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Impact of birth weight and gender on early postnatal hypothalamic energy balance regulatory gene expression in the young lamb

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Abstract

Intra-uterine growth restriction (IUGR) is involved in developmental metabolic programming and here we test the hypothesis that IUGR affects the developing hypothalamic energy balance regulatory pathways in a sex-specific manner. This experiment investigated early postnatal hypothalamic gene expression for six primary leptin- and insulin-sensitive neuropeptides and receptors in male and female IUGR (n = 8 and 9, respectively) and normal (N) birth weight lambs (n = 8 per gender) gestated and suckled by overnourished mothers. IUGR lambs were smaller at birth, had increased fractional growth rates (FGR), lower final body weight (11 weeks) and similar body fat content compared with N lambs, while males had higher final body weight and insulinemia but lower body fat and leptinemia than females. *In situ* hybridization revealed greater gene expression in the hypothalamic arcuate nucleus at 11 weeks for anorexigenic genes in females and orexigenic genes in males, with no effect of IUGR. Leptinemia correlated with gene expression for neuropeptide Y (NPY, negatively) in both sexes and pro-opiomelanocortin (POMC, positively) in females but with leptin receptor (negatively) only in males. Current FGR for girth correlated negatively with gene expression for NPY in males and POMC in females. Neither IUGR nor gender affected suckling activity (proxy for appetite) assessed at 3 weeks, but final NPY gene expression correlated with suckling weight gain in males. This study has revealed no effect of IUGR on early postnatal hypothalamic energy balance gene expression but a major effect of gender associated with major sex differences in adiposity and leptinemia.

Key words: developmental programming, intra-uterine growth restriction, adiposity, leptin

1. Introduction

The basic model of energy homeostasis in the mature animal includes peripheral metabolic feedback hormones leptin and insulin regulating the activities of opposing orexigenic and anorexigenic circuits in the hypothalamus (Schwartz et al., 2000). These neuronal circuits develop in the fetal brain, they are well established by birth in precocial species such as sheep and primates, and their development is influenced by the prenatal nutritional environment (Grayson et al., 2010). Changes in the developing hypothalamic circuitry may underlie the apparent programming of a predisposition to obesity and altered metabolic phenotype by intra-uterine growth restriction (IUGR), especially when such offspring are born into an unrestricted nutritional environment (Gluckman and Hanson, 2008). However, existing data largely come from studies of rodents in which hypothalamic neuroendocrine maturation occurs after birth (Breton, 2013); it is now appropriate to increase our understanding of prenatal nutritional programming of the hypothalamus in larger, precocial mammalian species.

Previously we have demonstrated the presence of key anorexigenic and orexigenic gene expression in the hypothalamic arcuate nucleus of the ovine fetus at mid as well as late gestation (Adam et al., 2008; Mühlhäusler et al., 2004). A consistent finding was the sensitivity of anorexigenic neuropeptides in the arcuate nucleus to fetal nutrient (glucose) supply, thus pro-opiomelanocortin (POMC) gene expression correlated with fetal glycemia at 81 days (term = 147 days; Adam et al., 2008), intra-fetal glucose infusion increased POMC gene expression at 140 days (Mühlhäusler et al., 2005), and cocaine- and amphetamine-regulated transcript (CART) gene expression correlated with fetal liver glycogen and was decreased in IUGR compared with normally-growing fetuses at 130 days (Adam et al., 2011a). It remains to be determined to what extent prenatal changes in hypothalamic neuropeptides persist in postnatal life when they may affect appetite and body weight

regulation in the free-living sheep. Findings from rodent offspring indicate early postnatal down-regulation of anorexigenic and up-regulation of orexigenic hypothalamic neuropeptides following prenatal fetal undernutrition caused by maternal undernutrition or placental insufficiency (Cripps et al., 2009; Desai et al., 2007; Huizinga et al., 2001). Earlier we reported in sheep that the presumed increase in fetal arcuate POMC gene expression induced in late gestation by maternal overnutrition and elevated glycemia was sustained in lambs at postnatal day 30 (Mühlhäusler et al., 2006). Now it is pertinent to investigate whether the presumed changes in anorexigenic hypothalamic gene expression in IUGR ovine fetuses are sustained postnatally.

Although insulin and leptin are present in the fetal circulation, there is little evidence for either hormone playing an adult-like nutritional signaling role in the hypothalamus. Precocial species like sheep lay down adipose tissue in the latter stages of gestation, which secretes leptin in proportional concentrations (Mühlhäusler et al., 2002), and the ovine fetal pancreas secretes insulin from mid gestation onwards (Aldoretta et al., 1998). Gene expression for receptors for both hormones is detected in the arcuate nucleus from mid gestation, but no correlation was found between circulating concentrations of either hormone and expression of key appetite regulatory genes in the fetal arcuate nucleus (Adam et al., 2008, 2011; Mühlhäusler et al., 2004). Circulating fetal insulin concentrations correlated negatively with arcuate insulin receptor (Ins-R) gene expression in an apparently adult-like ligand-receptor relationship, but circulating leptin concentrations were found to correlate positively with arcuate leptin receptor (OB-Rb) gene expression in late gestation (Adam et al., 2011). These latter findings were consistent with the substantial evidence for leptin playing a neurotrophic role in the neonate (Bouret, 2010). However by 5-6 months of age intracerebroventricular (ICV) leptin suppresses appetite in both male and female sheep indicating that a functional role for leptin signaling in the hypothalamus has developed by

this age (Adam et al., 2011b). Conversely, ICV-administered insulin had no effect on appetite in 5-6 month-old sheep (Adam et al., 2011b). In the present study, we examine relationships between hypothalamic arcuate neuropeptide and receptor gene expression, leptinemia and insulinemia at 3 months of age.

