Prenatal exposure to undernutrition and programming of responses to high-fat feeding in the rat

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Fetal undernutrition programmes risk of later metabolic disorders. Postnatal factors modify the programmed phenotype. This study aimed to assess the effects of a postnatal high-fat (HF) challenge on body weight gain, adiposity and gene expression following prenatal undernutrition. Pregnant rats were fed either a control diet or a low-protein (LP) diet, targeted at days 0–7 (LPE), days 8–14 (LPM), or days 15–22 (LPL) gestation. At 12 weeks of age offspring were either fed standard laboratory chow diet (4-13 % fat), or a 39-5 % fat diet, for 10 weeks. LP exposure had no effect on weight gain or abdominal fat in males. Females exposed to LP diet in utero exhibited a similar weight gain on HF diet as on the chow diet. Programming of fat deposition was noted in LPE females and males of the LPM and LPL groups (P<0.019). Hypothalamic expression of galanin mRNA was similar in all groups, but expression of the galanin-2 receptor was modified by LP exposure in female offspring. Hepatic expression of sterol response element binding protein (SREBP-1c) was decreased by LP at both the mRNA (P<0.008) and protein (P<0.001) level. HF feeding increased expression of SREBP-1c mRNA three-fold in controls, with little response noted in the LP groups. Interactions of factors such as postnatal diet, age and sex act together with prenatal factors to determine metabolic function and responsiveness at any stage of postnatal life. This study further establishes a role for prenatal nutrition in programming the genes involved in lipid metabolism and appetite regulation.

Fetal programming: Lipid metabolism: Rat: Gene expression: Transcription factors

The origins of the metabolic syndrome in humans are complex and multifactorial. The aetiology of all of the components of the syndrome (obesity, hyperinsulinaemia, dyslipidaemia, cardiovascular and renal disease) is likely to involve a variety of influences across the lifespan. The expression of genes that predispose to, or protect against any of these conditions will be modified through interactions with the postnatal lifestyle environment (diet, physical activity and smoking)1. Evidence for this prenatal component of disease risk comes from both epidemiological investigations and experimental studies in animals9.

Studies with animals show clearly that undernutrition in pregnancy is able to programme raised blood pressure, glucose intolerance, insulin resistance and obesity5. Exposure to a low-protein (LP) diet in fetal life programmes a phenotype that resembles the metabolic syndrome in ageing rats6,7. In such animals the expression of transcription factors regulating lipogenesis, and their downstream target genes are noted to be suppressed until nine months of age. Over-expression in later adulthood occurs coincident with the appearance of metabolic disorders including hepatic steatosis6,7.

The transcription factor, sterol response element binding protein (SREBP)-1c, is one of the key regulators of hepatic lipid metabolism8. SREBP-1c activates genes for enzymes involved in the biosynthesis of fatty acids and triacylglycerols and is therefore regarded as a promoter of lipogenesis. We have shown that programming the expression of SREBP-1c may play a critical role in determining the metabolic consequences of prenatal undernutrition. Increased hepatic lipogenesis, as noted in older animals subject to prenatal protein restriction, will promote steatosis and excess adiposity9,10.

Risk of obesity and related disorders may also be programmed in utero through changes in appetite, feeding behaviour and physical activity11,12. Earlier studies in our laboratory have shown that offspring of rats fed LP diets exhibit altered self-selection feeding behaviours. Whilst rats exposed to LP diets throughout fetal development showed an increased preference for fat13, the converse occurred when LP feeding was targeted at brief periods in early, mid or late gestation14. A number of neuropeptides are known to control feeding behaviour at the level of specific hypothalamic nuclei. In particular the galaninergic system is believed to regulate intake of fat15.

In contrast to older animals, young adult rats exposed to LP feeding during specific periods of fetal life appear resistant to

Abbreviations: gal2r, galanin-2 receptor; LP, low protein; LPE, LP diet targeted at early gestation; LPM, LP diet targeted at mid gestation; LPL, LP diet targeted at late gestation; SREBP, sterol response element binding protein.

