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Programming of hypothalamic neuropeptide gene expression in rats by maternal dietary protein content during pregnancy and lactation.

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ABSTRACT

Epidemiological studies show a link between low birthweight and increased obesity. In contrast, slow growth during the lactation period reduces obesity risk. This study investigates potential underlying mechanisms. Rats were established as follows: (a) controls (offspring of control dams fed a 20% protein diet), (b) recuperated animals (offspring of dams fed an isocaloric low-protein (8%) diet during pregnancy, nursed by control dams) and (c) postnatal low-protein animals (offspring of control dams nursed by low-protein dams). Serum and brains were collected from fed and fasted animals at weaning. Expression of hypothalamic energy balance genes was assessed using *in situ* hybridisation. Recuperated pups were smaller at birth, but caught up with controls by day 21 and gained more weight than controls between weaning and 12 weeks of age ($p<0.05$). At 21 days they were hypoleptinemic compared to controls in the fed state with generally comparable hypothalamic gene expression. Postnatal low-protein offspring had significantly lower body weight than controls at weaning and 12 weeks of age ($p<0.001$). At 21 days they were hypoglycemic, hypoinsulinemic and hypoleptinemic. Leptin receptor gene expression in the arcuate nucleus was increased in postnatal low-protein animals compared to controls. Consistent with the hypoleptinemia, hypothalamic gene expression for orexigenic neuropeptides, neuropeptide Y and, agouti-related peptide, was increased, and that for anorexigenic neuropeptides, proopiomelanocortin and cocaine-and amphetamine-regulated transcript was decreased. These results suggest that the early nutritional environment can affect the development of energy balance circuits and consequently obesity risk.

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INTRODUCTION

Over recent decades, there has been a large increase in the proportion of both adults and children that are either overweight or obese in developed countries. In the United States, 64.5% of adults had a body mass index (BMI) greater than 25kg m^{-2} in 2000, increased from 55.9% in 1988-94, while the proportion of adults that had a BMI over 30 kg m^{-2} had increased from 22.9% in 1988-94 to 30.5% in 2000 [1]. More worrying is the increase in childhood obesity with 22 million children under the age of 5 overweight and 10% of US preschool children obese [2]. The consequences of being overweight or obese are far reaching in terms of mortality and morbidity as well as escalating healthcare costs. Obesity increases the risk of type 2 diabetes, dyslipidaemia, hypertension and certain types of cancer such as those of the breast, colon and pancreas [3].

Many of the diseases where obesity is a risk factor have been linked by numerous epidemiological studies with low birth weight [4, 5]. These relationships have been found in populations worldwide but the mechanisms underlying this link are still unclear. However there is compelling evidence to suggest that the early environment and in particular early nutrition plays a key role. Studies of adults who were *in utero* during the 'Dutch Hunger Winter' provide direct evidence in humans for the importance of maternal nutrition on offspring's health. Men exposed to famine in early gestation had increased rates of obesity at 19 years of age while those exposed during late gestation and in the first months of life showed reduced rates of obesity [6]. Breast feeding provides infants with a lower plane of neonatal nutrition than formula feeding, and a meta analysis of studies investigating the effect of breast feeding showed that breast fed infants have a reduced risk of obesity compared to formula fed infants [7]. A variety of protocols have been established to investigate the effects of maternal diet on obesity risk in the offspring. The offspring of severely undernourished rat dams are hyperphagic and obese [8].Further evidence for the importance of the early postnatal period can be found in cross fostering studies. In mice, offspring of dams fed a reduced protein diet, cross fostered in small litters to control fed dams are born small, undergo catch up growth and are more susceptible to diet induced obesity. In contrast, offspring of control dams cross fostered onto a dam fed a reduced protein diet during lactation are resistant to the obesity inducing effects of a highly palatable diet [9, 10]. These studies show the long-term detrimental effects of "catch up" growth in early life while slow growth during this period appears to confer a protective effect, a programmed reduction in voluntary food intake.

The energy balance system is regulated by feedback from peripheral hormonal and metabolic signals onto central integratory circuits, which are concentrated in the hypothalamus. Within the hypothalamus, neuropeptides and their receptors are prominent. Leptin is a major energy balance signal derived from adipose tissue that relays long-term information to the brain about the size of body fat stores and shorter term information about the state of energy flux in adipose tissue. Leptin receptors are heavily expressed in the arcuate nucleus of the hypothalamus and colocalise with both the orexigenic (NPY/AgRP) and anorexigenic pathways (CART/POMC). The arcuate nucleus is a site of leptin feedback into the brain. Consequently, the leptin signal and its integration into the hypothalamic network are likely to be involved in the programming of energy balance systems by early life nutrition. A number of strands of evidence support this suggestion. *Ob/ob* mice, which lack leptin, show reduced neuronal outgrowth from the arcuate nucleus. Furthermore leptin caused neurite

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growth from neonatal arcuate explants but not adult ones [11]. During the lactation period it is thought that leptin is playing a developmental rather than an energy balance role. There is a leptin surge during this period [12] and this surge is earlier in undernourished mouse pups [13]. If control pups are given exogenous leptin, to mimic the premature leptin surge seen in the undernourished offspring, they show accelerated weight gain when placed on a high fat diet [13]. However in rats, if the offspring of undernourished dams are given exogenous leptin postnatally then the detrimental metabolic phenotype seen in adulthood can be reversed [14]. Alterations in neonatal leptin concentrations induced by changes in maternal nutrition could therefore affect hypothalamic development of energy balance circuits.