In addition to the effects of IUGR, we examine the influence of gender since there is evidence for sex differences in hypothalamic programming with respect to the hypothalamo-pituitary-adrenal 'stress' axis (Gardner et al., 2006; Wallace et al., 2011), and yet there is a lack of equivalent data with respect to the hypothalamic appetite regulatory axis. Furthermore, in the cohort of lambs used for the present study, whilst IUGR impacted postnatal fractional growth rates and glucose metabolism, gender had the overriding influence on body composition and metabolic hormone status (Wallace et al., 2013). Thus, in these lambs from our overnourished adolescent dam model of utero-placental insufficiency, IUGR led to increased fractional growth rates to 11 weeks of age and impaired glucose handling at 7 weeks compared with normal birth weight lambs whereas females had increased adiposity and leptinemia compared with males (Wallace et al., 2013). Importantly, IUGR and normal birth weight lambs in this cohort were both born to dams that had high dietary intakes throughout pregnancy and lactation, thus allowing us to examine their early postnatal phenotype without the confounding effect of differences in maternal nutrition. In order to estimate appetite drive and voluntary food intake in the lambs, suckling activity was assessed at 3 weeks of age.

This experiment therefore investigated the influences of IUGR and gender on the early postnatal phenotype with respect to the developing hypothalamic appetite regulatory pathways. Specifically, we examined gene expression for six primary leptin- and insulin-sensitive hypothalamic neuropeptides and receptors at 11 weeks of age in low and normal birth weight male and female lambs born to overnourished adolescent mothers. We test the

hypothesis that IUGR affects the developing hypothalamic energy balance regulatory pathways in a sex-specific manner.

2. Material and Methods

2.1 Animals

All procedures were licensed under the UK Animals (Scientific Procedures) Act 1986 and approved by local Ethical Review Committee. The derivation of the lambs is described in detail by Wallace et al. (2013). Briefly, growing adolescent recipient ewes (Dorset Horn x Mule) had been implanted with singleton embryos, derived from superovulated donors (Border Leicester x Scottish Blackface) and a single sire (Dorset Horn), and given a high quality complete diet *ad libitum* throughout pregnancy and lactation. The complete diet contained 12 MJ metabolizable energy and 140 g crude protein per kg dry matter and *ad libitum* intakes were calculated to promote rapid maternal growth during pregnancy leading to restricted placental growth, and hence restricted fetal growth in ~50% cases, followed by maximal milk yields during the 11-week lactation. In addition, lambs had access to their mothers' feed at all times. There was a continuous distribution of birth weights from which lambs were categorized as intra-uterine growth restricted (IUGR) or normal birth weight (N) (Wallace et al., 2013). The present study comprised 17 IUGR (n = 8 male, n = 9 female) and 16 N lambs (n = 8 per gender), with most of the individual embryo donors represented in both categories.

The lambs were weighed, measured and blood sampled mid-morning at 5-day intervals up to ~68 days of age and just before euthanasia at 77 days (11 weeks). Plasma leptin and insulin were determined by in-house radioimmunoassays (Marie et al., 2001 and MacRae et al., 1991, respectively) with all inter and intra-assay coefficients of variation less than 10% (as reported in Wallace et al., 2013). They were euthanized by lethal injection of

sodium pentobarbitone (10-15 ml Euthesate; 200 mg pentobarbitone/ml; Willows Francis Veterinary, Crawley, UK). Whole brains were removed, immediately frozen in isopentane over dry ice and stored at -80°C. Perirenal and visceral (omental and mesenteric) fat depots were dissected out and weighed.

2.2 Suckling activity assessment

Suckling activity was determined at 23 ± 0.9 days of age, coincident with the presumed peak lactation and prior to lambs showing any significant interest in eating their mothers' food. Ewes were milked by hand following intravenous oxytocin injection (Oxytocin-S® 10 i.u. per ewe; Intervet Ltd, Cambridge, UK) in order to empty the udder, the lamb was weighed and access to the udder was prevented using an udder cover. After 3 hours, the lamb was reweighed to determine fasting weight loss, the udder cover was removed and the number and duration of suckling bouts were determined for a period of 60 minutes. Lambs were weighed at 15-minute intervals throughout this observation period and again at 90 minutes.

2.3 Hypothalamic gene expression

The frozen brains were trimmed down to a mid-ventral block, mounted and sectioned by cryostat coronally through the hypothalamus from the mammillary body (caudal) to the optic chiasm (rostral). Sections (20 μm) were thaw-mounted onto poly-L-lysine-coated slides and stored at -80°C. Gene expression for neuropeptide Y (NPY), agouti-related peptide (AGRP), POMC, CART, OB-Rb and Ins-R was measured by *in situ* hybridization, using techniques described in detail elsewhere (Adam et al., 1997). The NPY riboprobe was generated from a rat cDNA (Adam et al., 1997), the CART probe from a cloned sheep cDNA (Barrett et al., 2001), and AGRP and POMC probes were generated from cloned Siberian hamster cDNAs (Mercer et al., 2000). A riboprobe complementary to fragments of the intracellular domain of OB-Rb was generated from a cloned sheep cDNA (Mercer et al.,

1998), and the Ins-R riboprobe was generated from a partial ovine cDNA (Archer et al., 2005). All probes have previously been validated in sheep brain (Adam et al., 2002; Archer et al., 2005) and corresponding sense probes showed no hybridization. Briefly, sections were fixed, acetylated, and hybridized overnight at 58 °C using ^{35}S-labelled cRNA probes (1–1·5 x 10^7 cpm/ml). They were then treated with RNase A, desalted, with a final high stringency wash (30 min) in 0·5 X SSC at 60 °C (Ins-R at 75 °C), dried and apposed for 7-10 days to Hyperfilm β-max (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK). Intensity and total area of hybridization were quantified in the hypothalamic arcuate nucleus on each autoradiographic image, using the Image-Pro Plus system (Media Cybernetics, Silver Spring, MD, USA). Example images are shown in Fig. 1. The integrated intensity of the hybridization signal (i.e. the optical density integrated over the total hybridization area) was computed using standard curves generated from ^{14}C autoradiographic micro-scales (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK). For each probe, up to 6 sections spanning the medial hypothalamus (i.e. in the region midway between the mammillary body and optic chiasm, further identified by third ventricle morphology) were examined from each brain. All reagents were obtained from Sigma (Sigma UK, Poole, Dorset, UK) unless otherwise stated.