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obesity. The aim of this study was to consider whether a postnatal challenge with a high-fat diet might overcome this resistance. As hepatic SREBP-1c is known to be up-regulated by the feeding of a diet rich in saturated fatty acids, the initial hypothesis for this study was that programmed suppression of expression of this transcription factor would be offset by a high-fat challenge, permitting a greater rate of lipogenesis. Given the altered preferences of LP-exposed rats for high-fat sources, the role of the galanergic system in this behaviour was evaluated by comparing the hypothalamic expression of galanin and the galanin-2 receptor (gal2r) under conditions of low- and high-fat intake.

Materials and methods

Animals

Experiments described in this paper were performed under licence from the UK Home Office in accordance with the 1986 Animals Act. Rats were housed in plastic boxes on a 12-h light cycle, at a temperature of 20 (SEM 2)°C. The rats had free access to food and water at all times. Twenty virgin female Wistar rats (Harlan Ltd, Belton, Leics., UK) were mated at weights between 180 and 220 g. Only two male studs were used to generate the pregnancies, thereby reducing genetic variation in the offspring produced. Upon confirmation of mating by the appearance of a semen plug samples using the TRIzol method (Invitrogen, Southampton, UK). The hypothalamus was dissected from the whole brain at the time of cull. Hypothalamus and liver were snap-frozen in liquid N2 and stored at −80°C prior to analysis of gene expression.

Determination of hypothalamic mRNA expression

Total RNA was isolated from snap-frozen hypothalamus samples using the TRIZol method (InVitrogen, Southport, UK). The RNA was treated with DNase (Promega, Southampton, UK) and subjected to phenol−chloroform extraction and ethanol precipitation. Total RNA (0.5 μg) was reverse-transcribed using MMLV Reverse Transcriptase (Promega, UK). Real-time PCR was performed using an ABI prism 7700 sequence detection system (Applied Biosystems, Warrington, UK). A template-specific primer pair and an oligonucleotide probe (Sigma-Genosys, Haverhill, UK) specific to each of galanin, gal2R and the house-keeping gene β-actin were designed using Primer Express version 1.5 (Applied Biosystems). The full sequences of the primers and probes are reported in Bellinger et al. All primer sets were tested under the Taqman PCR conditions using rat genomic DNA as a template. In all cases a single product of the appropriate size was detected by gel electrophoresis (data not shown). A negative template control and relative standard curve were included on every PCR run. The standard curve was prepared from a pool of sample cDNA at relative dilutions of 0.05, 0.1, 0.2, 0.4, 1.0, 2.5 and 5.0 Relative target quantity was calculated from the standard curve and all samples were normalized to β-actin expression. Hypothalamic β-actin mRNA expression did not vary significantly between the different experimental groups.

Determination of hepatic mRNA expression

Hepatic mRNA was isolated using the phenol−chloroform extraction method described by Chomczynski and Sacchi. cDNA was synthesized using the Taqman Reverse Transcription Reagents kit and then quantitative real-time PCR was performed using the ABI Prism 7700 Sequence detection system (Applied Biosystems). Fluorogenic probes were labelled with 6-carboxy-fluorescein at the 5’ end and with 6-carboxy-tetramethyl-rhodamine at the 3’ end. SREBP-1c mRNA expression was normalized relative to eukaryotic 18S rRNA. Hepatic 18S rRNA expression did not vary significantly between the different experimental groups. The SREBP-1c forward and reverse primers sequences were as reported by Kakuma et al.
The probe sequence was ACATGCTTCAGCTCATCAA-CAAC and designed using primer 3 www.cgi v 0.4 Software (Whitehead Institute for Biomedical Research, Cambridge, USA). 18S rRNA primers and probes were supplied by Applied Biosystems.

