

Maternal Nutrient Restriction During Late Gestation and Early Postnatal Growth in Sheep Differentially Reset the Control of Energy Metabolism in the Gastric Mucosa

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Fetal growth restriction followed by accelerated postnatal growth contributes to impaired metabolic function in adulthood. The extent to which these outcomes may be mediated centrally within the hypothalamus, as opposed to in the periphery within the digestive tract, remains unknown. In a sheep model, we achieved intrauterine growth restriction experimentally by maternal nutrient restriction (R) that involved a 40% reduction in food intake through late gestation. R offspring were then either reared singly to accelerate postnatal growth (RA) or as twins and compared with controls also reared singly. From weaning, all offspring were maintained indoors until adulthood. A reduced litter size accelerated postnatal growth for only the first month of lactation. Independently from postnatal weight gain and later fat mass, R animals developed insulin resistance as adults. However, restricted accelerated offspring compared with both the control accelerated and restricted restricted offspring ate less and had higher fasting plasma leptin as adults, an adaptation which was accompanied by changes in energy sensing and cell proliferation within the abomasum. Additionally, although fetal restriction down-regulated gene expression of mammalian target of rapamycin and carnitine palmitoyltransferase 1-dependent pathways in the abomasum, RA offspring compensated for this by exhibiting greater activity of AMP-activated kinase-dependent pathways. This study demonstrates a role for perinatal nutrition in the peripheral control of food intake and in energy sensing in the gastric mucosal and emphasizes the importance of diet in early life in regulating energy metabolism during adulthood. (*Endocrinology* 152: 2816–2826, 2011)

The low birth weight newborn, especially when experiencing rapid postnatal growth, can be at greater risk of developing the metabolic syndrome later in life (1, 2). This relationship can reflect long-term fetal and postnatal adaptations to the nutritional environment (3), although the consequences for the regulation of energy homeostasis

have yet to be established. Fetal growth is greatest over the final quarter of gestation, which is the time when endocrine functions linked to maturation of the hypothalamus are established (3, 4). It is also the period during which changes in maternal diet can have the greatest effect on birth weight in both sheep (5) and humans (6).

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Abbreviations: ACC α , Acetyl coenzyme A carboxylase; AdnR, adiponectin receptor; AMPK, AMP-activated kinase; AUC, area under the curve; C, control; CA, control accelerated; CPT1, carnitine palmitoyltransferase protein; dGA, day of gestation; FGR, fractional growth rate; FTO, fat mass and obesity-associated protein; IUGR, intrauterine growth restriction; MC4R, melanocortin 4 receptor; ME, metabolisable energy; mTOR, mammalian target of rapamycin; NPY, neuropeptide Y; ObR, leptin receptor; PCNA, proliferating cell nuclear antigen; PGC1, PPAR γ coactivator 1; PPAR, peroxysome proliferator-activated receptor; PTP1B, tyrosine-protein phosphatase nonreceptor; R, nutrient restriction; RA, restricted accelerated; RR, restricted restricted; SIRT1, sirtuin; TUNEL, terminal transferase-mediated 2'-deoxyuridine, 5'-triphosphate nick end labeling; UCP2, uncoupling protein 2.

Nutritionally programmed changes in the control of appetite due to interactions between trophic, energetic, hormonal, and epigenetic factors (7) have been described in both rodents (8, 9) and sheep (10), and these may ultimately determine the long-term regulation of energy balance. They involve changes in leptin sensitivity (11) and expression of a range of hypothalamic neuromediators, including proopiomelanocortin, neuropeptide Y (NPY), and the melanocortin 4 receptor (MC4R) (12). To date, these observations in sheep have been established after nutritional challenges specifically targeted during organogenesis of the fetal hypothalamus (*i.e.* early- to midgestation). Such early interventions do not, however, affect birth weight or postnatal growth (10). The influences of maternal nutrient restriction (R) during late gestation on the long-term control of food intake and metabolic health, therefore, remain to be fully established.

Currently, little is known about the potential programming outcomes in peripheral organs that also control appetite and regulate whole body energy homeostasis. This is surprising, considering the critical influence the perinatal period can have on the development of the gastrointestinal tract, a major complex organ actively involved in the control of energy balance (13). Indeed, the development of the stomach *in utero* is partly dependent upon fetal swallowing of amniotic fluid (14), which is regulated centrally and is confined to periods of fetal breathing that are, in turn, influenced by maternal energy intake (15) and neuroendocrine factors such as NPY (14). Immediately after birth, the gastrointestinal tract undergoes a pronounced transformation exhibiting rapid growth and a marked increase in acid production (16), at a time when milk intake and composition can both determine gastric barrier function (17). However, the extent to which changes in maternal food intake can contribute to long-term changes in gut function remains to be fully established.

Critically, both centrally, *i.e.* in the hypothalamus and in peripheral tissues, the control of food intake is regulated through common energy-sensing pathways that integrate cellular energy concentration (*i.e.* AMP:ATP ratio) and endocrine signals. For example, the leptinaemic signal to reduce food intake only occurs at high cellular concentrations of ATP (18, 19). Conversely, in the gastric mucosa, the hunger hormone ghrelin is only synthesized when ATP concentrations are depleted (20). This endocrine energy-sensing cross talk is governed by two proteins, AMP-activated kinase (AMPK) and the mammalian target of rapamycin (mTOR), which are also closely linked in the regulation of oxidative and inflammatory processes (19, 21, 22). The activity and intensity of AMPK and mTOR responses are dependent on mitochondrial activity that

involves the transcription factors carnitine palmitoyl-transferase protein (CPT1) (23) and peroxysome proliferator-activated receptor (PPAR) γ that favor fatty acid transport and β -oxidation into the mitochondria. In addition, the contents of mitochondria are regulated, in part, by the mitochondrial biogenitor PPAR γ coactivator 1 (PGC1) α (24). Furthermore, although it has been established that ghrelin production in the stomach is regulated by mTOR-dependent pathways (20, 22), whether this can be set in early life is unknown. The extent to which exposure to a chronic low caloric nutritional environment during late gestation, a critical period in the acquisition of fetal endocrine functions (3, 25), can program tissue energy sensitivity was a further focus of the present study.