2.4 Statistical analyses

Effects of birth weight category, gender and their interaction were examined by analysis of variance (General Linear Model; Minitab 16, Minitab Inc., State College, PA), and data are presented as means ± standard errors. Pearson product-moment correlation analyses and linear regression were used to explore relationships between variables where indicated (Minitab 16), and data are presented as correlation coefficients (r). Statistical significance was taken as $P < 0.05$, and a trend is indicated where $P = 0.06$ to 0.10.

3. Results

3.1 Postnatal growth, body composition, insulinemia and leptinemia

Detailed growth and metabolic data have been reported elsewhere for these lambs (n = 38 in Wallace et al., 2013) and relevant values are shown here for the representative subset of animals used in the present study (n = 33, Table 1). Birth weight was lower for IUGR versus N ($P < 0.001$) and for females versus males ($P < 0.05$), shoulder height at birth was lower for IUGR versus N ($P < 0.001$), but fractional growth rates (FGR, absolute growth rate relative to size at birth) for body weight, shoulder height, girth at the thorax and girth at the umbilicus were all higher in IUGR lambs ($P < 0.05$-0.001), with an effect of gender on FGR for girth at the thorax (females > males, $P < 0.001$) and a trend towards an effect of gender on FGR for girth at umbilicus (females > males, $P < 0.08$). Nonetheless, IUGR lambs had lower final body weight at 11 weeks compared with N lambs ($P < 0.001$), with no effect on internal body fat content, while males had higher final body weight than females ($P < 0.001$) but lower perirenal fat mass ($P < 0.01$) and lower proportional total internal fat mass ($P < 0.01$). Plasma insulin during days 65-73 (males > females; $P < 0.05$) and leptin both during days 65-73 (males < females; $P < 0.01$) and on final day 77 (males < females; $P < 0.001$) were influenced by gender but not IUGR (Table 1). For both males and females, plasma leptin concentrations in the days before euthanasia correlated positively with measures of internal fat mass ($P < 0.05$-0.001; Table 2) but plasma insulin only correlated with perirenal fat mass in males ($P < 0.01$).

3.2 Suckling activity

Weight loss during the 3-hour fast prior to the suckling assessment at 23 days of age, the number and duration of suckling episodes, and weight gain during suckling were independent of IUGR status and gender (Table 3). However, for all animals together, birth weight correlated positively with absolute fasting weight loss ($r = 0.407$, $P < 0.05$), post

fasting weight gain to 60 (r = 0.576, P < 0.001) and 90 minutes (r = 0.445, P < 0.01) and tended to correlate with the number of suckling episodes per 60 minutes (r = 0.335, P < 0.06). Similar and slightly stronger relationships were evident between current weight and absolute fasting weight loss (r = 0.540, P < 0.001), post fasting weight gain to 60 (r = 0.555, P < 0.001) and 90 (r = 0.503, P < 0.005) minutes. However, when expressed as relative (%) weight changes as opposed to absolute weight changes, or when males and females were examined separately, none of the foregoing correlations were significant.

3.3 Hypothalamic gene expression

In situ hybridization revealed greater gene expression in the hypothalamic arcuate nucleus for CART and POMC in females than males (P < 0.01-0.05), and greater gene expression for OB-Rb, NPY and AGRP in males than females (P < 0.001-0.01), but no effects of IUGR, and there was no effect of gender or IUGR on Ins-R gene expression (Figs 1 and 2).

Given the clear gender differences in gene expression, significant interrelationships and relationships with measures of growth, adiposity, metabolic hormones and appetite were explored within each sex. NPY and AGRP gene expression were positively correlated in both males (r = 0.807, P < 0.001) and females (r = 0.637, P < 0.01). In males, NPY and AGRP correlated negatively with plasma leptin (P < 0.01-0.001; Table 4 and Fig. 3A) and positively with OB-Rb (P < 0.05), and NPY correlated negatively with plasma insulin (P < 0.05); NPY, AGRP and OB-Rb all correlated negatively with perirenal fat mass (P < 0.05-0.001; Table 4). In females, POMC gene expression correlated positively with final plasma leptin (P < 0.05) and NPY correlated negatively with plasma leptin during the last week (P < 0.05), but none of the neuropeptide genes correlated with OB-Rb; AGRP correlated negatively with perirenal fat mass (P < 0.05; Table 4 and Figs 3 A,B). OB-Rb correlated negatively with plasma leptin in males but not females (Table 4 and Fig. 3C) and with plasma insulin in males but not

females (Table 4). Gene expression for ins-R and plasma insulin did not correlate in either sex.

None of the hypothalamic genes correlated with birth weight or final body weight within either sex but some correlations were found with final current FGR (CFGR, absolute growth rate relative to current weight at 68 days of age). Thus, within only males, NPY correlated negatively with thorax girth CFGR ($P < 0.05$) and there was a trend towards similar correlation for AGRP ($P < 0.07$; Table 4). Within only females, POMC correlated negatively with body weight CFGR ($P < 0.05$) and umbilical girth CFGR ($P < 0.05$; Table 4). Finally, in males only, NPY correlated positively with percentage weight gain to 90 minutes during the suckling assessment at 23 days of age ($P < 0.05$; Table 4). No other significant correlations were found between any of the hypothalamic genes and weight loss during fasting, weight gain during suckling or suckling activity during the assessment.

