy MiniKit (Qiagen). Samples were analysed by AGRF and were normalised using R-Bioconductor Software (www.bioconductor.org) and Partek Genomic Suite Software (for ANOVA analysis; Partek, Inc., St Louis, MO, USA).

Statistical analysis

All data are presented as means $\pm$ S.E.M. using GraphPad Prism 5 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Statistical comparisons between single parameters were made using one-way ANOVA analysis (Minitab 15, 2007; Sydney, NSW, Australia). For repeated measures, a general linear model (GLM) ANOVA was used

for comparison, and Tukey's *post hoc*-test was used to determine significance (Minitab 16.1.10, 2010). Significance was set at $P < 0.05$.

Results

Body weight and adiposity characteristics

DIO mice were significantly heavier than both DR and chow-fed mice from week 3 until week 6 ($P < 0.05$), whereas DR and chow-fed mice maintained similar body weights over the same time period (Fig. 1A). When body weight gain was calculated (Fig. 1B), DIO mice consistently gained more weight on the HED diet than did the DR mice on the same diet and the chow-fed mice ($P < 0.005$), and this weight gain was associated with increased adiposity across all of the fat depots ($P < 0.05$, Fig. 1C). DR mice had lower body weight gain as compared to chow-fed mice (Fig. 1B), and this was associated with similar subcutaneous and BAT masses and increased infra-renal and gonadal masses ($P < 0.05$, Fig. 1C), which reflected the increased fat intake of the DR mice.

Energy balance characteristics

DIO mice consumed significantly more food as compared to both DR and chow-fed mice (chow-fed: 3.76 ± 0.05 , DR: 3.60 ± 0.20 , DIO: 4.66 ± 0.44 , g/day; $P < 0.05$ DIO vs chow-fed and DR mice), but there was no difference between DR and chow-fed mice. With respect to energy intake, DIO mice still consumed more kilojoule than DR and chow-fed mice did ($P < 0.05$, Fig. 2A). DR mice consumed more kilojoule than the chow-fed mice did ($P < 0.05$) because of the increased energy density of the diet.

There were no significant differences in voluntary physical activity (Fig. 2B) or daytime REE (Fig. 2C) among the groups. Similarly, there was no significant preference for either fat or carbohydrate as a nutrient source among the groups as assessed by RQ (Fig. 2D) or when calculated as a rate of oxidation (fat oxidation, chow-fed: 50.95 ± 5.68 , DR: 49.33 ± 2.80 , DIO: 47.45 ± 4.00 ; glucose oxidation chow-fed: 24.98 ± 5.89 , DR: 17.51 ± 2.13 , DIO: 15.07 ± 2.65 , $P > 0.05$).

Plasma hormone levels

Non-fasted plasma insulin (Fig. 3A) was significantly elevated in the DIO mice as compared to the DR and chow-fed mice, yet glucose levels (Fig. 3B) were lower in both the DIO and DR groups as compared to the chow-fed mice. Plasma leptin (Fig. 3C) was, as expected, higher in the DIO group, and interestingly, plasma adiponectin was also significantly elevated as compared to both the DR and chow-fed mice (Fig. 3D). Plasma ghrelin, the main circulating appetite-stimulating hormone, was not different between the DIO and DR groups, but it was statistically lower in DIO as compared to chow-fed mice (Fig. 3E).