**Determination of hepatic protein expression**

The expression of SREBP-1c protein in the liver was quantified using Western blotting, as described previously. Briefly, hepatic protein was extracted using the method of Chomczynski and Sacchi. Isolated protein concentrations were quantified using the method of Lowry, adapted for use in a 96-well micro-assay plate. The protein sample (50–75 μg) was denatured by boiling for 5 min and samples were then loaded onto a SDS polyacrylamide gel for separation (40 milligrams for 2 h). Separated proteins were transferred on to Hybond ECL (enhanced chemiluminescence; Amersham-Pharmlia Biotech, Little Chalfont, UK) nitrocellulose membrane and after blocking of non-specific binding sites, membranes were incubated overnight with primary antibody solutions at 4°C. For SREBP-1c the primary antibody was SREBP-1 specific, developed in mice (ATCC, Middlesex, UK). Expression of α-actin protein was used to normalize SREBP-1c expression. For this a rabbit anti-α actin was used as the primary antibody (Sigma-Aldrich, St Louis, USA).

After this incubation, further washing and blocking steps were followed by incubation with a horseradish peroxidase-labelled secondary antibody to allow imaging of antibody binding using AIDA Image Analyser software (Raytest, GmbH Straubenhardt). For SREBP-1c the secondary antibody was peroxidase conjugated rabbit anti-mouse (Dako, Ely, Cambridgeshire, UK). For α-actin the secondary antibody was peroxidase conjugated swine anti-rabbit (Dako).

**Statistical analysis**

Results are presented as means with their standard errors. All data were analysed using 2- or 3-way ANOVA as appropriate, to consider effects of maternal diet, offspring sex and postnatal diet. As groups were of unequal size type III analyses were performed. ANOVA was followed by a least significant difference test as a post hoc test for univariate effects, where appropriate. Post hoc tests cannot be performed to determine group to group differences where interactions of factors are noted, for example maternal diet and sex. As multiple pups from the same dam were included in the experimental groups a mixed model analysis was performed, to adjust for within-litter effects as recommended by Festing. P < 0.05 was accepted as statistically significant.

**Results**

The weights of the 12-week old animals in each group were similar at the start of the trial, although females were significantly (P < 0.05) lighter than males (data not shown). Over the 10 weeks of the high-fat feeding trial the animals were still growing and therefore significant (P < 0.05) weight gain was seen in all cases. Weight gain in the high-fat groups was generally significantly (P < 0.05) higher than in the Chow-fed animals, although this difference was less marked in the female animals (35–79% greater in high-fat-fed males compared to Chow groups, 8–44% greater in high-fat-fed females compared to Chow groups; Table 1). Females exposed to LP in utero and fed high-fat diet did not gain more weight than their Chow-fed counterparts. In absolute terms (g food intake/d, data not shown), females had lower food intakes than males and accordingly gained less weight. Table 1 shows food intake corrected for body weight to examine differences independently of the greater weight achieved by the high-fat-diet-fed groups. On these terms food intake was not influenced by the maternal diet and was greater in females than in males. High-fat feeding resulted in a decreased food intake (29–40% lower) in all groups as the animals rapidly adjusted intake to compensate for the greater energy density of the diet.

At the end of the trial it was noted that the high-fat diet had reduced the size of the liver relative to body weight (10–22% reduction) in males and females of all groups (Table 2).

<table>
<thead>
<tr>
<th>Table 1. Weight gain and food intake of rats exposed to diets of differing composition in utero and fed chow or high-fat diets in adult life. (Values are means with their standard errors for n observations per group)</th>
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</thead>
<tbody>
<tr>
<td>Maternal diet group*</td>
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<tr>
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<tr>
<td>Weight gain (g)</td>
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<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Food intake (g/d per kg body wt)</td>
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* ANOVA indicated that weight gain was influenced by postnatal diet (P < 0.001), sex (P < 0.001) and the interaction of sex and postnatal diet (P < 0.001). The interaction of postnatal diet, sex and maternal diet approached significance (P = 0.051). Food intake was determined by postnatal diet (P < 0.001) and sex (P < 0.001).