The aim of this study was therefore to investigate the effect of fetal growth restriction followed by catch up growth, or normal fetal growth followed by slowed growth during neonatal life on anatomically resolved expression of a panel of neuropeptide and receptor genes known to be involved in energy balance regulation. An early time point was chosen allowing the elucidation of possible mechanisms rather than later life consequences.

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METHODS

Animals and experimental protocol

All procedures involving animals were carried out under the UK Home Office Animals (Scientific Procedures) Act, 1986. Virgin female Wistar rats weighing 240-260g were housed individually and maintained at 22ºC on a 12h light-dark cycle (lights on 0700-1900). They were mated and day 1 of gestation was taken as the day on which vaginal plugs were expelled. Rats were then fed either a control (20% protein) diet or an isocaloric low protein (8%) diet. These were purchased from Arie Blok (Woerden, Netherlands) and their composition has been described previously [15]. The diet has no effect on litter size (control 13 ± 0.3 vs low protein 13 ± 0.4, p=0.64), sex ratio (Control 48 ± 2.3 % males, Low Protein 53 ± 4.4 % males, p=0.33) or pup survival. Maternal weight and food intake were measured daily until weaning. After spontaneous birth on day 21, the control dams were either continued on the control diet with their own litter culled to 8 offspring ('control') or received and suckled 4 randomly selected offspring from the litter of a low protein dam ('recuperated'). The culling of this litter to only 4 served to maximise the plane of nutrition received by these pups during suckling and also the rate of catch up growth. Low protein dams that had donated their pups to a control dam received that dam's litter (both male and female pups) unculled ('postnatal low protein') (litter size 13±0.41). This litter was left unculled to minimise the amount of nutrition during suckling and therefore the rate of growth. For the pups killed on postnatal day 22, weights were taken on postnatal days 3,7,14 and 21.

Pups were weaned at 21 days. One cohort of male animals was immediately killed (at 0900) and a separate group of male animals were starved overnight and killed on day 22 (at 0900). This enabled study of the animals in both fed and fasted states. No more than 2 pups were taken from each litter and each cohort of animals studied used separate groups of dams giving 4-8 dams used for each group of offspring studied at each timepoint. On the day of the experiment, animals were weighed and then killed by CO_2 inhalation and exsanguination. Blood serum was collected. Brains were removed, immediately frozen on dry ice and then stored at -80°C for *in situ* hybridisation. Retroperitoneal and mesenteric fat pads were weighed. An additional cohort of animals were studied up to 12 weeks of age after weaning onto a standard laboratory chow diet at 21 days of age (LAD1, SDS, Witham, UK). Body weights and food intakes were measured weekly. At 12 weeks of age this cohort was killed by CO_2 inhalation and exsanguinations. The fat pads were dissected and weighed.

Circulating hormones and metabolites.

Blood glucose was measured (5µl) using a blood glucose analyser (Hemocue AB, Ängelholm, Sweden). Leptin concentrations in serum were measured using an ELISA assay from CrystalChem (Il, USA), the sensitivity of the assay is reported as 12.5 nmoles/litre and the intra-assay coefficient was 4.9%. Serum insulin concentrations were measured using an ultra sensitive rat/mouse ELISA assay from Mercodia (Uppsala, Sweden). The sensitivity of the assay is reported as 3.5 pmoles/litre and the intra-assay coefficient was 1.7%.

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Hypothalamic gene expression

Hypothalamic gene expression for a number of energy balance related neuropeptides and receptors was quantified using *in situ* hybridization techniques, which are described in detail elsewhere [16]. Riboprobes complementary to partial fragments of Agouti related protein (AgRP), pro-opiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), melanocortin-4 receptor (Mc4R) and cannabinoid receptor 1 (CBR1) were generated from cloned cDNAs as previously described [17-19]. Neuropeptide Y (NPY) riboprobes were generated using a partial rat cDNA sequence [20]. Riboprobes for suppressor of cytokine signaling-3 (mSOCS-3) and leptin receptor (OB-Rb, long form) were generated using partial mouse and rat cDNA sequences, respectively [21, 22].