Hypothalamic ARC neuropeptide gene expression

To determine if changes in the expression of hypothalamic neuropeptides were associated with physiological differences, we assessed ARC gene expression of the orexigenic neuropeptides *Npy* and *Agrp*, the anorexigenic pro-hormone *Pomc* and a marker of leptin insensitivity, *Socs3*. *Npy* (Fig. 4A) and *Agrp* (Fig. 4B) mRNA levels were not significantly different between the groups. *Pomc* mRNA

Figure 1

Absolute body weights (g) (A), body weight gain (g/day) and average body weight gain over 6 weeks (B) in female B6 mice fed either a chow diet (Chow) or an HED diet and stratified into diet-induced obese (DIO) and diet-induced resistant (DR) groups over 6 weeks of feeding. (C) Absolute fat pad mass (g) from individual white adipose tissue depots (subcutaneous,

infra-renal and gonadal) and total brown adipose tissue (BAT) from Chow, DR and DIO groups. Results are presented as means $\pm$ S.E.M. ($n = 20$ – 24 per group). $^aP < 0.005$ vs DR; $^bP < 0.005$ vs Chow (one-way ANOVA for single parameters and GLM ANOVA for repeated measurements). Sub, subcutaneous; Infra, infra-renal; Gon, gonadal.

Figure 2

(A) Daily total energy intake (kJ/g per day) in female B6 mice fed either a chow diet (Chow) or an HED diet and stratified into diet-induced obese (DIO) and diet-induced resistant (DR) groups over 6 weeks of feeding. The inset panel shows the average energy intake over 6 weeks in the Chow, DR and DIO groups. (B) Physical activity (revolutions/day) as measured by wheel running in Chow, DR and DIO groups after 6–7 weeks of feeding. (C) Resting energy expenditure (REE; kcal/day per kg) as measured by

indirect calorimetry in Chow, DR and DIO groups after 6 weeks of feeding. (D) Respiratory quotient (RQ) levels in Chow, DR and DIO groups after 6 weeks of feeding. Results are presented as means $\pm$ s.e.m. ($n=11$ –24 per group for food intake and energy intake measurements, and $n=4$ for energy expenditure measurements). ^a $P<0.005$ vs DR; ^b $P<0.005$ vs Chow (one-way ANOVA for single parameters and GLM ANOVA for repeated measurements).

levels, however, were significantly elevated in the DIO mice as compared to the DR and chow-fed mice ($P<0.05$, Fig. 4C). *Socs3* mRNA levels (Fig. 4D) were not significantly different between any of the groups. PVN sections showed no difference among the groups in any of the genes measured, and levels were significantly lower in these sections as compared to the ARC sections (data not shown).

Hypothalamic ARC microarray analysis

Microarray comparisons with significant changes of ≥ 1.25 -fold revealed a total of 101 differentially expressed (DE) genes between the DIO and DR groups (85 up and 16 down in the DIO group); 60 DE genes between the DIO and chow-fed groups (26 up and 34 down in the DIO group); and 188 DE genes between the DR and chow-fed groups (12 up and 176 down in the DR group). The gene ontologies of these DE genes within each comparison are presented in Supplementary Tables 2, 3 and 4, see section on supplementary data given at the end of this article and are separated into biological processes, cellular components and molecular functions. Genes that

were deemed to be potential candidates and were unique within each comparison (i.e. not shared with the other comparisons) are shown in Tables 1 and 2 (DIO and DR) and in Supplementary Tables 5 (DIO and chow-fed) and 6 (DR and chow-fed). Of these, 58 genes were regulated between the DIO and DR mice (46 genes up and 12 genes down in the DIO group); 44 genes were regulated between the DIO and chow-fed mice (19 genes up and 25 genes down in the DIO group); and 112 genes were regulated between the DR and chow-fed mice (nine genes up and 103 genes down in the DIO group). All data available online at: <https://lims.agrf.org.au/ClientSubmission/Logon.aspx>.