4. Discussion

This study has revealed no effect of birth weight but a major effect of gender on gene expression for hypothalamic energy balance regulatory pathways in young ovine offspring. Thus females had higher anorexigenic gene expression (POMC, CART) but males had higher orexigenic gene expression (NPY, AGRP, OB-Rb) in the arcuate nucleus. Gender also had a major effect on adiposity since females had higher levels of body fat and leptinemia, with no effect of birth weight. We therefore reject the hypothesis that IUGR affects the developing hypothalamic energy balance regulatory pathways in a sex-specific manner at this early postnatal life stage, since gender itself had the overriding influence irrespective of birth weight.

Growth and body composition data from the present subset of lambs was wholly representative of the total number reported in Wallace et al. (2013) whereby low birth weight (categorized as IUGR) was a direct reflection of reduced placental size. IUGR decreased birth weight and final body weight at 11 weeks and increased postnatal FGR, but absolute catch-up growth was not shown. However, gender had the major effect on final body weight (males > females), adiposity (females > males), leptinemia (females > males) and insulinemia (males > females). Importantly, plasma leptin was an accurate circulating indicator of adipose reserves in both sexes. By contrast, plasma insulin showed a weaker relationship with adiposity (only with perirenal fat mass) and only in males. Despite birth weight and on-going body weight differences, there was no evidence for IUGR or gender affecting postnatal appetite drive since there were no group effects on weight gain or frequency or duration of suckling bouts during the suckling assessment at 3 weeks of age. By contrast, De Blasio et al. (2007) observed that IUGR lambs (obtained by pre-mating maternal carunclectomy) had increased suckling (feeding) activity at 15 days of age, indicative of increased appetite. The difference may be attributable to the younger age and/or the more rapid catch-up growth in De Blasio's lambs compared with our model. Although correlation analysis of our data revealed that heavier lambs (at birth and current) consumed more, with higher absolute weight gain during suckling, this relationship did not hold true for relative (percentage) weight gain, suggesting that there was indeed no effect on appetite drive. On the other hand, these data are consistent with the original premise that milk yields (i.e. available food for the offspring) would be the same between IUGR and N mothers receiving the same nutritional regime in our model.

Given the lack of sex differences in postnatal appetite drive and body weight FGR, the clear sex differences in underlying hypothalamic appetite-regulatory neuropeptides were perhaps surprising, albeit examined at a later age. However, a recent review of sex differences in developmental programming, based largely on phenotype and peripheral

molecular outcomes, identifies temporal, spatial and biochemical differences between male and female pre- and postnatal development (Aiken and Ozanne, 2013). Our data have now additionally shown important sex differences in hypothalamic neuroendocrine development. The reasons for these differences may include inherent (genetic) sex differences or differences in timing of development, but also the influence of different steroid hormone exposure and, in the present study, differences in adiposity. Gonadal steroid hormone secretion is initiated early in ovine gestation (Quirke et al., 2001) and so development pre- and postnatally occurs in an environment of high testosterone for males and high estrogen for females. Consistent with the increased orexigenic/anorexigenic balance in gene expression in the present males, testosterone is known to up-regulate NPY gene expression in adult male sheep (Dobbins et al., 2004), increase AGRP immunoreactivity in prenatally androgenized adult ewes (Sheppard et al., 2011) and decrease POMC gene expression in perinatally androgenized female mice (Nohara et al., 2011). Consistent with the increased anorexigenic/orexigenic balance in the females, estradiol stimulates POMC and inhibits NPY and AGRP gene expression in young rats (Santollo et al., 2012). Exposure to the different steroid milieux during development may program the different ratios of orexigenic/anorexigenic expression between males and females to produce a similar appetite phenotype, in other words producing a sex difference in hypothalamic 'set point' for a given food intake.

Apart from the presumed difference in circulating sex steroids, the major significant gender difference observed in the present study was in body composition. The importance of the hypothalamus-adipose axis in developmental programming has recently been recognized (Breton, 2013), and the present sex differences in adiposity may have been in part causally related to the differences in hypothalamic gene expression. Thus females had greater amounts of adipose tissue, circulating leptin, and hypothalamic POMC and CART gene expression

whereas males had less adipose tissue and leptin but more NPY, AGRP and OB-Rb gene expression. Both scenarios fit the basic model of adult energy balance regulation (Schwartz et al., 2000), but some additional sex differences were revealed by the correlation analyses. Both NPY and AGRP gene expression correlated with OB-Rb gene expression (positively) and plasma leptin (negatively), consistent with their adult-like regulation by leptin via its receptor (Adam et al., 2002), but only in males. Meanwhile in females, although gene expression for NPY (negatively) and POMC (positively) correlated with leptin in an adult-like manner, there was no correlation with OB-Rb gene expression, which was inconsistent with neuropeptide regulation by leptin via its receptor. Nonetheless, females had overall lower OB-Rb expression and higher plasma leptin values and the absence of receptor-ligand correlation may have been because OB-Rb levels were already baseline and unable to respond to further increases in leptinemia. It would seem that changes in post-receptor sensitivity may underlie the present association between higher leptin and higher POMC gene expression in the female lambs.