Materials and Methods

Animals and experimental design

All animal procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986 with approval from the Local Ethics Committee of the University of Nottingham (Nottingham, UK).

Experimental design

A summary of the animal protocol is illustrated in Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>.

Dietary intervention in pregnancy

At 100 d of gestation (dGA), 26 Bluefaced Leicester cross Swaledale twin bearing sheep were individually housed, and on dGA 110, they were randomly allocated to one of two nutrition groups until normal delivery at term ($\sim 145 \pm 2$ d). Thus, a control (C) group of nine pregnant sheep was fed to completely meet their metabolisable energy (ME) requirements (*i.e.* from $0.46 \text{ MJ/kg} \cdot \text{body weight}^{0.75}$ at dGA 110 increasing to $0.72 \text{ MJ/kg} \cdot \text{body weight}^{0.75}$ at dGA 130) (26), whereas the remaining 17 pregnant sheep were all R and fed a diet 60% of this amount. Each pregnant sheep was weighed on a weekly basis before feeding, after which their total food requirements were adjusted as necessary. The diet comprised a mix of 40% concentrated pellets and 60% straw nuts (Manor Farm Feeds, Oakham, UK) that had an estimated ME content of 12.6 MJ/kg and a crude protein content (nitrogen $\times 6.25$) of 162 g/kg and 8.6 MJ/kg and a crude protein content of 69 g/kg dry matter, respectively. In addition, all pregnant sheep had free access to a mineral block to ensure adequate micronutrient supply.

Lactation

All mothers were fed to fully meet their ME requirements throughout lactation. Within 12 h of birth, each pair of twins was either separated, with only one twin being reared by its mother to accelerate postnatal growth, or reared together by their mother as twins (R). All nine animals successfully completed the study in each of the control accelerated (CA) and restricted accelerated (RA) groups (CA: four females, five males; RA: seven females, two males). Both offspring born to restricted restricted

(RR) mothers were kept together with their mothers through to weaning, but only one twin from each mother was subsequently placed in an obesogenic environment (see below, six females and two males). Offspring were weighed twice a week during the first month of postnatal life and once a week thereafter until weaning. For ethical considerations intended to reduce the number of animal used in such studies, the remaining twin of the R pair was not used in this experiment but allocated to an independent study (see Supplemental Fig. 1).

Postweaning

All offspring were weaned at 3 months of age. All were raised in the same restricted indoor environment thereafter to promote obesity (27) and weighed monthly.

Fractional growth rate (FGR) from birth to 40 d of age and from 3 to 7 months of age was determined in each animal using the following formula:

$$\text{FGR 1–40 d} = (\text{weight at 40 d} - \text{weight at birth}) / \text{weight at birth}$$
$$\text{FGR 3–7 months} = (\text{weight at 7 months} - \text{weight at 3 months}) / \text{weight at 3 months}$$

Blood samples (5 ml) were collected in the morning before feeding at 3, 7, and 16 months of age from each animal, collected from the jugular vein into heparinized and K+EDTA-coated tubes. The plasma was immediately separated by centrifugation ($2500 g \times 10 \text{ min}$ at 4 C) and stored at -80 C until analysis. Additional blood sampling over a 24-h period, at 2, 4, 8, and 24 h after feeding, was undertaken at 16 months of age. In addition, glucose tolerance tests were undertaken on all offspring at 8 and 17 months of age after an overnight fast and the area under the curve (AUC) calculated (10).

Measurement of food intake

At 16 months of age, for 2 wk before the end of the study, all offspring were housed individually indoors in United Kingdom Home Office designated floor pens (3 m^2) to monitor food intake and appetite. For each animal, daily energy intake was assessed through weighed intake and food refusal when offered sufficient energy for the 24-h period based on a mix of low (straw nuts, 8.9 MJ/kg) and 800 g of high (concentrate pellets, 12.6 MJ/kg) energy-dense food.

Physical activity

The level of spontaneous physical activity at adulthood was determined using uniaxial accelerometers (Actiwatch; Linton Instrumentation, Diss, UK) (10). A ratio between physical activity and food intake was calculated at 16 months of age as previously described (10).

Body composition

Total body fat, fat free mass, and bone mineral density was determined at 8 and 16 months of age when the animal was sedated (im injection of $1.5 \text{ mg} \cdot \text{kg}^{-1}$ ketamine with $0.1 \text{ mg} \cdot \text{kg}^{-1}$ xylazine) and scanned in a transverse position using a Lunar DPX-L (fast-detail whole body smartscan).

Postmortem analysis

At 17 months of age, the animals were humanely euthanized by electrical stunning and exsanguination after an overnight fast. All the major organs were weighed and representative samples

snap frozen in liquid nitrogen and stored at -80 C as was the entire hypothalamus of each animal (10).

Laboratory analysis

Plasma metabolite and hormones

Glucose, nonesterified fatty acid and triacylglycerol concentrations were determined by colorimetric assays (Randox, Crumlin, UK). Insulin was assayed using an ovine-specific ELISA (Mercodia; Diagenics Ltd., Milton Keynes, UK), and leptin was determined by a radio-immunoassay (10) as was cortisol (Diagnostic Products Corporation coat-a-count; Siemens, Camberley, UK).