Indicates a significant difference between animals of same sex related to postnatal diet. Post hoc testing was only applied to univariate effects, so differences arising through interactions of factors are not indicated in the table.
The size of the gonadal fat pad was increased (20–76% larger) in animals that had been fed the high-fat diet, and although males of the LPM group and females of the LPE group showed no significant change at this fat depot, no significant influence of maternal diet was noted. However, for the perirenal depot (Table 2) more complex influences were noted. Among male animals fed chow, the LPM group had significantly (15–20%) less fat at this site than animals in all other groups. On feeding a high-fat diet, males of the LPL group showed a particularly strong response and deposited 32% more fat at the perirenal depot than control males. Among females, the LPE group had 22% less perirenal fat than control animals after the feeding of a high-fat diet. Non-fasting plasma insulin concentrations (Fig. 1) differed greatly between males and females (P<0.001), particularly among rats fed a high-fat diet. Whereas high-fat-fed males tended to increase plasma insulin by 44–62% compared to chow-fed rats, females maintained a similar insulin concentration (Fig. 1). Maternal diet had significant effects upon insulin in males, but not females. LPM males had high plasma insulin (2.0-fold higher than controls) when fed chow (Fig. 1(A)), whilst among high-fat-fed males both LPM and LPL were hyperinsulinemic relative to the controls (Fig. 1(B)).

The hepatic expression of SREBP-1c mRNA differed markedly between the different maternal dietary groups (Fig. 2(A)). Among chow-fed animals exposed to maternal LP diet in utero, expression was approximately half that seen in controls, irrespective of the timing of protein restriction. Control animals exhibited a major (3-fold) increase in expression when fed a high-fat diet for 10 weeks, and whilst the same response was noted among the LP-exposed rats, this was blunted and expression remained significantly (P<0.05) below that noted in control animals. SREBP-1c is synthesized as an immature 125 kDa protein that is inserted into the endoplasmic reticulum and nuclear membrane. The mature form of the protein (60 kDa) is produced in response to insulin stimulation and is the active transcription factor. Expression of both forms of the SREBP-1c protein (only mature expression data shown) essentially mirrored that of the mRNA (Fig. 2(B)), although the differences between expression between chow and high-fat feeding were not significant for the LPM and LPL groups.

The hypotalamic expression of galanin mRNA was unrelated to maternal diet, offspring sex or the postnatal diet (Table 3). However, expression of the gal2r was influenced by interactions of all three factors (maternal diet × sex, P=0.037; maternal diet × postnatal diet, P=0.015). In males expression tended to be lower (15–20%) in the high-fat-fed animals than in chow-fed rats. Among females, LPM rats fed chow tended to have high gal2r expression, which declined by 37% on feeding high-fat diet. LPL exposed females exhibited a 50% increase in expression when comparing high-fat-fed rats to chow-fed animals of the same maternal dietary group, a response not noted in any of the other groups.

**Discussion**

Interest in the potential for prenatal nutrition to influence long-term adiposity and hence risk of associated metabolic disorders originally arose from the outcomes of follow-up studies of individuals subject to intrauterine famine in the Dutch Hunger Winter. Moderate restriction of rations during specific periods of pregnancy was found to increase risk of obesity in individuals subject to restriction in early gestation,
whilst those subject to undernutrition during the final trimester appeared relatively obesity-resistant. In keeping with these observations some animal experiments have demonstrated prenatal programming of obesity, most notably those of Vickers and colleagues\textsuperscript{11}, who showed that restriction of pregnant rats to 30\% of \textit{ad libitum} intakes produced gross adiposity in their mature offspring. However, some nutritional manipulations in rodent pregnancy that are known to produce cardiovascular and metabolic disturbances have not been found to induce a greater rate of fat deposition\textsuperscript{27}. An important limitation of our study was the comparison of a synthetic high-fat diet with an open formula chow diet. These were not equivalent in their formulation, presentation (chow was \textit{pelletted}, high-fat diet was provided as large balls) and palatability. Although the main difference in composition was the fat content, it must be acknowledged that other differences in composition and ingredients might explain the observed results.