Wallace et al. (2013) found no evidence for IUGR leading to decreased neonatal leptin in these lambs, unlike in rats (Coupé et al., 2010), perhaps reflecting a difference between precocial and altricial species. All the lambs had similar (baseline) plasma leptin concentrations at birth which increased rapidly for the first week; thereafter IUGR and N groups had similar values throughout but the sexes diverged (females > males) with the difference significant from 4 weeks (Wallace et al., 2013). Compared to males, the female hypothalamus therefore had prolonged postnatal exposure to greater amounts of leptin as well as the significantly greater leptinemia in the week before and on the day of euthanasia reported here. Leptin concentrations in early life are important for the development of the adult metabolic profile (Granado et al. 2012) and these authors concluded that reported differences in the effects of postnatal hyperleptinemia may be attributed to critical time points

of sensitivity and/or prenatal influences; however, our present data suggest that gender could also be a major influencing factor.

The evidence for leptin's critical role in neonatal programming of metabolic neuroendocrine hypothalamic pathways comes from altricial rodent models (Bouret and Simerly, 2007), and it is likely that the temporal importance of leptinemia may be different in precocial species like sheep in which hypothalamic development and adipogenesis occur prenatally. Moreover, although sex differences in adult leptinemia (female > male) have long been recognized (Kennedy et al., 1997; Watanobe and Suda, 1999), the influence of gender on leptin's neurotrophic actions is unreported. There are few studies of neonatal leptin concentrations in lambs, with no gender comparisons (Bispham et al., 2002; Long et al., 2011; McFadin et al., 2002), but the sex difference in leptinemia in the present lambs was unequivocal (Wallace et al., 2013). Higher than normal leptin concentrations occurring earlier than normal in neonatal rodents decrease hypothalamic leptin receptors (Toste et al., 2006) and lead to leptin resistance in adulthood (Ahima and Hileman, 2000; Yura et al., 2005). The present female lambs, exposed to elevated leptinemia from soon after birth, may have been showing signs of leptin resistance (i.e. lack of correlation between leptin and its receptor and low expression of leptin receptor) whereas the males exposed to lower leptinemia exhibited adult-like hypothalamic neuropeptide and receptor responses and no sign of leptin resistance. Nonetheless, in spite of the observed sex differences in the developing hypothalamic leptin signaling system at 3 months, both sexes show a decreased food intake response to intra-hypothalamic leptin indicative of adult-like functionality at 5-6 months of age (Adam et al., 2011b).

In early postnatal life (11 weeks), the expression of hypothalamic energy balance genes was not affected by IUGR and none were correlated with birth weight or final weight in either sex. NPY gene expression correlated positively with the percentage weight gain to

90 minutes during the suckling assessment, indicative of increased orexigenic drive with higher amounts of food intake, but only in the males. Since the suckling assessment was made 8 weeks before brains were taken for gene expression analysis, current fractional growth rates (CFGR) just prior to euthanasia may provide a more temporally relevant indicator of overall energy balance in these lambs. However, the opposite relationship was seen, with NPY gene expression in males correlating negatively with thorax girth CFGR, suggesting that orexigenic drive decreased with increasing anabolic state (as in adult mammals; Schwartz et al., 2000). Conversely, POMC gene expression in female lambs correlated negatively with body weight CFGR and umbilical girth CFGR, suggesting that increased body energy status was associated with decreased anorexigenic drive (unlike in adults; Schwartz et al., 2000). In other words, the dominant orexigenic pathway in males appears to down-regulate in order to limit positive energy balance whereas the dominant anorexigenic pathway in females appears to down-regulate in order to facilitate positive energy balance. These findings may reflect sex differences in the stage of maturity for the developing neuroendocrine hypothalamus, as seen in other organ systems (Aiken and Ozanne, 2013).

Unlike leptinemia, insulinemia was higher in males than females in this study. In males, insulinemia correlated with perirenal fat mass, providing an additional adiposity signal to the brain where it correlated negatively with NPY and OB-Rb gene expression, in agreement with the basic model of adult energy balance regulation (Schwartz et al., 2000). There was no evidence for an association between insulinemia and hypothalamic gene expression in the females, and indeed studies in adult rats have shown that the male brain is more sensitive to insulin than the female brain (Clegg et al., 2003). Conversely, the female brain is more leptin-sensitive than that of the male, with estrogen increasing leptin sensitivity and decreasing insulin sensitivity (Clegg et al., 2006). There was no evidence for insulin

signal regulation through its hypothalamic receptor in either sex in this study, in contrast to the late gestation fetus (Adam et al., 2011a), but a negative correlation with OB-Rb gene expression in the males agrees with the inhibitory action of insulin on OB-Rb previously demonstrated in adult sheep (Daniel et al., 2000). Altogether, our data would tend to indicate that insulin may play a significant hypothalamic signaling role in addition to leptin in the young postnatal male but not in the female. However, in terms of the food intake response to intrahypothalamic insulin, neither the male nor female brain appeared sensitive to insulin at 5-6 months of age (Adam et al., 2011b).

Therefore, while the present data do not preclude an effect of IUGR in later life, this study has revealed no effect of IUGR on early postnatal hypothalamic energy balance gene expression but a major effect of gender which was associated with major sex differences in adiposity and leptinemia. Furthermore, the data strongly indicate that the sexes should be considered separately in studies of developmental hypothalamic programming.

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Fig. 1. Example autoradiographic images of coronal hypothalamic sections hybridized to radiolabelled riboprobes for POMC, CART, NPY, AGRP, OB-Rb and Ins-R in (A, C, E, G, I, K) male and (B, D, F, H, J, L) female lambs. 3V = third ventricle. ARC = arcuate nucleus. Bar = 2 mm.