Gene expression

This was determined by real-time RT-PCR. Total RNA was extracted from entire hypothalamus, abomasum, omental, and sc adipose tissue using the Chomczynski and Sacchi method (28, 29) with deoxyribonuclease treatment (Rneasy Plus mini kit; QIAGEN, Crawley, UK). It was then reverse transcribed (Superscript II reverse transcriptase; Invitrogen Ltd., Pasley, UK) and cDNA amplified on a real-time thermocycler (Quantica; Techne, Inc., Barloword Scientific Ltd., Stone, UK) using SYBR green based *Taq* polymerase reaction mix (ABsolute blue QPCR SYBR green; Thermo Scientific, Epsom, UK). Product specificity for every pair of primers was confirmed by sequencing and the efficiency of the primer sets, within the range of requested DNA amplification, assessed and optimal efficiency established ($2.00 \pm 5\%$). Hypothalamus were analyzed for insulin receptor, leptin receptor (ObR), tyrosine-protein phosphatase nonreceptor (PTP1B), adiponectin receptors (AdnR) (AdnR1 and AdnR2), suppressor of cytokine signaling, ghrelin receptor, melanocortin receptors (MC4R and MC3R), agouti-related peptide, NPY, fat mass and obesity-associated protein (FTO), AMPK α , and mTOR. Abomasal samples were analyzed for ghrelin, leptin, AMPK α 2, acetyl coenzyme A carboxylase (ACC α), dinucleotide methyl transferase type I, FTO, mTOR, CPT1, ObR, insulin receptor, uncoupling protein 2 (UCP2), PPAR γ , PGC1 α , and sirtuin (SIRT1). Omental and sc adipose tissues were analyzed for leptin, adiponectin, IL-6, IL-18, TNF α , toll-like receptor 4, glucose-related peptide 78, FTO, and macrophage chemoattractant protein 1. Ribosomal 18S RNA and ribosomal protein large P0 were used as housekeeping genes. Gene expression was determined by using the $2^{-\Delta\Delta\text{CT}}$ calculation.

Protein abundance

AMPK α and phospho-AMPK α were analyzed by immunoblotting. Total proteins were extracted from abomasal samples (30) using ice-cold lysis buffers (Sigma, Hertfordshire, UK) and protease inhibitors. After reduction and denaturation with sodium dodecyl sulfate at 100 C, 10 μg of protein extracts were separated by vertical, polyacrylamide-based electrophoresis. After migration and electrical protein transfer, nitrocellulose membranes were blocked overnight and blotted with antibodies for either AMPK α , phospho-AMPK α (catalog no. 23A3 and Thr172, respectively; Cell Signaling, Hitchin, UK), or β -actin (Abcam, Cambridge, UK). Relative protein abundance was then determined using chemiluminescence (Immobilon Western Chemiluminescence; Millipore, Watford, UK) and the image analyzed (Aida Image Analyzer; Raytek Scientific Ltd., Sheffield, UK).

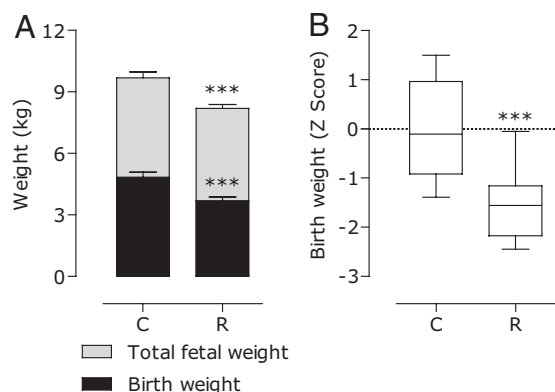


FIG. 1. Maternal R in late gestation induced IUGR. Influence of a 40% R on birth weight of (A) siblings and (B) standardized Z score for birth weight. Values are mean \pm SEM and significant differences between groups, ***, $P < 0.001$.

Immunohistochemistry

Tissue fixation

Snap-frozen abomasal samples that had been stored at -80°C were fixed. Before fixation, each sample was gradually thawed by first placing it at -20°C for 24 h and then for 12 h at 4°C . They were fixed with 10% saline formaldehyde solution and embedded in paraffin wax with $5\text{-}\mu\text{m}$ microtome sections used for

subsequent analysis. Hematoxylin and eosin staining was performed to confirm that tissue integrity was maintained.

Cell proliferation

These sections were also immunostained to determine the relative expression of the proliferating cell nuclear antigen (PCNA) (Abcam) (27). Staining was carried out on the Bondmax histology system using Bond Polymer Refine Detection System (DS9800; Vision Biosystems, Mount Waverley, Australia) and Bond software version 3.4A. Briefly, slides are heated and stained as follows: 5 min on a peroxide block, 15 min with primary antibody, 8 min with secondary antibody, 10 min with 3,3-diaminobenzidine, and 5-min counterstaining with hematoxylin and eosin. Negative slides were run in parallel, with the exclusion of the primary antibody. Slides were imaged using a Nikon Eclipse 90i microscope (Nikon, Surrey, UK) with charge-coupled device high-speed color camera (Micropublisher 3.3RTV; Qimaging, Surrey, British Columbia, Canada) and analyzed using Volocity 4 (version 4.2.1; Improvision, Coventry, UK) quantification software. To ensure uniformity of staining using the automated protocol, all animals were analyzed at the same time with the investigator blinded to nutritional group.

Detection of apoptosis

Terminal transferase-mediated 2'-deoxyuridine, 5'-triphosphate nick end labeling (TUNEL) was determined using a fluorescence Nikon Eclipse 90i microscope using a similar protocol to that described above.