White adipose tissue gene expression

To determine if WAT gene changes were associated with physiological differences, we measured mRNA expression of leptin, adiponectin and the pro-inflammatory marker *Tnf α* in each fat depot. Leptin mRNA expression was not significantly different among the groups in either subcutaneous or gonadal fat (Fig. 5A). However, there was a

Figure 3

Non-fasted plasma levels of insulin (A), glucose (B), leptin (C), adiponectin (D) and ghrelin (E) in Chow, DR and DIO groups after 6 weeks of feeding. Results are presented as means $\pm$ s.e.m. ($n=14-20$ per group). ^a $P < 0.005$ vs DR; ^b $P < 0.005$ vs Chow (one-way ANOVA).

significant reduction in the infra-renal depot of DIO as compared to DR mice but not as compared to chow-fed mice. Moreover, DR mice had significantly elevated infra-renal leptin mRNA expression as compared to chow-fed mice ($P < 0.05$). Adiponectin mRNA expression (Fig. 5B) showed DIO mice trending towards reduced expression in the subcutaneous fat as compared to both DR and chow-fed mice ($P=0.09$ for both), but it was significantly reduced in the gonadal depot ($P < 0.05$). Infra-renal adiponectin expression was not different between the DIO and chow-fed mice; however, DR mice had significantly elevated levels as compared to both groups ($P < 0.05$). Subcutaneous and infra-renal but not gonadal *Tnfa* expression was significantly reduced in DIO mice relative to DR and

chow-fed mice ($P < 0.05$), but no differences were seen between DR and chow-fed mice in any fat depot.

Ileum gene expression

To determine if DIO and DR mice have differential expression patterns in genes involved in fatty acid-induced gut peptide secretion, we measured the fatty acid-specific G protein-coupled receptors (*Gpr*) and glucagon-like peptide 1 (*Glp1*) mRNA in gut ileum. *Glp1* mRNA expression was significantly reduced in DIO mice as compared to both DR and chow-fed mice (Fig. 6A), whereas DR mice had the same expression level as chow-fed mice. When GPR gene levels were assessed, DIO mice

Figure 4

Central mRNA expression levels of *Npy* (A), *AgRP* (B), *Pomc* (C) and *Socs3* (D) in ARC samples from Chow, DR and DIO groups after 6 weeks of feeding. *Rn18s* was used as the housekeeping gene. Results are presented as

means $\pm$ s.e.m. ($n=8-13$ per group) relative to Chow mice. ^a $P < 0.005$ vs DR; ^b $P < 0.005$ vs Chow (one-way ANOVA).

Table 1 Summary of hypothalamic ARC genes up-regulated ≥ 1.25 -fold in DIO mice as compared to DR mice ($n=4$ per group)