The maternal LP diet protocol used in this experiment is long-established as a model system for the investigation of blood pressure programming\textsuperscript{28}. A similar protocol generates offspring which develop insulin resistance\textsuperscript{29}. We and others have previously reported that when fed standard laboratory chow (low-fat) diets, body-weight gain and fat deposition is similar in young adult rats exposed to LP \textit{in utero} compared to control animals\textsuperscript{13,27}. This is in contrast to the reports of Vickers \textit{et al.}\textsuperscript{11} and may be explained by differences in the metabolic profile programmed by different intrauterine exposures.

Whilst we have noted young offspring of low-protein fed dams to have similar body composition to controls\textsuperscript{13}, Jones and Friedman\textsuperscript{30} reported that LP diets in pregnancy induce excess adiposity in the offspring if combined with a postnatal high-fat diet. The present experiment set out to explore the
hypothesis that weight, body fat, regulation of food intake and metabolic response to a hyperenergetic, high-fat diet would be altered by prenatal protein restriction. Our initial hypothesis was not supported by the data, as we observed that feeding a high-fat diet to mature rats for 10 weeks produced no clear programmed effect upon fat deposition. However, despite the lack of variation in adiposity it was clear that short, specific periods of protein restriction in utero did promote hyperinsulinaemia and altered expression of SREBP-1c. In addition to programming of basal expression, the nutritional regulation of SREBP-1c is subject to prenatal programming influences.

Rats exposed to LP diet in utero exhibit altered preferences for macronutrients when allowed to self-select. Targeting of LP diet to the early, mid and late periods of gestation, as in this paper, was associated with selection of a low-fat carbohydrate diet. As galanin acts within the hypothalamus to regulate fat intake, and the gal2r has a key role in controlling appetite for fat, we studied hypothalamic gene expression for both peptides. No programmed differences in galanin expression were noted in that previous study. This is in keeping with earlier work and the current observations of no programmed changes in galanin expression. Expression of gal2r was also unaltered in chow-fed animals, but interestingly the high-fat diet challenge did reveal some programming effects. LPL females had increased receptor expression whilst LPM females exhibited a decrease. The food intakes of both groups were identical and were similar to those of controls. These findings are consistent with earlier work and the current observations of no programmed changes in galanin expression. Expression of gal2r was also unaltered in chow-fed animals, but interestingly the high-fat diet challenge did reveal some programming effects. LPL females had increased receptor expression whilst LPM females exhibited a decrease. The food intakes of both groups were identical and were similar to those of controls.

In rats fed the Chow diet we found that hepatic expression of SREBP-1c was suppressed by prenatal protein restriction at any stage of fetal development. The expression of this transcription factor is modulated by nutritional signals, including insulin and glucagon. High-fat feeding in mice induces SREBP-1c expression, as noted in the control group in the present experiment. Saturated fatty acids and cholesterol mediate this effect, whilst PUFA reduce mRNA expression and post-translational processing of the protein. However, as all groups of rats fed Chow in the present study consumed the same diet and had similar food intake, the suppression of SREBP-1c expression by prenatal LP feeding cannot be explained by variance in nutrition-related signals.

Exposure to LP diet at any of the three stages of gestation was associated with an attenuated up-regulation of hepatic SREBP-1c expression in the liver. As SREBP-1c favours fat synthesis and deposition the immediate implications of the findings for rats of this age are likely to be a relative resistance to obesity. This may explain differences in adiposity between offspring of protein-restricted mothers and those subject to global undernutrition in utero. Expression of SREBP-1c increases markedly with age in LP-exposed rats, consistent with the development of insulin resistance. The present study found that insulin was elevated in males of the LPM group when fed the Chow diet, and in the LPL group on high-fat feeding. These observations may provide an early sign of the metabolic disturbances that develop with age in the prenatally undernourished animal. Up-regulation of SREBP-1c in older LP-exposed rats may be driven by their developing hyperinsulinaemia.