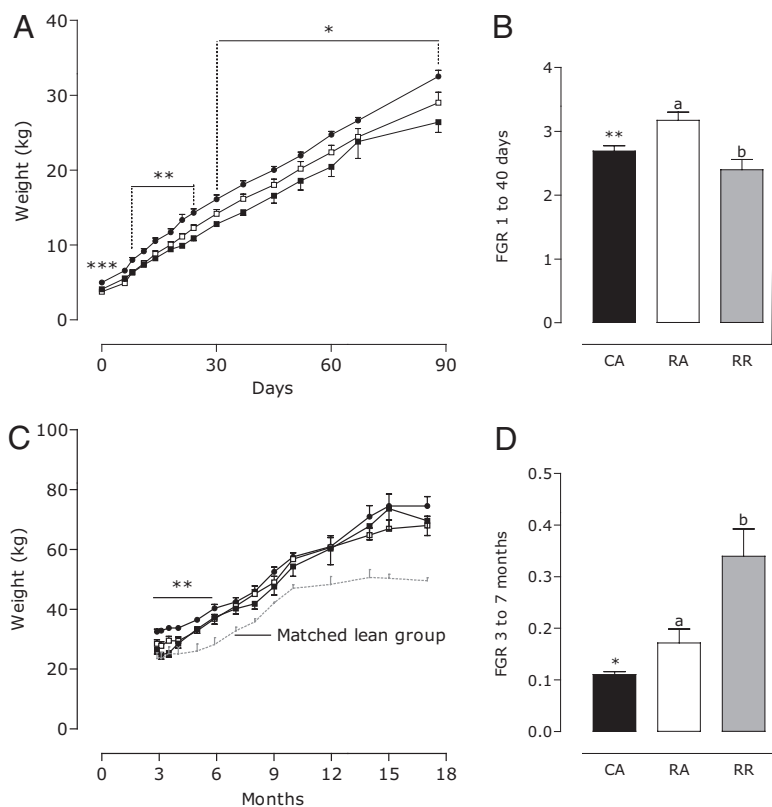


FIG. 2. Postnatal growth of offspring born to C-fed sheep or those R from dGA 110 up to term and then reared as a singleton [CA (closed circles), RA (open squares)] or as twins [RR (closed squares)] as measured (A) during and (C) after the weaning period, *i.e.* 3 months of age. FGR during (B) lactation and (D) weaning are also illustrated. Values are mean \pm SEM and significant differences between groups, CA and RA: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; RA and RR; a vs. b, $P < 0.05$; a vs. c, $P < 0.01$.

Statistical analysis

Statistical analysis of the data were performed using PASW statistics software (version 17.02; IBM, Chicago, IL). Kolmogorov-Smirnoff tests were realized on every parameter analyzed to determine the Gaussian distributions of the variables. The influence of maternal nutrition (R vs. C) or early postnatal growth (RA vs. RR) was determined, according to parametric distribution, using unpaired Student's *t* or Mann-Whitney *U* tests. Data are expressed as mean values with their SE. To address the limitations of multiple testing, statistical trend was accepted with a 95% interval of confidence ($P < 0.05$), and significance was accepted with a confidence interval of 99% ($P < 0.01$). Each variable was tested for gender. Only body weight and fat mass after birth differed between males and females, and no other factors were found to be affected. Moreover, comparison for each variable between groups for females only demonstrated similar outcomes.

Results

Birth weight and postnatal growth

A 40% caloric restriction in late gestation had no effect on length of gestation

TABLE 1. Influence of fetal growth restriction and accelerated postnatal growth on insulin sensitivity and fat mass

	Age (months)	CA	RA	RR	IUGR	Postnatal growth
AUC insulin (a.u.)	8	38.3 ± 10	64.0 ± 11	32.6 ± 6.9	<0.05	<0.05
	16	53.1 ± 15	107 ± 37	91.1 ± 47	<0.05	—
AUC glucose (a.u.)	8	1118 ± 121	1015 ± 189	1233 ± 227	—	—
	16	602 ± 322	831 ± 345	803 ± 461	—	—
Total fat mass (%)	8	11.7 ± 7.2	15.3 ± 3.2	15.1 ± 3.2	—	—
	16	18.2 ± 7.3	21.8 ± 7.0	20.0 ± 3.3	—	—
Postmortem analysis						
Total perirenal fat (g)	17	960 ± 162	1069 ± 240	939 ± 113	—	—
Total omental fat (g)		1353 ± 182	1563 ± 381	1509 ± 195	—	—

Values are means ± SEM. a.u., Arbitrary units. Full details of pre- and postnatal nutritional interventions are included in *Materials and Methods*.

(C, 145 ± 0.5 d; R, 145 ± 1.1 d). The siblings in each set of twins were of similar body weight, and offspring of R mothers were lighter with a negative mean Z score for birth weight (Fig. 1). From birth to weaning, R offspring remained lighter than C and as expected reduced litter size promoted early growth (Fig. 2A). RA offspring grew faster than both RR and CA groups over first month of postnatal life, with very similar growth rates in these two groups (Fig. 2B).

After weaning at 3 months of age, all offspring born to R mothers showed accelerated weight gain, which approached that of CA animals by 7 months of age (Fig. 2, C and D). RR offspring exhibited the fastest rate of postweaning growth (Fig. 2D). Nevertheless, no offspring born to R mothers grew faster than the C, so that, at 17 months of age, mean body weight was similar between groups.

Body composition and metabolic parameters

Offspring born to C and R mothers exhibited similar total body composition as determined by dual-energy x-

ray absorptiometry scanning at 8 and 16 months, and there was no difference between groups in the weight of visceral fat depots (*i.e.* omental, perirenal, and pericardial) at postmortem (Table 1). At 8 months of age, RA offspring exhibited signs of insulin resistance with an enhanced insulin, but not glucose, AUC, which was maintained up to 16 months of age when both the RA and RR offspring were insulin resistant compared with C. Fasting blood samples were analyzed to assess whether the circulating metabolic profile was programmed by prenatal diet, and R animals exhibited raised plasma insulin, but reduced triacylglycerol, only as adults (Table 2).

Characteristics of energy homeostasis in the adult offspring

With increased age and fat mass, plasma leptin rose in all groups. However, in the adults, fasting plasma leptin further increased in RA offspring (Table 2), a difference that persisted over the 24-h sampling period (Fig. 3A).