Briefly, hypothalamic sections were collected from the very caudal extent of the arcuate nucleus (ARC) through to the rostral extent of the paraventricular nucleus (PVN) onto 2 sets of 10 slides spanning the full extent of the ARC, approximating –4.52 to –2.30 mm, relative to Bregma, according to the atlas of the rat brain [23], and continuing through to –1.40 mm relative to Bregma and including both rostral and caudal extents of the PVN. Sections were fixed, acetylated, and hybridized overnight at 58°C using ^{35}S-labeled antisense riboprobes (1–1.5 x 10^{10} dpm/L). Slides were treated with RNase A to remove unhybridized probe and then desalted with a final high-stringency wash in 0.1X SSC at 60°C for 30 min. The slides were air dried and exposed to Kodak BioMax MR autoradiography film (Amersham Pharmacia Biotech UK). Autoradiographic images were quantified using the Image-Pro Plus system (Media Cybernetics). This determined the intensity and area of the hybridization signal on the basis of set parameters; the integrated intensity was then computed using standard curves generated from ^{14}C autoradiographic microscales (Amersham UK). Depending on the probe used, image analysis was performed on up to 5 sections spanning the ARC and 2 or 3 sections from the PVN, dorsomedial nucleus (DMH) and ventromedial nucleus (VMH).

This experiment was initially conducted on overnight fasted animals at 22 days of age. However to remove the possibly confounding effect of differential susceptibility to fasting or weaning stress between the groups it was then repeated on *ad libitum* fed animals at 21 days of age. As these experiments were not conducted at the same time, it is not possible to directly compare animals in the fed and fasted state.

Statistical Analysis

Data are expressed as mean ± SEM except for serum insulin, which is shown as geometric means (95% confidence intervals). Statistical differences between means were assessed using one-way ANOVA with maternal diet as the independent variable followed by Duncans post hoc test where appropriate. Serum insulin data was log transformed prior to testing as it was not normally distributed, and so is presented as geometric means (95% confidence intervals). Differences between means were considered significant at values of $p<0.05$.

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RESULTS

Maternal Weight and Food Intake

Low protein dams were smaller than control fed dams by the end of gestation (388.9 ± 10.4g vs. 426.0 ± 4.8g, p<0.01). Total maternal food intake during gestation was not significantly different (Low protein dam intake 525.9 ± 24.2g vs. control dam intake 561.5 ± 13.0g). After cross fostering, there are 3 experimental groups of dams. During lactation, the weight of the low protein dams decreased further so by the end of lactation they were 30% smaller than control dams (254.3 ± 5.1g vs. 355.6 ± 5.7g, p<0.001) while the control dams nursing either control or recuperated litters maintained their weight.

Growth of pups until weaning

Data are shown in Figure 1. At birth, pups whose mothers were fed the low protein diet were significantly smaller than the control pups (p<0.001). This is a mild intervention and did not significantly affect litter size (Control 13.0 ± 0.3, Low Protein 13.0 ± 0.4, p=0.64) or the sex ratio of the offspring (Control 48 % ± 2.3 males, Low Protein 53% ± 4.4 males). Recuperated pups caught up with control pups by day 14 and postnatal low protein pups were smaller than control pups by day 7 (p<0.001). Postnatal low protein pups remained smaller throughout lactation and weighed less than 50% of control pups on day 21 (p<0.001).

Organ weights of fasted 22 day old offspring

The weight of the brain (p<0.01), retroperitoneal (p<0.001) and mesenteric fat pads (p<0.001) were all significantly lower in the postnatal low protein animals compared to controls (Table 1). However the reduction in brain weight and the fat pad weights were not in proportion with the reduction in body weight (Table 1). The growth of the brain (p<0.001) was relatively spared whereas the reduction in fat masses (both p<0.001) were greater than overall body weight (Table 1).

Characteristics of offspring blood/serum

There was a significant overall effect of maternal diet (p<0.001) on blood glucose concentrations. Postnatal low protein animals had lower glucose concentrations compared to controls (p<0.05 for fed animals, p<0.001 for fasted rats) (Table 2). There was a significant overall effect of maternal diet manipulation (p<0.001) on serum leptin concentrations. Leptin concentrations were lower in postnatal low protein rats compared to controls (p<0.001 for fed animals, p<0.05 for fasted rats). In fed rats, recuperated animals had lower serum leptin concentrations compared to controls (p<0.001). However in the fasted state, recuperated male animals had higher serum leptin concentrations compared to controls (p<0.05) (Table 2). Maternal diet also altered insulin concentrations (p<0.001). Insulin concentrations being lower in postnatal low protein rats compared to controls (p<0.001 for fed animals, p<0.05 for fasted rats) (Table 2).