Gene ^a	Gene name	Fold change	P value
<i>Zfp533</i>	Zinc finger protein 533	1.624	0.046
<i>Arrdc3</i>	Arrestin domain containing 3	1.576	0.026
<i>Etv1</i>	Ets variant 1	1.517	0.033
<i>Grm5</i>	Glutamate receptor, metabotropic 5	1.517	0.020
<i>Mal</i>	Myelin and lymphocyte protein, T cell differentiation protein	1.505	0.041
<i>Trim59</i>	Tripartite motif-containing 59	1.477	0.019
<i>Slc44a1</i>	Solute carrier family 44, member 1	1.472	0.037
<i>B230208H17Rik</i>	RAB, member RAS oncogene family-like 6	1.446	0.046
<i>LOC227393</i>	Predicted gene 1833	1.425	0.040
<i>Prr18</i>	Proline rich 18	1.411	0.014
<i>Papola</i>	Poly (A) polymerase α	1.388	0.044
<i>scl0001118.1_0</i>		1.388	0.012
<i>Chd2</i>	Chromodomain helicase DNA binding protein 2	1.388	0.014
<i>Sox10</i>	SRY (sex determining region Y)-box 10	1.377	0.027
<i>Actb</i>	Actin, β	1.369	0.017
<i>Ppargc1b</i>	Peroxisome proliferative activated receptor, γ , coactivator 1 β	1.369	0.045
<i>scl0002785.1_49</i>		1.366	0.046
<i>Zmiz1</i>	Zinc finger, MIZ-type containing 1	1.344	0.039
<i>BC017647</i>	Family with sequence similarity 222, member B	1.342	0.004
<i>Klf4</i>	Kruppel-like factor 4 (gut)	1.340	0.030
<i>Mpp5</i>	Membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5)	1.328	0.003
<i>Irs2</i>	Insulin receptor substrate 2	1.317	0.013
<i>Mcp1</i>	Microcephaly, primary autosomal recessive 1	1.308	0.031
<i>Tle1</i>	Transducin-like enhancer of split 1, homolog of Drosophila E(spl)	1.304	0.034
<i>Ttc3</i>	Tetratricopeptide repeat domain 3	1.297	0.007
<i>Sypl</i>	Synaptophysin-like protein	1.295	0.014
<i>Ank2</i>	Ankyrin 2, brain	1.295	0.037
<i>LOC331507</i>	Predicted gene 1866	1.294	0.029
<i>BC057371</i>	Ribosomal modification protein rimK-like family member A	1.287	0.005
<i>AI314180</i>	Expressed sequence AI314180	1.284	0.028
<i>Foxq1</i>	Forkhead box Q1	1.283	0.032
<i>Bbx</i>	Bobby sox homolog (Drosophila)	1.282	0.018
<i>Kcnma1</i>	Potassium large conductance calcium-activated channel, subfamily M, α member 1	1.281	0.040
<i>Lhfp13</i>	Lipoma HMGIC fusion partner-like 3	1.278	0.049
<i>Riok1</i>	RIO kinase 1 (yeast)	1.276	0.032
<i>AI427122</i>	Plastin 1 (I-isoform)	1.273	0.048
<i>Pvrl3</i>	Poliovirus receptor-related 3	1.271	0.008
<i>Gsk3b</i>	Glycogen synthase kinase 3 β	1.269	0.023
<i>5830415L20Rik</i>	Family with sequence similarity 133, member B	1.267	0.024
<i>Slc12a2</i>	Solute carrier family 12, member 2	1.267	0.027
<i>Tmem184c</i>	Transmembrane protein 184C	1.265	0.018
<i>Setd8</i>	SET domain containing (lysine methyltransferase) 8	1.264	0.027
<i>Insc</i>	Inscuteable homolog (Drosophila)	1.262	0.046
<i>Map2k2</i>	Mitogen-activated protein kinase kinase 2	1.261	0.036
<i>Slc35b1</i>	Solute carrier family 35, member B1	1.253	0.037
<i>Bace1</i>	β -site APP cleaving enzyme 1	1.251	0.0005

^aGenes of interest are in red font.

displayed significantly more *Gpr40* mRNA expression than both DR and chow-fed mice (Fig. 6B), and *Gpr120* (Fig. 6C) was significantly lower as compared to DR and chow-fed mice. Similar levels were observed between the latter two groups. In contrast, *Gpr119* expression was reduced in both DIO and DR mice as compared to chow-fed, with DIO mice displaying an even more marked reduction as

compared to DR mice ($P < 0.05$, Fig. 6D). Similarly, *Gpr41* was also reduced in both the DIO and DR groups relative to the chow-fed group and also a greater reduction in the DIO vs the DR group (Fig. 6E). *Gpr43* only showed a trend for reduced expression in the DIO group as compared to the DR group ($P = 0.07$, Fig. 6F), but there was a significant reduction as compared to the chow-fed group.

Table 2 Summary of hypothalamic ARC genes down-regulated ≥ -1.25 -fold in DIO mice as compared to DR mice

Gene	Gene name	Fold change	P value
1700001C02Rik	RIKEN cDNA 1700001C02 gene	-1.366	0.011
Gm1673	Predicted gene 1673	-1.331	0.035
1700027A23Rik	Primary cilia formation	-1.325	0.016
9030607L17Rik	IZUMO family member 4	-1.316	0.037
Slc6a7	Solute carrier family 6 (neurotransmitter transporter, l-proline), member 7	-1.313	0.040
Bola2	BolA-like 2 (<i>E. coli</i>)	-1.313	0.043
Rarres2	Retinoic acid receptor responder (tazarotene induced) 2	-1.311	0.012
LOC623121	PREDICTED: similar to Interferon-activatable protein 203 (Ifi-203) (interferon-inducible protein p203)	-1.278	0.015
Ppm1f	protein phosphatase 1F (PP2C domain containing)	-1.271	0.031
Nme5	NME/NM23 family member 5	-1.257	0.032
3010026O09Rik	RIKEN cDNA 3010026O09 gene	-1.253	0.048
Lhb	Luteinizing hormone β	-1.250	0.014