TABLE 2. Plasma concentrations of metabolites and hormones as measured at immediately prior to daily feeding at 0900 h in the growing offspring at 3, 8, and 16 months of age

	Age (months)	CA	RA	RR	IUGR	Postnatal growth
Glucose (mM)	3	3.0 ± 0.17	2.8 ± 0.10	2.6 ± 0.10	—	—
	8	3.4 ± 0.34	4.0 ± 0.18	3.0 ± 0.42	—	—
	16	2.9 ± 0.25	2.8 ± 0.34	3.1 ± 0.09	—	—
NEFA (nM)	3	0.76 ± 0.44	0.76 ± 0.31	0.78 ± 0.44	—	—
	8	1.34 ± 0.07	1.01 ± 0.11	1.27 ± 0.08	—	—
	16	0.67 ± 0.05	0.48 ± 0.07	0.48 ± 0.08	—	—
Triacylglycerol (mM)	3	0.46 ± 0.06	0.38 ± 0.04	0.36 ± 0.09	—	—
	8	0.18 ± 0.01	0.17 ± 0.01	0.18 ± 0.04	—	—
	16	0.19 ± 0.02	0.09 ± 0.01	0.12 ± 0.02	<0.01	—
Cortisol (nM)	3	34.1 ± 3.4	48.6 ± 13.8	49.2 ± 10.3	—	—
	8	66.8 ± 18.0	62.1 ± 12.6	59.5 ± 10.3	—	—
	16	38.8 ± 8.4	55.5 ± 13.9	45.0 ± 10.0	—	—
Leptin (ng/ml)	3	0.7 ± 0.11	0.5 ± 0.10	0.7 ± 0.10	—	—
	8	1.8 ± 0.22	2.4 ± 0.45	2.1 ± 0.40	—	—
	16	3.8 ± 0.60	6.2 ± 0.94	3.7 ± 0.47	<0.05	<0.05
Insulin (pM)	3	12.0 ± 1.7	12.0 ± 2.0	13.7 ± 1.7	—	—
	8	25.8 ± 2.1	29.2 ± 2.0	27.5 ± 2.4	—	—
	16	17.2 ± 2.1	25.8 ± 1.9	25.8 ± 6.8	<0.05	—

Values are means ± SEM. Full details of pre- and postnatal nutritional interventions are included in *Materials and Methods*. NEFA, Nonesterified fatty acid.

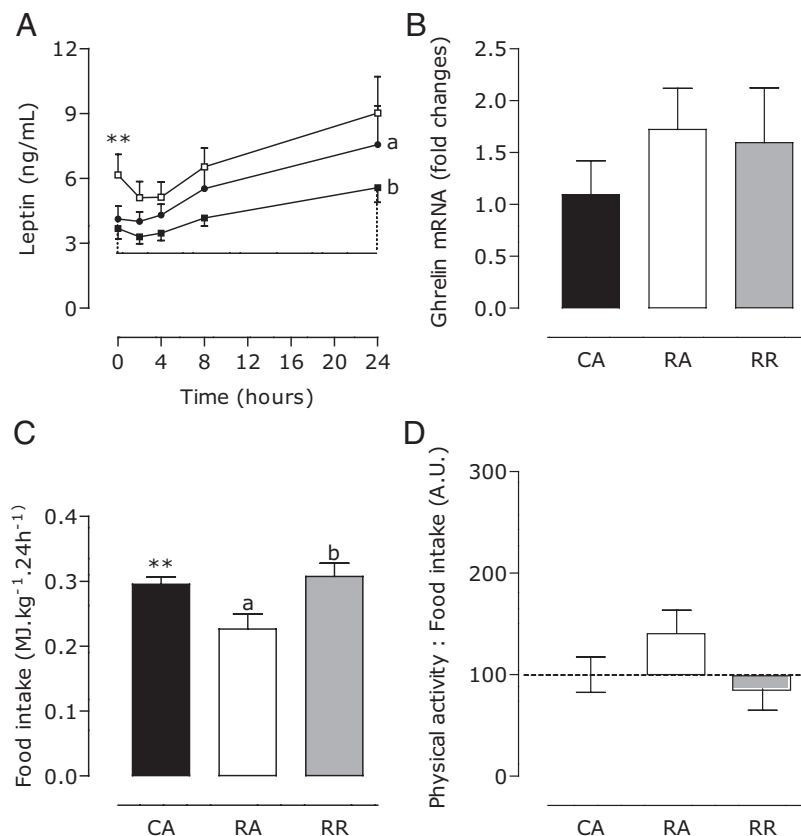


FIG. 3. Fetal IUGR and postnatal growth differentially programmed the control of food intake in the resulting adult at 16 months of age. A, Plasma leptin profiles in CA (closed circles; born to C-fed sheep and reared as a singleton), RA (open squares; born to R sheep and reared as a singleton), and RR (closed squares; born to R sheep and reared as a twin) offspring. Abomasal ghrelin mRNA abundance (B), daily food intake (C), and relative energy balance (D) as determined from measurements of food intake and physical activity in arbitrary units (A.U.). Values are mean \pm SEM and significant differences between groups, CA and RA: *, $P < 0.05$; RA and RR: a vs. b, $P < 0.05$.

In addition, a significant positive correlation between plasma leptin with early growth was observed (CA: $R^2 < 0.01$, $P = 0.9$; RA: $R^2 = 0.77$, $P = 0.001$; RR: $R^2 < 0.01$, $P = 0.7$). In contrast, gastric ghrelin and leptin mRNA expression were not affected by either maternal diet or early postnatal growth (Fig. 3B and Table 3). Measurement of daily food intake and 24-h activity profiles demonstrated that the RA group consumed less food at 16 months of age (Fig. 3C), which is likely to be mediated by the raised plasma leptin in these animals compared with CA and RR groups (Fig. 3A). There were no differences in physical activity or the ratio of physical activity to food intake (Fig. 3D). Although in rodent models of diet-induced obesity reduced insulin sensitivity frequently arises from a chronic proinflammation that originates from hyperplastic adipose tissue (31), gene expression analysis in both the sc and omental fat depots in the present study was similar between groups as was adipocyte size, and there are no obvious signs of exaggerated crown-like structures in any samples (data not shown).