Hypothalamic gene expression

Receptors and SOCS-3

Data are shown in Figure 2. There was a significant overall effect of maternal diet on OB-Rb gene expression in the ARC of *ad libitum* fed rats (p<0.001) and overnight fasted rats (p<0.01). In each case, ARC OB-Rb gene expression was elevated in postnatal low protein rats compared to controls, but this difference was more

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substantial in fed rats than in fasted ones. In the VMH, there were no effects on OB-Rb gene expression in *ad libitum* fed rats but there was a significant overall effect of maternal diet ($p<0.001$) in overnight fasted animals. OB-Rb gene expression being high in the VMH of postnatal low protein animals compared to control animals in the fasted state.

There was a significant overall effect of maternal diet ($p<0.001$) on SOCS3 gene expression in the ARC of *ad libitum* fed animals. Both recuperated and postnatal low protein animals had reduced SOCS3 expression compared to control animals in the fed state. There was also an effect of maternal diet ($p<0.01$) on SOCS3 expression in overnight fasted animals. However, the effect of maternal diet was only observed in postnatal low protein animals, which had lower SOCS3 gene expression compared to control animals.

There was an overall effect of maternal diet ($p<0.01$) on MC4R gene expression in the PVN in *ad libitum* fed animals, with postnatal low protein animals having lower MC4R gene expression. However this effect was not observed in fasted animals.

Orexigenic peptides

Data are shown in Figure 3. There was a significant effect of maternal diet ($p<0.001$), on NPY gene expression in the ARC of both fed ($p<0.001$) and fasted rats ($p<0.05$). In each case, ARC NPY gene expression was elevated in postnatal low protein rats compared to controls. This difference was more marked in the fed rats than in the fasted animals. In the DMH there was no effect of maternal diet on NPY gene expression in either fed or fasted conditions. There was a significant effect of maternal diet on AgRP gene expression in the ARC of both fed ($p<0.001$) and fasted ($p<0.01$) rats. In each case, ARC AgRP gene expression was elevated in postnatal low protein rats compared to controls. This difference was again more marked in fed rats than in fasted.

Anorexigenic peptides

Data are shown in Figure 4. There was a significant effect of maternal diet on POMC gene expression in the ARC of fed ($p<0.001$) and fasted ($p<0.01$) rats. POMC gene expression was lower in postnatal low protein rats compared to controls. The down regulation of POMC in the postnatal low protein group was more substantial in fed rats than in fasted.

There was a significant effect of maternal diet on CART gene expression in the ARC of fed ($p<0.001$) and fasted ($p<0.01$) rats, with CART gene expression being lower in postnatal low protein rats compared to controls. This effect was not as substantial as that observed in fed rats.

Growth and energy intake post weaning

There was a significant effect of maternal diet on body weight gain from weaning to 12 weeks of age ($p<0.001$). Recuperated animals gained more weight than controls ($p<0.05$), while postnatal low protein animals were gained less weight ($p<0.001$) (Table 3). This led to recuperated animals being heavier than control animals ($p<0.05$) at 12 weeks of age and postnatal low protein animals were lighter ($p<0.001$) (Table 3). There was a significant effect of maternal diet on cumulative energy intakes during this period with the same pattern, recuperated animals consumed more energy ($p<0.05$) compared to control animals while postnatal low protein animals consumed less energy ($p<0.001$) (Table 3).

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The weights of both the retroperitoneal and epididymal fat pads were affected significantly by maternal diet in adult rats ($p<0.001$ for both). Postnatal low protein animals had smaller fat pads than control animals while there was a tendency ($p=0.06$ for both) for the recuperated animals to have heavier fat pads than control animals (Table 3). When expressed relative to body weight, there was still an overall effect of maternal diet ($p<0.001$ for both fat pads) with postnatal low protein animals having lower relative fat masses ($p<0.001$ for both) but the recuperated animals relative fat pad weights were not significantly different from control animals (Table 3).

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DISCUSSION

Alterations in maternal dietary protein content during gestation and lactation have permanent effects on susceptibility to weight gain and longevity in rodents [9, 10, 24], but the molecular mechanisms that underlie these programming events remain to be resolved. The current study examined alterations in leptin and the expression of neuropeptides and receptors implicated in energy balance control after manipulation of maternal diet protein content and the plane of nutrition received during lactation. In addition, the effect of fasting on these young animals and their energy balance related hypothalamic gene expression was examined.

Long term effects on weight gain could be mediated by changes in energy intake and/or energy expenditure. In this model, recuperated animals consumed more energy than control animals and this was accompanied by a larger increase in body weight while postnatal low protein animals consumed less energy and so gained less weight in adulthood. It is therefore clear that simple alterations in early life nutrition had long-term effects on energy balance via energy intake. Currently it is unknown whether there are similar effects on energy expenditure.