Fig. 2. Gene expression in the hypothalamic arcuate nucleus for POMC, CART, NPY, AGRP, OB-Rb and Ins-R in (A) IUGR (dark grey bars; n = 17) versus normal birth weight (N, pale grey bars; n = 16) lambs and (B) female (solid bars; n = 17) versus male (open bars; n = 16) lambs. Results are shown as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 3. Relationships between plasma leptin concentration (final sample) and hypothalamic arcuate nucleus gene expression for (A) NPY, (B) POMC and (C) OB-Rb in female (solid symbols) and male (open symbols) lambs, irrespective of birth weight. Pearson correlation analysis: males r = -0.776 (P < 0.001) and females r = -0.185 (not significant, NS) for A; males r = 0.090 (NS) and females r = 0.494 (P < 0.05) for B; males r = -0.542 (P < 0.05) and females r = 0.061 (NS) for C.

Table 1. Size at birth, fractional growth rates, final body weight, body fat depot weights and plasma leptin and insulin concentrations in lambs at 11 weeks of age.

	Females		Males		P value		
	N n = 8	IUGR n = 9	N n = 8	IUGR n = 8	N v. IUGR	Female v. male	Interaction
Birth weight (g)	4662 ± 137	3131 ± 221	5254 ± 198	3402 ± 167	**<0.001**	**0.028**	0.399
Shoulder height at birth (cm)	22.8 ± 0.30	18.7 ± 0.76	23.9 ± 0.95	19.5 ± 1.04	**<0.001**	0.253	0.862
FGR bodyweight (%/day)	8.42 ± 0.235	11.31 ± 0.699	8.53 ± 0.378	10.83 ± 0.220	**<0.001**	0.688	0.518
FGR shoulder height (%/day)	1.20 ± 0.022	1.61 ± 0.080	1.22 ± 0.061	1.53 ± 0.076	**<0.001**	0.647	0.489
FGR thorax girth (%/day)	1.37 ± 0.018	1.60 ± 0.052	1.25 ± 0.043	1.41 ± 0.040	**<0.001**	**0.001**	0.423
FGR umbilical girth (%/day)	1.75 ± 0.072	1.82 ± 0.044	1.60 ± 0.054	1.76 ± 0.058	**0.043**	0.077	0.432
Final bodyweight (kg)	33.7 ± 0.53	29.6 ± 1.25	39.4 ± 0.628	32.8 ± 1.49	**<0.001**	**<0.001**	0.254
Perirenal fat (g)	462 ± 50.7	444 ± 48.9	338 ± 28.5	296.4 ± 48.5	0.514	**0.006**	0.797
Internal fat (g/kg)[1]	47.4 ± 2.24	50.3 ± 3.53	34.5 ± 3.25	35.4 ± 2.47	0.733	**<0.001**	0.528
Leptin (ng/ml during final week) [2]	3.90 ± 0.640	4.03 ± 0.779	1.58 ± 0.551	1.76 ± 0.578	0.817	**0.002**	0.972
Leptin (ng/ml, final day) [3]	4.04 ± 0.484	2.93 ± 0.429	1.48 ± 0.422	1.68 ± 0.514	0.336	**<0.001**	0.166
Insulin (ng/ml during final week)[2]	2.52 ± 0.209	2.78 ± 0.417	3.71 ± 0.609	3.70 ± 0.417	0.782	**0.022**	0.766

Values are means ± SEM. N, normal birth weight. IUGR, intra-uterine growth-restricted. FGR, fractional growth rate. [1]Combined omental and mesenteric fat depots, g/kg empty bodyweight. [2]Average of three samples taken on days 65-73. [3]Sample taken on day 77. Data from Wallace et al. (2013).

Table 2. Relationships between plasma leptin and insulin concentrations and body fat depot weights in male and female lambs at 11 weeks of age, irrespective of birth weight.

	Females n = 16		Males n = 17	
	Perirenal fat (g)	Internal fat (g/kg) [3]	Perirenal fat (g)	Internal fat (g/kg) [3]
Leptin (ng/ml during final week) [1]	**0.778*** **	**0.723*** **	**0.833*** **	**0.783*** **
Leptin (ng/ml, final day) [2]	**0.559***	0.288	**0.704****	**0.830*** **
Insulin (ng/ml during final week) [1]	0.011	0.005	**0.658****	0.197

Values are Pearson correlation coefficients. Significant correlations shown in bold. *P<0.05; **P<0.01; ***P<0.001. [1]Average of three samples taken on days 65-73. [2]Sample taken on day 77. [3]Combined omental and mesenteric fat depots (g/kg empty bodyweight). Data derived from Wallace et al. (2013).

Table 3. Lamb body weight changes and suckling activity during assessment at 23 days of age.

	Female		Male		*P*-value		
	N	IUGR	N	IUGR	N v. IUGR	Female v. male	Interaction
	n = 8	n = 9	n = 8	n = 8			
Weight loss during 3h fast (g)	171 ± 16.9	199 ± 22.8	147 ± 14.7	171 ± 13.7	0.143	0.143	0.933
Weight loss during fasting (%)	1.3 ± 0.11	1.4 ± 0.14	1.3 ± 0.12	1.6 ± 0.08	0.161	0.341	0.892
Suckling episodes per 60 min (n)	5.9 ± 0.83	6.5 ± 1.33	4.9 ± 1.13	5.4 ± 0.96	0.341	0.614	0.950
Suckling duration per 60min (s)	120 ± 21.5	154 ± 35.9	130 ± 26.0	129 ± 15.4	0.758	0.522	0.509
Weight gain to 60 min after fast (g)	298 ± 53.1	388 ± 39.9	217 ± 60.6	289 ± 24.7	0.069	0.103	0.866
Weight gain to 90 min after fast (g)	291 ± 46.0	349 ± 44.1	260 ± 45.4	278 ± 14.5	0.214	0.360	0.524
Weight gain to 90 min after fast (%)	2.7 ± 0.19	2.4 ± 0.23	2.5 ± 0.41	2.6 ± 0.13	0.469	0.654	0.902

Values are means ± SEM. N, normal birth weight. IUGR, intra-uterine growth-restricted.