Discussion

Despite many years of effort, the mechanisms involved in the variability of body weight gain in response to an energy-rich diet remain unresolved. By using the C57BL/6J (B6) mouse strain, the present study has endeavoured to make further inroads into this understanding. Whereas others have shown this weight gain variability predominantly in male B6 mice (Huang *et al.* 2003a,b, Enriori *et al.* 2007, Peyot *et al.* 2010), the present study demonstrated the same phenomenon within a female population. Although reports have suggested that energy expenditure increases with obesity development (Leibel *et al.* 1995, MacLean *et al.* 2004), we showed no evidence of such changes at either a physical activity or an REE level to account for this group variance. However, because we did not directly measure NEAT (or fidgeting) in our mice, it may still be possible that this component could contribute to the variation, given the previous evidence from Levine *et al.* (1999) which demonstrated the importance of NEAT in weight gain responses to increased energy loads. Nonetheless, based on the present results, energy intake appears to be the major contributor to this weight gain difference in B6 mice.

Hypothalamic gene alterations, particularly in the ARC, have been suggested to be responsible for obesity susceptibility and resistance, particularly because of its critical role in appetite regulation (Huang *et al.* 2003a,b, Enriori *et al.* 2007). However, the present gene expression analysis showed that neither of the major orexigenic neuropeptides (*Npy* or *Agrp*) were different between the DIO and DR mice; this finding contrasts earlier studies (Huang *et al.* 2003a,b). Even the expression of *Socs3*, a factor involved in central leptin insensitivity (a potential mechanism for obesity susceptibility), which was shown previously to be up-regulated in a similar B6 DIO cohort

(where *Npy* and *Agrp* expression was unchanged) (Enriori *et al.* 2007), was unaltered in the present model. Differences in dietary composition could be a contributing factor, with our diet being rich in sweetened condensed milk-based fatty acids and fructose (Levin *et al.* 1997) compared to the others which were coconut and sunflower oil based (Huang *et al.* 2003a,b), as well as the fact that we used female mice, whereas previous reports have used males. In this respect, it is important to highlight the differences in fat distribution following HF feeding between the sexes: male mice deposit fat in all of the depots (Funkat *et al.* 2004), whereas female mice preferentially gain subcutaneous fat (Fig. 1C). Indeed, we were surprised to find that *Pomc*, the major melanocortin pathway precursor involved in appetite suppression, was significantly elevated in the present DIO mice. Although it is paradoxical, this has been reported previously in other HF fed mouse models (Ziotopoulou *et al.* 2000, Torri *et al.* 2002). We speculate that this up-regulation of the melanocortin pathway is meant to produce more of the active product, α -MSH, in an attempt to suppress the excess energy intake and to limit weight gain in DIO mice. However, the mice are clearly unresponsive. Defects downstream from *Pomc* (splicing into α -MSH or melanocortin 4 receptor expression/function defects) may account for this failure to regulate, although we did not directly measure these in the present study. Interestingly, the DR mice had similar neuropeptide expression as that of the chow-fed control mice, which indicates that hypothalamic changes are not associated with weight gain resistance. It is important to note that we did not assess protein levels to verify the gene expression data. Nevertheless, our findings imply that the known key regulators in the ARC may not directly contribute to the differences between DIO and DR mice and that the

Figure 5

Peripheral mRNA expression levels of *leptin* (A), *adiponectin* (B) and *Tnfα* (C) in subcutaneous, infra-renal and gonadal WAT depots from Chow, DR and DIO groups after 6 weeks of feeding. *Rn18s* was used as the housekeeping gene. Results are presented as means $\pm$ S.E.M. ($n=8-13$ per group) relative to Chow mice. ^a $P<0.05$ vs DR; ^b $P<0.05$ vs Chow (one-way ANOVA). Sub, subcutaneous; Infra, infra-renal; Gon, gonadal.

changes in DIO mice may be consequential rather than causative.