In this study we addressed mechanisms by which these programmed changes in energy intake could occur by studying animals at weaning. By choosing an early time point, programmed changes that could lead to the development of the phenotype rather than changes as a consequence of the phenotype can be examined. At this age the recuperated animals did not differ from the control animals in terms of body weight. We demonstrated that the serum concentration of leptin was altered by manipulation of maternal protein intake. Leptin during the lactation period is a candidate for programming of the appetite system [11, 25, 26]. Postnatal low protein animals had substantially reduced blood leptin concentration compared to control animals under both fed and fasted conditions. This reduction is likely to be a consequence of the dramatically reduced adiposity in this group. Recuperated animals had lower leptin concentration than controls in the fed state, despite similar body weights, suggesting a possible alteration in the control of leptin secretion. This could lead to an increased drive to eat and could increase their susceptibility to obesity, as seen by their higher cumulative energy intakes and body weights from weaning to 12 weeks of age. In addition there was a tendency for increased fat pad mass in the recuperated animals at 12 weeks of age when compared to control animals although thiswas in proportion with the increased body weight. After an overnight fast, the leptin concentration in recuperated animals did not show the expected large drop seen in both control and postnatal low protein animals. This resulted in recuperated animals having anelevated leptin concentration compared to the control animals in the fasted state. Recuperated animals at this age therefore seem to have a defect in their ability to alter their plasma leptin concentration in response to feeding status.

Differences in long term susceptibility to weight gain may be a consequence of changes in central leptin sensitivity. The mRNA of the signalling form of the leptin receptor was more highly expressed in the arcuate nucleus of postnatal low protein animals compared to control animals in both fed and fasting conditions. This, combined with the lower expression of the negative regulator of leptin signalling, SOCS3, may increase leptin sensitivity in these animals, which may contribute to their long term reduction in weight gain compared to control animals. It has been shown previously that low leptin concentrations can lead to upregulation of the leptin receptor in the ARC [27]. The fed recuperated animals had low concentrations of circulating leptin compared to the control animals, however, this hypoleptinemia was not accompanied by an increase in the expression of the leptin receptor in the ARC.

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This contrasts with the postnatal low protein animals and suggests there is a difference in the response to low leptin levels between these two groups of animals. High levels of leptin are presumed to signal positive energy balance and counteract this state through two pathways. The expression of anorexigenic genes such as CART and POMC are increased in response to leptin while the expression of orexigenic genes such as NPY and AgRP are decreased [28]. In the fed state, expression of the mRNAs for the orexigenic peptides, NPY and AgRP, in the ARC was higher in the postnatal low protein animals and expression of the mRNAs for the anorexigenic peptides, CART and POMC, was lower compared to control animals. These changes are appropriate for the low serum leptin concentration in this group. This suggests that these animals register the negative energy balance they are in i.e. their low body weight compared with developmentally appropriate controls. However these postnatal low protein animals remain small even when fed *ad libitum*, and so do not respond appropriately to this multi-component signal. This suggests that these animals have a fault downstream of leptin signalling.

This study reinforces the suggestion, which is growing in the literature, that fetal and early life nutrition affects energy balance systems. Small litter size animals that go on to become obese show reduced arcuate nucleus gene expression of OB-Rb and increased arcuate mRNA expression of the orexigenic peptides NPY and AgRP post weaning [29]. Neonatally overnourished rats at weaning have been shown to have alterations in the paraventricular nucleus of the hypothalamus with decreased numbers of CCK-positive neurons [30] and increased numbers of galanin positive neurons [31]. There is also evidence for a change to the response of the ventromedial hypothalamic nucleus to leptin. In neonatally normally nourished rats, leptin increases firing from ventromedial hypothalamic neurons while in neonatally overnourished animals, leptin inhibits firing [32]. Other models of altered early nutrition have shown altered expression of energy balance neuropeptides in the hypothalamus; maternal high fat feeding leads to increased expression of galanin in the paraventricular hypothalamic nucleus and orexin in the lateral hypothalamus [33]. While the offspring of food restricted rat dams have a lower level of hypothalamic POMC expression [34]. This could cause an increase drive to eat in these offspring. Maternal obesity, as a consequence of the genetic background of the dam, during lactation of the pups has been shown to influence expression of energy balance neuropeptides in adult offspring [35].

This study has shown that hypothalamic mRNA expression of regulatory neuropeptides and signalling proteins of energy balance systems are altered by maternal dietary protein manipulations during pregnancy and lactation. Those animals that grew slowly during lactation show evidence of changes in expression of neuropeptides that should signal that they are in a state of negative energy balance. However, the anticipated physiological response to these signals, namely behavioural and energetic compensation does not develop, and the animals stay small during adolescence and adult life when fed *ad libitum* with lower energy intakes over this period than control animals. This could be a result of the action of the neuropeptides being blocked further downstream or a complete alteration of the wiring of the system. These animals that remain lean show changes in expression of leptin signalling molecules that could indicate increased sensitivity to this important regulator of energy balance. Animals that underwent *in utero* growth restriction followed by catch up growth showed similar expression of examined neuropeptides to control animals but reduced leptin in the fed state compared to control animals. This could provide an increased drive to eat, even when fed, thus providing a possible

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mechanism for their increased weight gain post weaning and indicates a potential peripheral defect in theses animals. This study provides new insight into the mechanistic basis that underlies the link between low birth weight and obesity and slow growth and leanness in adulthood.