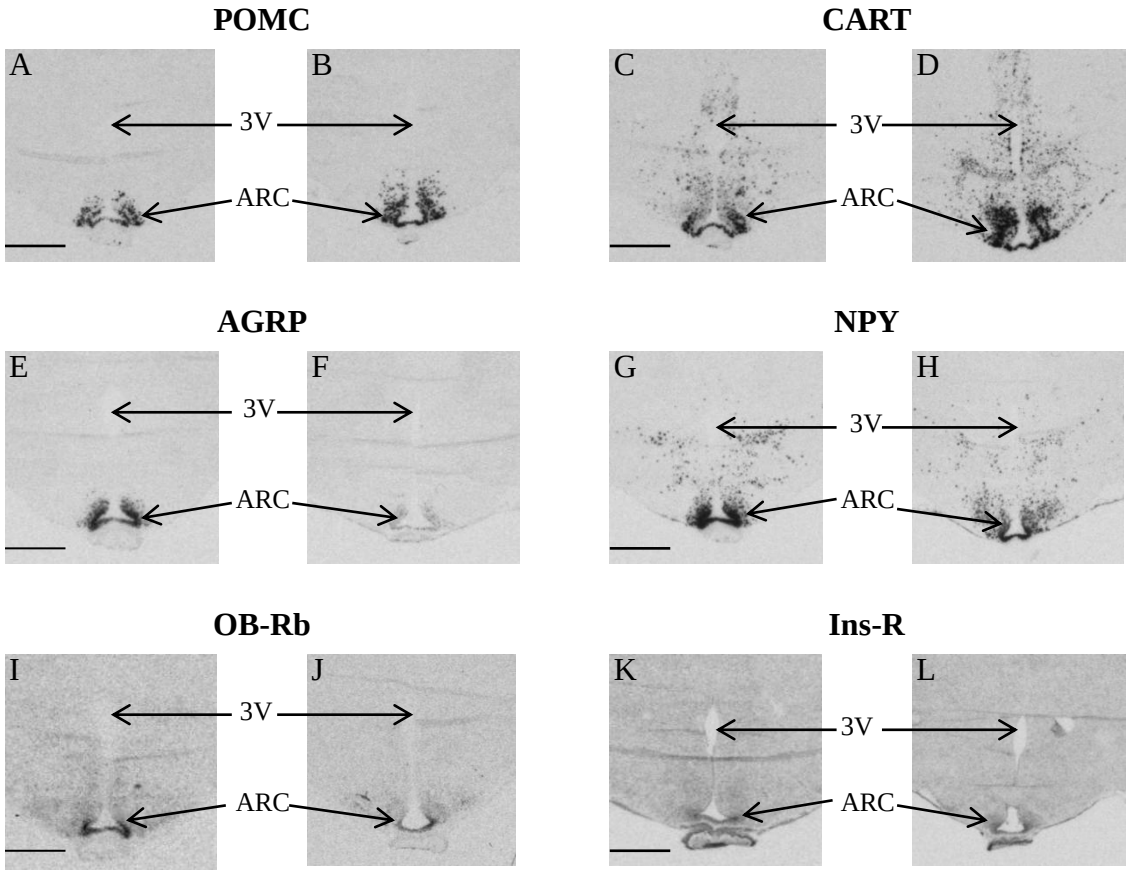
Table 4. Relationships between arcuate nucleus expression of energy balance regulatory genes, body fat, leptinemia, insulinemia, weight gain during a suckling activity assessment, and final current fractional growth rates for body weight and girth in lambs at 11 weeks of age.

	Gene expression (arbitrary densitometry units)					
	POMC	CART	NPY	AGRP	OBRb	Ins-R
Females						
OB-Rb gene expression	-0.234	-0.420	0.346	0.350	-	-
Ins-R gene expression	0.214	-0.057	0.009	0.069	-	-
Perirenal fat (g)	0.405	0.171	-0.362	**-0.520***	-0.110	0.331
Internal fat (g/kg)[1]	0.159	0.205	-0.313	-0.456	0.009	0.088
Leptin (ng/ml, final day)[2]	**0.494***	0.128	-0.185	-0.364	0.061	0.317
Leptin (ng/ml, during last week)[3]	0.303	0.204	**-0.510***	-0.440	-0.162	0.046
Insulin (ng/ml, during last week)[3]	-0.386	0.268	0.040	-0.001	-0.324	-0.396
Suckling % weight gain to 90min[4]	-0.232	-0.065	-0.263	0.263	0.061	-0.025
CFGR body weight (%/day)[5]	**-0.490***	-0.123	-0.195	0.167	0.369	0.015
CFGR thorax girth (%/day)[5]	-0.290	-0.019	0.231	0.212	0.392	0.122
CFGR umbilical girth (%/day)[5]	**-0.547***	-0.340	0.324	0.237	-0.002	-0.389
Males						
OB-Rb gene expression	-0.147	-0.242	**0.535***	**0.507***	-	-
Ins-R gene expression	-0.261	0.164	0.315	0.146	-	-
Perirenal fat (g)	0.257	-0.045	**-0.784*****	**-0.683****	**-0.529***	-0.324
Internal fat (g/kg)[1]	0.245	-0.295	**-0.751*****	**-0.596***	-0.281	-0.481
Leptin (ng/ml, final day)[2]	0.090	-0.287	**-0.776*****	**-0.670****	**-0.542***	-0.383
Leptin (ng/ml, during last week)[3]	0.237	-0.180	**-0.798*****	**-0.632****	-0.472	-0.344
Insulin (ng/ml, during last week)[3]	-0.128	0.127	**-0.501***	-0.433	**-0.643****	0.130
Suckling % weight gain to 90min[4]	0.195	-0.094	**0.623****	0.264	0.329	0.114
CFGR body weight (%/day)[5]	-0.129	-0.277	-0.248	-0.287	0.216	0.423
CFGR thorax girth (%/day)[5]	-0.022	-0.236	**-0.564***	-0.466	-0.089	0.199
CFGR umbilical girth (%/day)[5]	-0.013	-0.191	-0.393	-0.302	-0.106	0.385

Values are Pearson correlation coefficients. Significant correlations shown in bold. *P < 0.05; **P < 0.01; ***P < 0.001. [1]Combined omental and mesenteric fat depots (g/kg empty bodyweight); [2]Sample taken on day 77; [3]Average of three samples taken on days 65-73; [4]Suckling assessment at 23 days; [5]CFGR (current fractional growth rate) measurements at 68 days of age.