This surprising finding led us to perform microarray analysis in ARC samples of the mice to identify other genes that may be differentially regulated. A comparison between DIO and DR identified 58 genes in total, whereas a comparison with the chow-fed group highlighted 44 genes in the DIO and 112 genes in the DR group. This suggests that the susceptibility to diet-induced obesity is associated with a smaller number of ARC gene changes, whereas resistance to weight gain and maintaining the

same level of body weight as the chow-fed group requires a larger number of genes to be DE. Of the candidates identified in the DIO/DR screen, several have previously been implicated in obesity development in both human subjects and in mouse models. Specifically, the arrestin domain containing three gene (*Arrdc3*) (up-regulated 1.6-fold in the present DIO mice) has been shown through genome-wide association scans (GWAS) to be positively associated with increased BMI in human obesity and to play a primary role in adipose tissue in regulating energy expenditure (Patwari *et al.* 2011). Mice deficient in this gene have an obesity resistance phenotype via its positive effects on physical activity and thermogenesis (Patwari *et al.* 2011). Peroxisome proliferative activated receptor, γ , coactivator 1 β (*PPARGC1 β* , or *PGC-1 β*) has also been positively associated with obesity development in humans through the existence of at least 20 single nucleotide variants in its coding region (Andersen *et al.* 2005), and it has been found to be highly expressed in tissues such as skeletal muscle, BAT, heart and brain (Meirhaeghe *et al.* 2003). The present DIO mice indicate an ~ 1.4 -fold increase in the expression of *Ppargc1 β* as compared to DR mice. Glycogen synthase 3 kinase β (*Gsk3 β*) was also found to have a significant difference between the DIO and the DR mice (about 1.3-fold in favour of the DIO mice). Although it has more commonly been found in skeletal muscle to inhibit cellular responses to insulin, this gene has also been found to be present in neural tissues (Clodfelder-Miller *et al.* 2005). In fact, hypothalamic ARC activity of *Gsk3 β* has been shown to be increased in HF-fed and HF/high-fructose-fed mice (Benzler *et al.* 2012, Anderson *et al.* 2013), whereas overexpression in the mediobasal hypothalamus has been shown to lead to hyperphagia and obesity in mice that were challenged with an HF diet (Benzler *et al.* 2012). The insulin receptor substrate 2 (*IRS2*) gene, another important modulator of insulin signalling, has been associated with severe human obesity (Lautier *et al.* 2003), whereas the central ablation of *Irs2* in mouse models has been shown to lead to obesity (Lin *et al.* 2004, Sadagurski *et al.* 2012). However, a recent study has shown an opposite phenotype, with obesity induced by an HF/high-fructose diet leading to an increase in *Irs2* expression (Anderson *et al.* 2013). This may be in line with the present data, which indicates a ~ 1.3 -fold increase in the DIO group as compared to the DR group. Finally, another two potential candidates are the epigenetic modifiers chromodomain helicase DNA binding protein 2 (*Chd2*) and SET domain containing (lysine methyltransferase) 8 (*Setd8*); the former is involved in chromatin remodelling, and the latter is involved in

Figure 6

Peripheral mRNA expression levels of *Gp1* (A) and the fatty acid responsive receptors *Gpr40* (B), *Gpr120* (C), *Gpr119* (D), *Gpr41* (E) and *Gpr43* (F) in whole ileal tissue from Chow, DR and DIO groups after 6 weeks of feeding.