Differential central (hypothalamus) and peripheral (gastric) programming

To assess the influence of the early nutritional environment on the hypothalamus and stomach, gene expression profiles were determined at 17 months of age using quantitative RT-PCR. In the hypothalamus, maternal R resulted in raised gene expression for the *MC3R*, *AMPK α* , *mTOR*, *PTP1B*, *AdnR2*, and the *FTO* gene (Table 3 and Fig. 4A) with no effect of postnatal growth. In the abomasum, maternal R resulted in reduced gene expression of *mTOR*, *CPT1*, *SIRT1*, and *UCP2*, whereas the RR offspring in whom postnatal growth was reduced exhibited a pronounced increase in mRNA abundance for *PGC1 α* , *PPAR γ* , *AMPK α* , *ACC α* , glucose transporter 1, *DMNT1*, *ObR*, and *FTO* (Table 3 and Fig. 4B). No difference, however, was seen in the protein abundance of AMPK α or in the relative abundance of active phosphorylated-AMPK α (data not shown). Finally, histological examination of the abomasum (Fig. 5) revealed a marked reduction in PCNA with enhanced postnatal growth after maternal nutrition, without alteration in TUNEL staining.

Discussion

The present study demonstrates that, in a large animal model known to share important similarities with humans in the outcomes of fetal programming, growth rate immediately after birth was an important determinant of adult energy homeostasis. Growth-restricted fetuses born to R mothers were, therefore, insulin resistant and exhibited specific impairments in the regulation of food intake that were accompanied with long-term changes in the activity of the abomasum.

The animal model

The strength of sheep as a model for the human lies in the fact that time courses of fetal and perinatal development are comparable, particularly in the establishment of hypothalamic neural networks and the rapid growth of adipose tissue during late gestation (32). It must, however, be noted that ruminant sheep differ from monogastric om-

TABLE 3. Fold changes in mRNA abundance in the hypothalamus and gastric mucosa sampled from adult offspring as measured at 17 months of age

	CA	RA	RR	IUGR	Postnatal growth
Hypothalamus					
<i>IR</i>	1.0 ± 0.24	1.2 ± 0.17	1.5 ± 0.30	—	—
<i>PTP1B</i>	1.0 ± 0.13	1.5 ± 0.12	1.5 ± 0.20	<0.05	—
<i>AdnR1</i>	1.0 ± 0.08	1.3 ± 0.14	1.1 ± 0.10	—	—
<i>AdnR2</i>	1.0 ± 0.13	1.5 ± 0.13	1.4 ± 0.18	<0.05	—
<i>ObR</i>	1.0 ± 0.19	0.9 ± 0.09	0.8 ± 0.15	—	—
<i>SOCS3</i>	1.0 ± 0.18	1.4 ± 0.22	1.6 ± 0.32	—	—
<i>GHSR</i>	1.0 ± 0.13	1.1 ± 0.13	0.8 ± 0.08	—	—
<i>AgRP</i>	1.0 ± 0.10	1.5 ± 0.13	1.2 ± 0.24	—	—
<i>MC4R</i>	1.0 ± 0.15	1.1 ± 0.09	1.2 ± 0.14	—	—
<i>NPY</i>	1.0 ± 0.24	1.2 ± 0.24	0.7 ± 0.11	—	—
<i>FTO</i>	1.0 ± 0.24	1.4 ± 0.12	1.4 ± 0.10	<0.05	—
Stomach					
<i>IR</i>	1.0 ± 0.11	0.7 ± 0.20	0.8 ± 0.16	—	—
<i>GLUT1</i>	1.0 ± 0.15	1.0 ± 0.45	3.2 ± 0.42	—	<0.01
<i>ObR</i>	1.0 ± 0.61	0.5 ± 0.22	3.4 ± 1.00	—	<0.001
<i>Leptin</i>	1.0 ± 0.34	2.8 ± 1.08	2.5 ± 1.10	—	—
<i>Ghrelin</i>	1.0 ± 0.32	1.7 ± 0.39	1.6 ± 0.52	—	—
<i>ACCα</i>	1.0 ± 0.14	0.9 ± 0.31	3.7 ± 0.87	—	<0.05
<i>SIRT1</i>	1.0 ± 0.09	0.7 ± 0.08	0.7 ± 0.10	<0.05	—
<i>DNMT1</i>	1.0 ± 0.08	0.8 ± 0.21	3.3 ± 0.70	—	<0.01
<i>FTO</i>	1.0 ± 0.25	1.1 ± 0.35	5.2 ± 1.06	—	<0.001

Values are means ± SEM. *AgRP*, Agouti-related peptide; *DNMT1*, dinucleotide methyl transferase 1; *GHSR*, ghrelin receptor; *GLUT1*, glucose transporter 1; *IR*, insulin receptor; *SOCS3*, suppressor of cytokine signalling. Full details of pre- and postnatal nutritional interventions are included in *Materials and Methods*.

nivorous humans in their short-term control of feeding. Nevertheless, the fourth stomach chamber in sheep, the abomasum, acts as does the stomach of monogastric mammals and is the main site for ghrelin production (33), although there is limited information in sheep. The similarities between humans and sheep in the nutritional control of abomasal ghrelin synthesis clearly suggest a role in the control of food intake (34). Critically, during the early postnatal period when young sheep only feed on milk, an esophageal groove exists, and they only use their true stomach (*i.e.* the abomasum) to digest milk making them comparable with human infants. Both pregnant women and sheep are prone to maternal catabolism in the last third of gestation (5, 35), and this may act to maintain fetal growth and invoke common pathways by which reduced birth weight results in long-term adverse consequences.

As previously observed in sheep (36), and supported by retrospective analysis in the Dutch famine birth cohort, late gestational R results in low birth weight and adult insulin resistance (6). The risk of developing the metabolic syndrome in small birth weight offspring can be amplified in those individuals exhibiting rapid postnatal growth (37). However, the results from our study do not readily support such a relationship, because when all offspring were raised in an obesogenic environment, they became equally obese. Although preterm infants develop greater relative fat mass probably as a consequence of increased postnatal nutrition (38), the relationships between specific

changes in fat mass and insulin sensitivity and postnatal growth in the term growth restricted newborn are less clear. The results of the present study suggest that intra-uterine growth restriction (IUGR) and rapid postnatal growth may not represent separate risk factors for insulin resistance later in life.