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TABLE 1. Absolute and relative weights of fat pads and brain in 22 day old male rat pups

	CONTROL	RECUPERATED	POSTNATAL LOW PROTEIN
Absolute retroperitoneal fat pad weight (g)	0.20 ± 0.01	0.20 ± 0.03	0.03 ±0.01 ***
Absolute mesenteric fat pad weight (g)	0.18 ± 0.01	0.20 ± 0.03	0.05 ± 0.01 ***
Absolute brain weight (g)	1.50 ± 0.02	1.47 ± 0.03	1.35 ± 0.05 **
Relative retroperitoneal fat pad weight (%)	0.37 ± 0.02	0.40 ± 0.05	0.14 ± 0.02 ***
Relative mesenteric fad pad weight (%)	0.35 ± 0.2	0.41 ± 0.05	0.20 ± 0.02 ***
Relative brain weight (%)	2.86 ± 0.07	3.0 ± 0.14	6.14 ± 0.2 ***

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TABLE 2 Blood/serum data at 21 days (fed) and 22 days (fasted) of age

	Control	Recuperated	Postnatal low protein
Fed blood glucose (mM)	9.1 ± 0.6	10.0 ± 0.6	6.2 ± 0.8 *
Fasted blood glucose (mM)	5.0 ± 0.1	5.3 ± 0.1	4.3 ± 0.1 ***
Fed serum leptin (μM)	0.23 ± 0.021	0.11 ± 0.014 ***	0.09 ± 0.013 ***
Fasted serum leptin (μM)	0.06 ± 0.004	0.09 ± 0.009 *	0.03 ± 0.007 *
Fed serum insulin (pM)	95.8 (73.5-131.7)	65.7 (42.3-117.9)	3.9 (3.2-4.9) ***
Fasted serum insulin (pM)	20.0 (9.4-36.3)	10.4 (6.4-21.5)	7.7 (4.0-16.7) *

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TABLE 3 Body weight and energy intakes at 12 weeks of age

	Control	Recuperated	Postnatal low protein
Body weight gain from weaning to 12 weeks (g)	322.2 ± 6.6	347.3 ± 8.4 *	282.3 ± 5.9 ***
Body weight at 12 weeks	403.2 ± 7.5	425.8 ± 9.4 *	322.0 ± 5.1 ***
Cumulative energy intake from weaning to 12 weeks (kJ)	16641 ±224	17237 ± 467 *	13853 ± 258 ***
Absolute epididymal fat pad weight (g)	5.33 ± 0.32	6.20 ± 0.44 p=0.06	3.08 ± 0.18 ***
Absolute retroperitoneal fat pad weight (g)	6.56 ± 0.42	7.81 ± 0.54	3.27 ± 0.35 ***
Relative epididymal fat pad weight (%)	1.34 ± 0.08	1.48 ± 0.07 p=0.06	0.95 ± 0.06 ***
Relative retroperitoneal fat pad weight (%)	1.65 ± 0.11	1.88 ± 0.10	1.01 ± 0.11 ***

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LEGENDS

TABLE 1

Tissue weights shown as absolute weight (g) and weight relative to body weight (%) collected from overnight fasted animals. n=8 per group. Data expressed as mean ± SEM and examined using a one-way ANOVA with appropriate Duncan's post hoc tests. p values shown are compared to control animals. ** $p<0.01$ and *** $p<0.001$ when compared to control animals.

TABLE 2

Serum and plasma characteristics of males pups at both 21 days of age (fed state) and 22 days of age (fasted state). n=8 per group. Data expressed as mean ± SEM and examined using a one-way ANOVA with appropriate Duncan's post hoc tests. p values shown are compared to control animals. Insulin data was log transformed prior to examination and is shown as geometric mean (confidence intervals). * $p<0.05$ and *** $p<0.001$ when compared to control animals.

TABLE 3

Bodyweight (g) at 12 weeks, bodyweight gain (g) from weaning until 12 weeks, cumulative energy intake over this period and absolute and relative fat pad weights at 12 weeks in male rats. n=10 per group. Data expressed as mean ± SEM and examined using a one-way ANOVA with appropriate Duncan's post hoc tests. * $p<0.05$ and *** $p<0.001$ when compared to control animals.

FIGURE 1

Growth curves of male rats during lactation. n=10 per group. Data expressed as mean ± SEM and examined using one-way ANOVA with appropriate Duncan's post hoc tests. *** $p<0.001$ when compared to control animals of the same age.

■ CONTROL, ■ RECUPERATED and □ POSTNATAL LOW PROTEIN

FIGURE 2

Gene expression of OB-Rb, SOCS3 and MC4R in indicated regions of the hypothalamus in fed 21 day old males (A) and fasted 22 day old males (B). n=8 per group. Data expressed as % of control and shown as mean ± SEM and examined using a one-way ANOVA with appropriate Duncan's post hoc tests. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ when compared to control animals.