Rn18s was used as the housekeeping gene. Results are presented as means $\pm$ s.e.m. ($n=8-13$ per group) relative to Chow mice. $^aP<0.05$ vs DR; $^bP<0.05$ vs Chow (one-way ANOVA).

histone methylation. Both of these candidates were increased by 1.4- and 1.3-fold respectively in the present DIO mice. *CHD2* has previously been associated with T2D in human subjects in two GWAS studies (Diabetes Genetics Initiative of Broad Institute of Harvard and MIT *et al.* 2007, Zeggini *et al.* 2007), and *Chd2* was DE in a mouse model of HF-induced islet failure (Dreja *et al.* 2010). More recently, a study has shown increased whole-brain *Chd2* expression in an obese congenic strain (Sarahan *et al.* 2011). Similarly, increased expression levels of *Setd8* have been documented in the adipose tissue of both DIO and *ob/ob* mouse models and was found to be regulated by *Ppar γ* ; in these studies, adipogenesis occurred through chromatin modification (Wakabayashi *et al.* 2009, Okamura *et al.* 2010). Although these genes appear to be interesting potential candidates based on peripheral expression studies, more extensive work is required to identify their importance centrally and to determine whether they play a significant role in the differentiation between susceptibility and resistance.

Although hypothalamic regulation has always been viewed as the master controller of body weight, we cannot discount the importance of other central regions, including the brainstem. A recent study showed that exposure to HF during the perinatal period compromises the excitability and responsiveness of the gastric-projecting dorsal motor nucleus of the vagus (Bhagat *et al.* 2015). Furthermore,

lateral pontine parabrachial nucleus leptin receptor activation leads to reduced food intake (Alhadeff *et al.* 2014). Thus, there may well be differences between DIO and DR mice in the way that peripheral signals (e.g. leptin, adiponectin, *Gp1* and ghrelin) are processed in the hindbrain.

Of the major circulating factors, the adipokines leptin and adiponectin are key contributors to appetite and body weight regulation. As was expected, the present DIO mice showed elevated plasma leptin levels, and similar levels were seen between the DR and chow-fed mice. Interestingly, there appears to be a disconnect with gene expression levels: the DIO mice demonstrated similar expression to that of the chow-fed mice across all three fat depots, whereas the DR group displayed a twofold increase in infra-renal fat. This same difference between obesity-prone and resistant mice has been reported previously in obese B6 vs lean A/J mice following HF feeding (Watson *et al.* 2000). However, the fact that our DIO mice have the same level of expression as the chow-fed mice implies that susceptibility is not associated with defects in leptin gene expression, which is similar to other findings (Ahren *et al.* 1997, Van Heek *et al.* 1997). This increase in plasma leptin is likely reflective of total fat mass. In contrast, the present finding of increased expression in DR mice has to our knowledge not been reported previously. With respect to adiponectin, which in obesity and insulin-resistant states

is generally found to be reduced (Arita *et al.* 1999, Weyer *et al.* 2001), our DIO mice exhibited elevated plasma levels. However, there is some evidence to suggest that this is not always the case: one study showed no difference (Barnea *et al.* 2006), and another showed an increase in plasma levels (Perez-Echarri *et al.* 2005). At the gene level, adiponectin expression was reduced in the gonadal and infra-renal depots of the DIO mice, but there were no changes in the subcutaneous depot. Interestingly, the reports that have shown dissociation between obesity and plasma adiponectin also reported reduced adiponectin gene expression (Barnea *et al.* 2006), but they were not specific about the depot involved. Moreover, adiponectin is known to have an inverse relationship with the pro-inflammatory cytokine *Tnf α* ; increased plasma adiponectin suppresses *Tnf α* expression in adipose tissue (Maeda *et al.* 2002). The present results are also in line with this, in that they showed a reduction in *Tnf α* mRNA expression in the subcutaneous and infra-renal depots of the DIO mice. The DR mice, in contrast, had the same plasma adiponectin levels as the chow-fed mice and showed the same gene expression pattern of leptin, which suggests that the DR mice may experience increases in the amount of gene required to maintain the same plasma levels as the chow-fed group. Nonetheless, the present data collectively imply that adipose tissue may not be associated with susceptibility; rather, it may be an adaptive tissue in resistance.