Despite having a modest effect on the onset of insulin resistance, weight gain in the first month of life specifically influenced the regulation of energy balance. Adult offspring born to R mothers ate less than C, an outcome that was abolished in the RR offspring, *i.e.* those in which early postnatal growth was reduced. In addition, RA offspring exhibited higher plasma leptin, which could partially explain their lower food intake. Future studies will now be needed to determine whether these adaptations are mediated by changes in leptin production from adipose tissue and/or the development of leptin resistance. We found no influence of perinatal weight gain on either total or visceral fat mass, adipocyte morphology or gene expression that would support a programmed response in white adipose tissue. No change in hypothalamic expression of the ObR or any other neurotransmitters was observed to indicate the development of central leptin resistance. Moreover, despite exhibiting enhanced gene expression for *AMPK*, *mTOR*, *PTP1B*, *MC3R*, and *FTO* in relation to IUGR, the activity of the hypothalamus remained surprisingly unaffected by postnatal growth. The hypothalamic changes we observed may, in fact, be secondary to those in

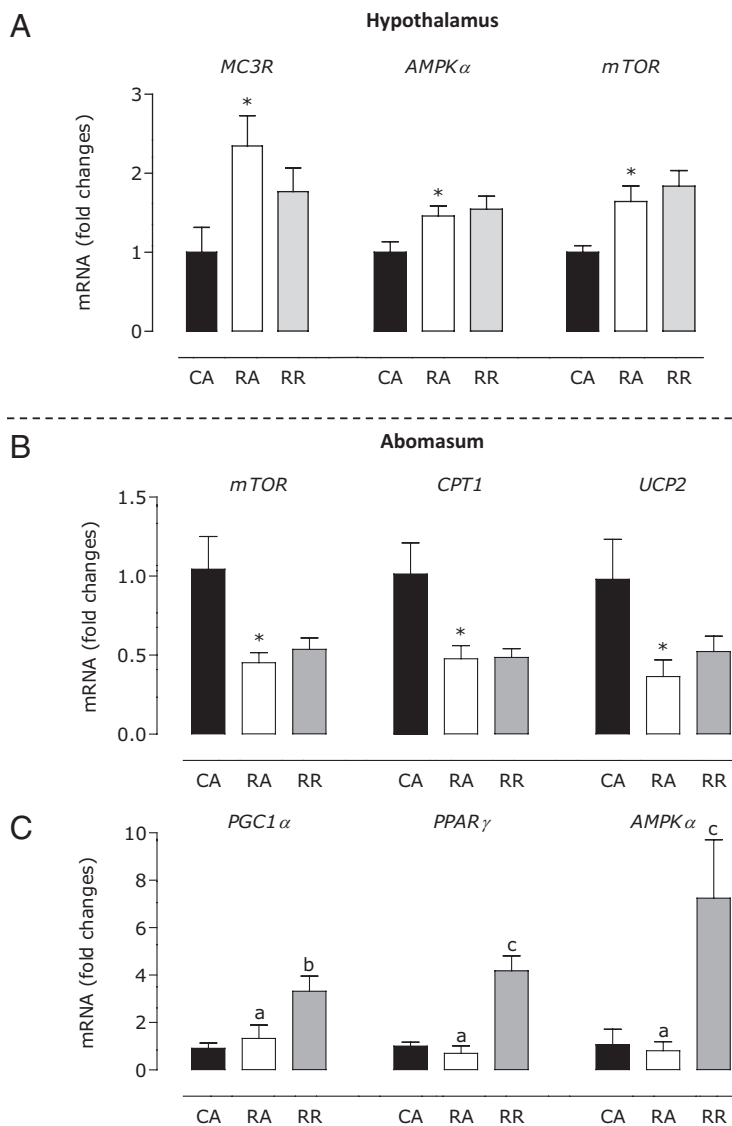


FIG. 4. Perinatal programming of energy sensing in the adult offspring at 17 months of age. Influence of maternal nutrition on gene expression in the (A) on the adult hypothalamus and (B and C) abomasum. CA, Born to C-fed sheep and reared as a singleton; RA, born to R sheep and reared as a singleton; and RR, born to R sheep and reared as a twin. Values are mean \pm SEM and significant differences between groups, CA and RA: *, $P < 0.05$; RA and RR: a vs. b, $P < 0.05$; a vs. c, $P < 0.01$.

plasma leptin and insulin and thus be indicative of an indirect metabolic effect. This lack of long-term effect within the hypothalamus may be important for improving our understanding of organ plasticity and critical windows of susceptibility. We have previously observed that a similar nutritional challenge imposed earlier in gestation induced long-term programming effects on hypothalamic gene expression (10). The only difference between these experiments was the timing of the nutritional challenge, *i.e.* either during the early period of hypothalamic organogenesis or later when it was much more developed and perhaps less plastic (39). This raises the question as to whether the fully mature hypothalamus has lost its capacity to be epigenetically programmed in late gestation. We

must, however, acknowledge that in both these experiments, the entire hypothalamus was used. This has been adopted by several other groups (40, 41), and future analysis may need to focus on specific regions within the different regulatory nuclei.

IUGR reduces gastric energy sensitivity

We have demonstrated the influence of the early life nutritional environment on programming of the gastrointestinal tract as gene expression of constitutive *mTOR*, plus *SIRT1*, *CPT1*, and *UCP2* were all halved in offspring born to R mothers, irrespective of postnatal growth rate. These adaptations are likely to be critical as *mTOR* signaling integrates cellular pathways regulating gastric ghrelin synthesis and proinflammatory processes (42–44). When energy production increases within the gut, a parallel rise in *mTOR*, by inhibiting ghrelin synthesis, reduces food intake (20). Our findings, therefore, suggest that chronic energy restriction during late gestation reduces *mTOR* action in the stomach with the potential to either increase energy intake or enhance the rate of nutrient exchange across the gut.