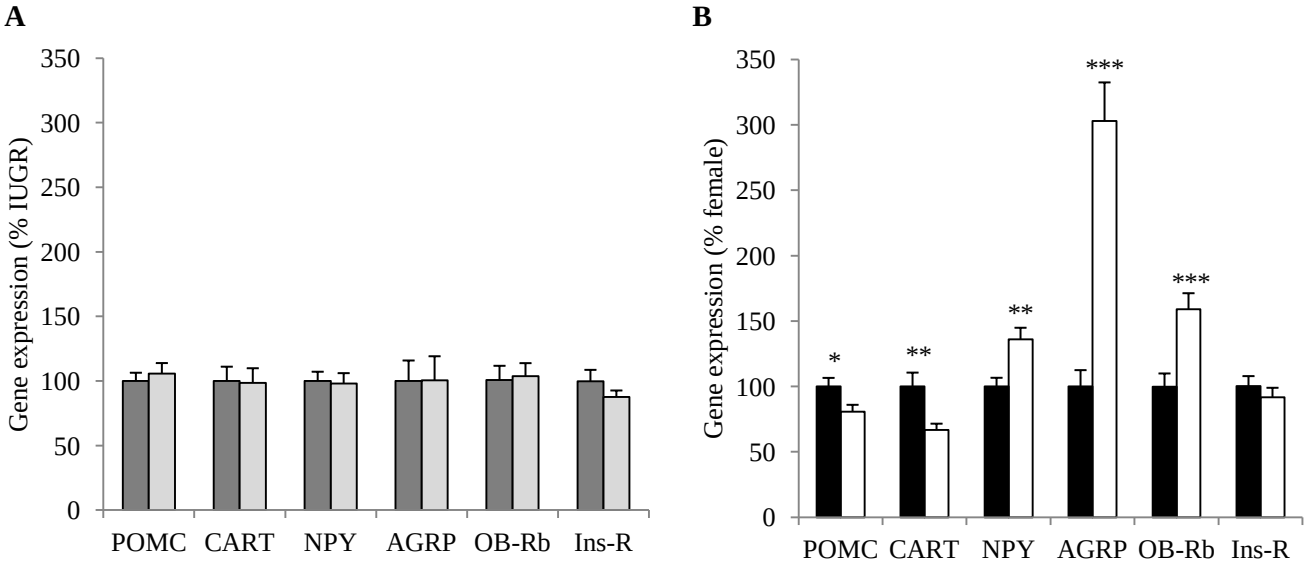
Figure(s)

Fig. 1



Figure(s)

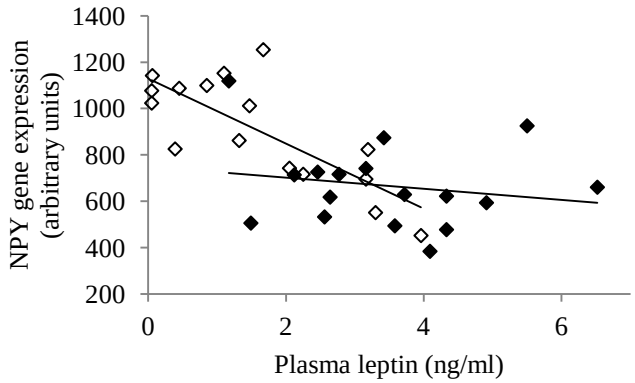
Fig. 2



Figure(s)

Fig. 3

A



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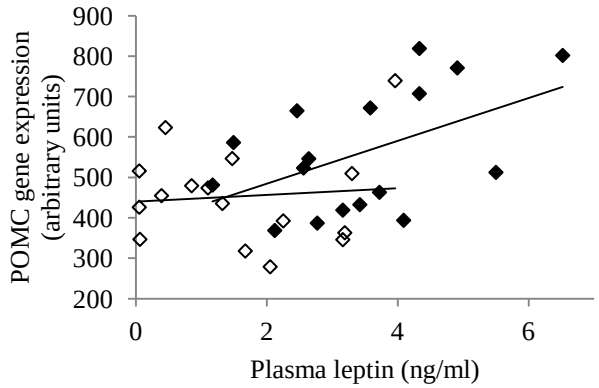
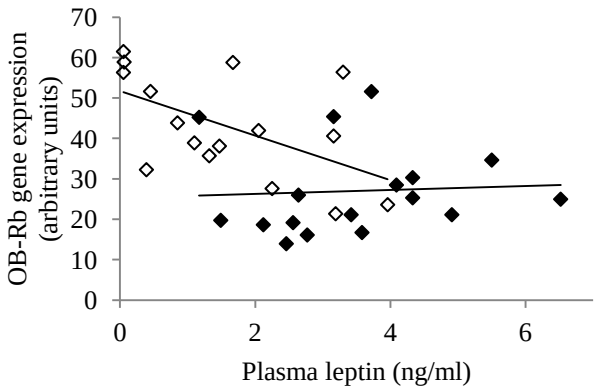
C