■ CONTROL, ■ RECUPERATED and □ POSTNATAL LOW PROTEIN

FIGURE 3

Gene expression of NPY and AgRP in indicated regions of the hypothalamus in fed 21 day old males (A) and fasted 22 day old males (B). n=8 per group. Data expressed as % of control and shown as mean ± SEM and examined using a one-way ANOVA

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with appropriate Duncan's post hoc tests. * $p<0.05$ and *** $p<0.001$ when compared to control animals.

■ CONTROL, ■ RECUPERATED and □ POSTNATAL LOW PROTEIN

FIGURE 4

Gene expression of POMC and CART in the arcuate nucleus of the hypothalamus in fed 21 day old males (A) and fasted 22 day old males (B). n=8 per group. Data expressed as % of control and shown as mean ± SEM and examined using a one-way ANOVA with appropriate Duncan's post hoc tests. ** $p<0.01$ and *** $p<0.001$ when compared to control animals.

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Figure 1

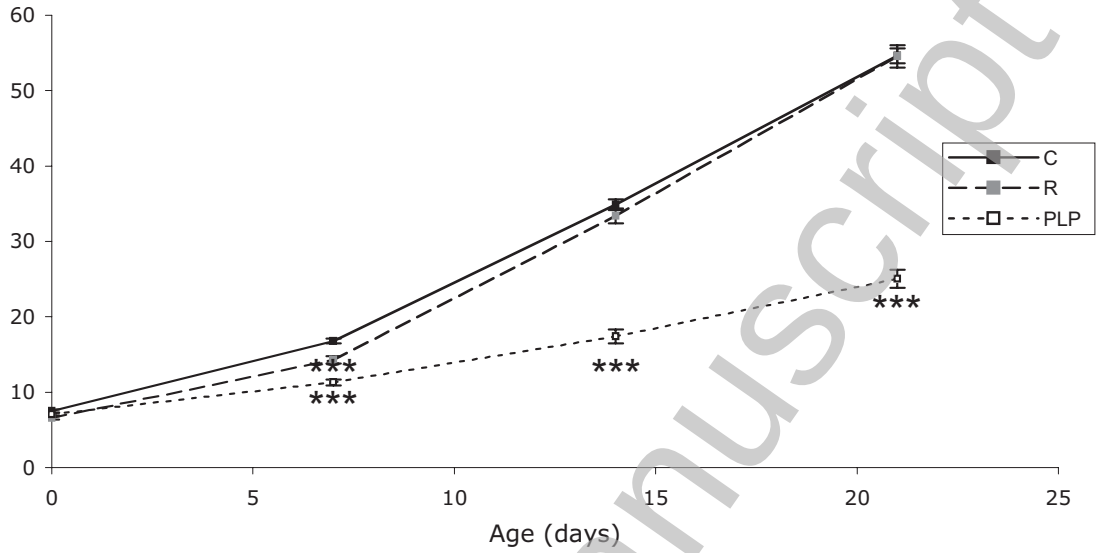

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Figure 2

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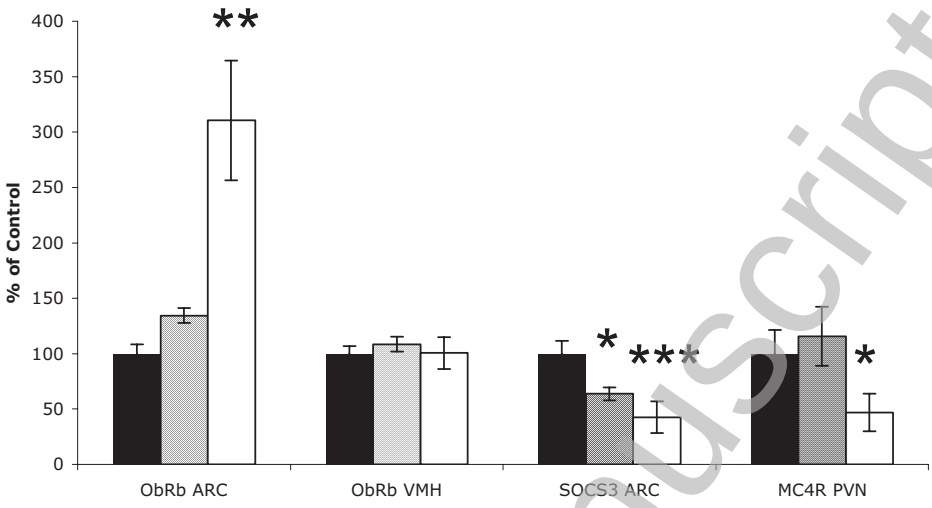