By generating groups of offspring subject to protein restriction at different stages of development we intended to explore the possibility that programming of gene expression in the target organs occurs during a specific critical window during fetal life. The finding that protein restriction generated a broadly similar phenotype regardless of the timing of the insult was an important observation allowing different inferences to be made. It is possible that the expression of SREBP-1c and sensitivity to nutritional signals is equally sensitive to nutritional programming at any stage of fetal life. Alternatively it may be argued that the sensitive period for programming of lipid metabolism and for appetite regulation lies in mid to late gestation as the few variations seen between LP groups, for example insulin concentrations in males or gal2r expression in females, tended to occur in LPM and LPL groups.

### Table 3. Hypothalamic expression of galanin and galanin-2 receptor mRNA in rats exposed to diets of differing composition in utero and fed Chow or high-fat diets in adult life.

(Values are means with their standard errors for n animals per group.)

<table>
<thead>
<tr>
<th>Maternal diet group†</th>
<th>Control</th>
<th>LPE</th>
<th>LPM</th>
<th>LPL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td><strong>Postnatal diet</strong></td>
<td><strong>Mean SEM</strong></td>
<td><strong>Mean SEM</strong></td>
<td><strong>Mean SEM</strong></td>
</tr>
<tr>
<td><strong>Galanin (arbitrary units)</strong></td>
<td><strong>Male</strong></td>
<td><strong>Chow</strong></td>
<td>0.91 0.09</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>High fat</strong></td>
<td>0.72 0.20</td>
<td>0.68 0.21</td>
</tr>
<tr>
<td></td>
<td><strong>Female</strong></td>
<td><strong>Chow</strong></td>
<td>0.86 0.09</td>
<td>0.99 0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>High fat</strong></td>
<td>1.08 0.28</td>
<td>0.89 0.38</td>
</tr>
<tr>
<td><strong>Galanin-2 receptor (arbitrary units)</strong></td>
<td><strong>Male</strong></td>
<td><strong>Chow</strong></td>
<td>1.39 0.30</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>High fat</strong></td>
<td>1.09 0.28</td>
<td>0.95 0.17</td>
</tr>
<tr>
<td></td>
<td><strong>Female</strong></td>
<td><strong>Chow</strong></td>
<td>1.05 0.01</td>
<td>1.23 1.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>High fat</strong></td>
<td>1.15 0.45</td>
<td>1.00 0.09</td>
</tr>
</tbody>
</table>

LPE, low-protein feeding targeted to days 0–7 of gestation; LPM, low-protein feeding targeted to days 8–14 of gestation; LPL, low-protein feeding targeted to days 15–22 of gestation; ND, not determined.

* For n see Table 1.

† All data was normalised for the expression of β-actin mRNA, which was unaltered by treatments. ANOVA indicated that expression of galanin-2 receptor was influenced by the interaction of postnatal diet and sex (P = 0.001), the interaction of maternal diet and postnatal diet (P = 0.015) and by the interaction of maternal diet and sex (P = 0.037).
Sex-specific effects of protein restriction were noted in this study. Hypothalamic expression of gal2r appeared sensitive to prenatal influences only in female offspring. To some extent this is consistent with data relating to feeding behaviour in other studies, where programming of appetite was greater in females than in males\textsuperscript{12,14}. We also noted that only males exposed to LP in utero developed hyperinsulinaemia. Ozanne and colleagues have also provided evidence of sex-specific programming of insulin and glucose metabolism. In their studies whilst males exposed to LP diets in fetal life exhibited frank diabetes by 17 months of age\textsuperscript{42}, females only developed impaired glucose tolerance and at a greater age\textsuperscript{43}.

It is clear from this study that complex interactions of factors such as postnatal diet, age and sex will act together with prenatal factors to determine metabolic function, responsiveness and susceptibility to disease at any stage of postnatal life. This is consistent with data emerging from epidemiological studies which also suggest that fetal growth retardation followed by rapid catch-up growth and excessive fat gain in adolescence may be the strongest predictors of adult metabolic syndrome\textsuperscript{44,45}. This study further establishes a role for prenatal nutrition in programming the genes involved in lipid metabolism. The mechanism through which this occurs remains to be resolved although a role for tissue remodelling can be invoked.

Acknowledgements

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