A current focal area in the pathophysiology of obesity is the gut (Blad *et al.* 2012), which is responsible for the secretion of a number of hormones in response to nutrient intake and is important in appetite and body weight regulation. Whereas energy extraction and obesity have been associated with changes in the gut microbiome (Turnbaugh *et al.* 2006, 2008), the present study did not measure the microbiota profile of the mice or its metabolite by-products (such as long- and short-chain fatty acids). However, it is known that these by-products can affect the functional expression of intestinal nutrient-responsive *Gpr* and may suggest a role for gut microbiota in modulating satiation. Therefore, we measured ileal gene expression of *Gpr40*, *Gpr120*, *Gpr119*, *Gpr41* and *Gpr43*, all of which are modulated by fatty acids. *Gpr40* is known to be involved in the β -cell response of secreting insulin following fatty acid stimulation (Kebede *et al.* 2008), whereas all of these *Gpr* variants have been shown to be associated with *Glp1* secretion from the gut (Hirasawa *et al.* 2005, Edfalk *et al.* 2008, Lauffer *et al.* 2009, Blad *et al.* 2012). However, little is known about the contribution of these receptors to diet-induced weight gain. The present

data demonstrate that DIO mice have increased gut *Gpr40* gene expression but reduced *Gpr120* and *Gpr119* expression relative to DR and chow-fed mice, and *Gpr40* and *Gpr120* were similarly expressed between the DR and chow-fed mice. *Gpr41* also showed the same reduced expression pattern in the DIO mice, with a trend in *Gpr43* expression. A study by Duca *et al.* (2012) reported similarly increased *Gpr40* but reduced *Gpr120* and *Gpr41* gene expression in DIO rats as compared to DR rats; however, this was in response to an intragastric lipid load, and there were no chow-fed controls for comparison. The reduction in *Gpr* expression in the present study may also explain why we saw a reduction in *Glp1* mRNA expression in our DIO mice. It is clear that *Glp1* affects satiety by acting centrally (Lamont & Andrikopoulos 2014), and the reduced gut expression of this incretin may well contribute to the increased energy intake of the DIO mice. Although it is not entirely clear why *Gpr40* is markedly elevated in DIO mice, there may be a compensatory mechanism in place that activates this *Gpr* when the others are reduced. If and how this occurs is not known, however. The variable expression patterns that occur following exposure to the same diet could also infer polymorphic differences between DIO and DR mice in the transcriptional regulators of *Gpr*. Although it is uncertain how these *Gpr* changes interact and contribute to the obese or resistant phenotype, what is evident is that receptor expression differences appear to be important in the ability of B6 mice to respond to incoming nutrients, which implies a significant role of the gut in diet-induced weight gain and weight gain resistance.

Although the present study is descriptive in nature, the fact remains that we still do not have a firm grasp on why genetically similar mice that consume the same HED diet in the same environment can have vastly different body weight gain responses. We have endeavoured to shed some light on this by reporting the greater involvement of peripheral pathways (fat and gut) than hypothalamic pathways. It is indeed an important exercise to provide such descriptive reports, seeing as dietary composition, length of diet intervention and even the sex of the animal used can provide different outcomes from those already published, as is evident by the present study. Nonetheless, we demonstrated that susceptibility and resistance to diet-induced obesity is quite complex, and given the increased interest in gut microbiota in the development of obesity and its associated comorbidities, the changes in *Gpr* that were demonstrated in the present study provide us with a novel and plausible mechanism by which these differences may occur.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-15-0131>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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