Fetal growth restriction was also accompanied by a reduction in *SIRT1*, *UCP2*, and *CPT1* gene expression, together with histological evidence of impaired gastric mucosal integrity. Taken together, these adaptations are indicative of a prooxidative status with *SIRT1*, *UCP2*, and *CPT1* acting

to reduce intracytoplasmic reactive oxygen species concentration (45), although the precise mechanisms involved remain an area of intense debate (46, 47). Importantly, our study provides novel mechanistic insights into how epidemiological findings of a link between low birth weight, gastroesophageal reflux, or carcinoma (48–50) may occur.

Protective effect of postnatal growth restriction on later gastric function

One further outcome in our study was that, despite gene expression of *mTOR*, *SIRT1*, *UCP2*, and *CPT1* being down-regulated in all offspring born to R mothers, the

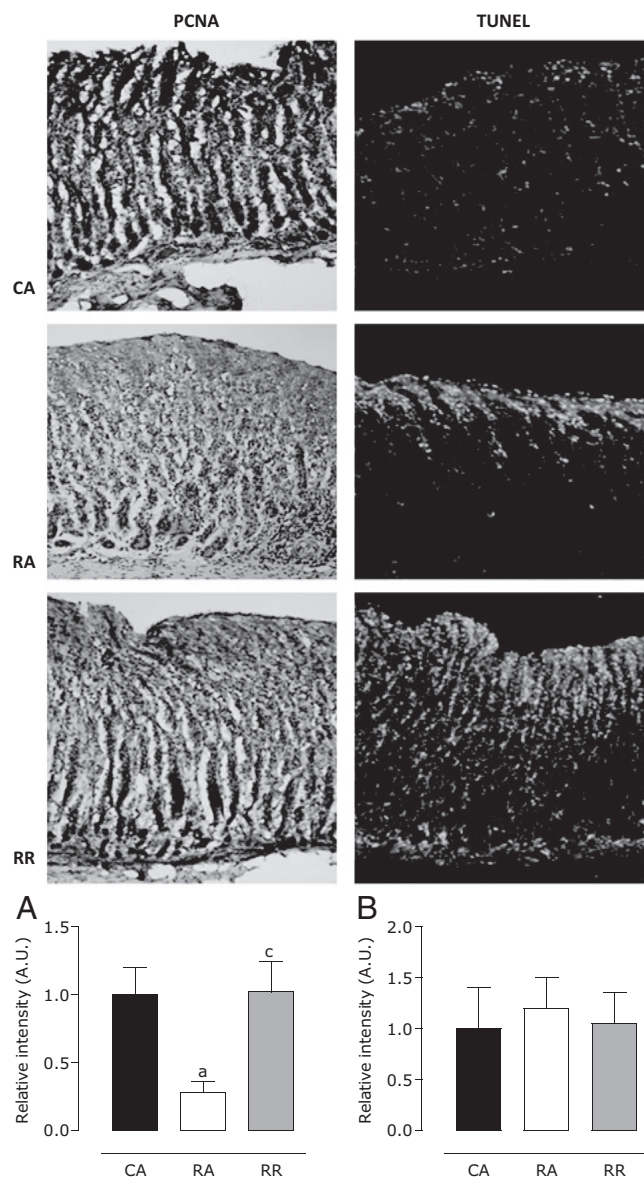


FIG. 5. Fetal IUGR and accelerated postnatal growth compromise gastric structure. Tissue remodeling as determined in the 17-month-old offspring. Representative examples ($\times 10$ magnification) of (A) PCNA and (B) TUNEL staining are shown together with relative intensity analysis. CA, Born to C-fed sheep and reared as a singleton; RA, born to R sheep and reared as a singleton; and RR, born to R sheep and reared as a twin. Values are mean \pm SEM and significant differences between groups, RA and RR: a vs. b, $P < 0.05$.

adverse histological outcomes in terms of PCNA staining were not found in those animals, which did not increase their growth during early lactation. We propose that this apparent protective effect was related to adaptations in gene expression regulating gastric energy metabolism as indicated by overexpression of those involved in AMPK/ACC-related pathways. As a consequence, these offspring would be able to stimulate mitochondrial biogenesis after increased *PGC1 α* and cellular energy intake and oxidation due to raised glucose transporter 1 and *PPAR γ* . At the same time, enhanced leptin sensitivity induced by a higher

ObR abundance would ultimately reduce prooxidative pathways and favor gastric integrity (44, 51). The mechanistic basis for differential epigenetic resetting as suggested by constitutive changes in gene expression remains largely unexplained, and control mechanisms appear to differ greatly between genes in the same tissue (52). Intriguingly, we found these transcriptional changes to be associated with dinucleotide methyl transferase type I and *FTO*, two factors that can regulate DNA methylation (53, 54). Furthermore, *FTO* has been established to encode a 2-oxoglutarate DNA demethylase and provides a link between energy-sensing pathways (55–57) that could include be further modulated by changes in short-chain fatty acid production (58), which are the main products of rumination.

In conclusion, restricted maternal food intake during late gestation, which is sufficient to induce IUGR, resulted in a long-term resetting of energy homeostasis. Small birth weight offspring were predisposed to insulin resistance, a response which was not amplified with rapid postnatal growth but was accompanied with long-term changes in the control of food intake. This was linked to a programmed adaptation in the abomasum but not in the hypothalamus. Critically, both fetal growth restriction and the timing of postnatal growth promotion in the IUGR offspring had a large impact upon mTOR and AMPK-dependent energy-sensing pathways, which could influence cell proliferation in the abomasum. These observations could be clinically relevant and suggest that very early nutrition should have a major long-term effect in the gastrointestinal organs involved in the control of food intake.

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