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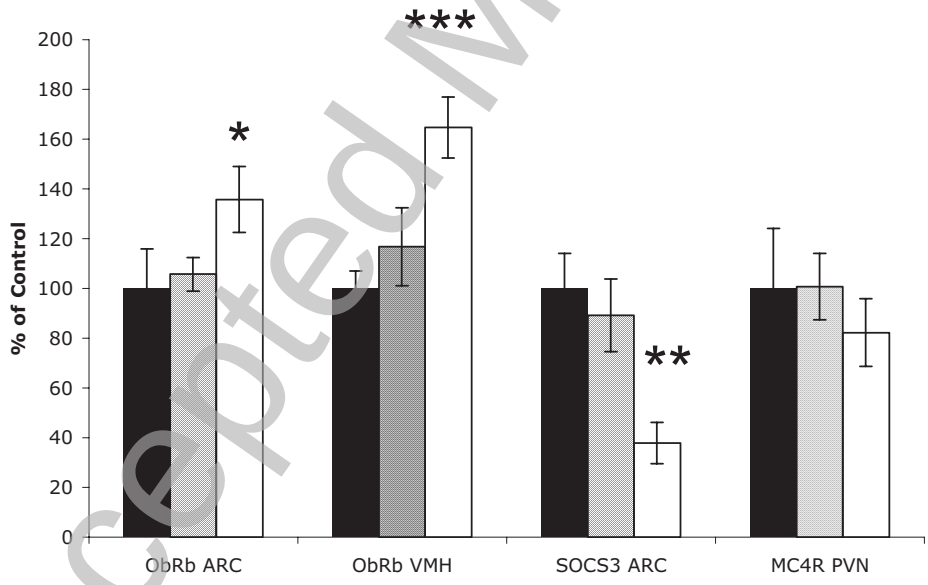

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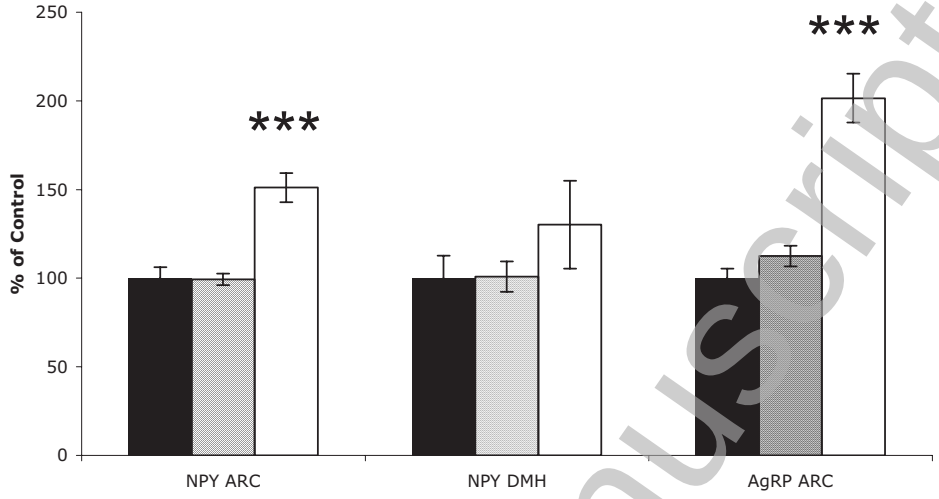
Figure 3

A



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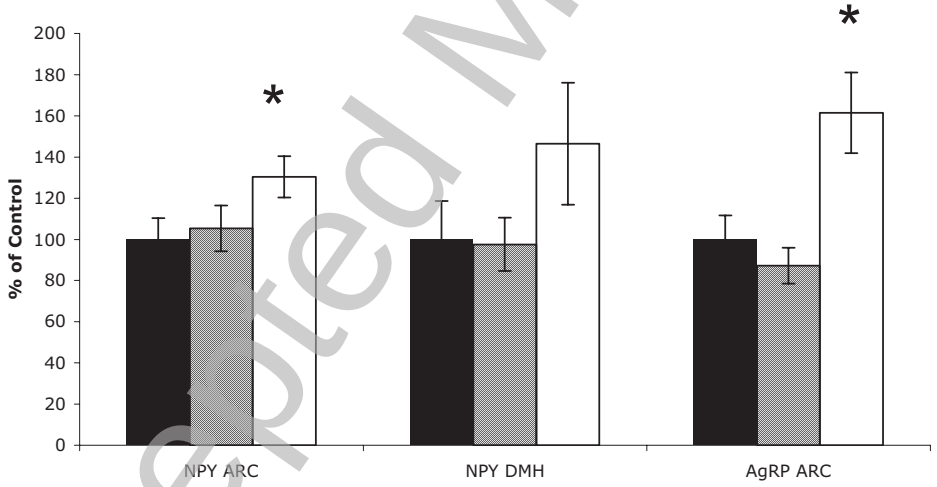

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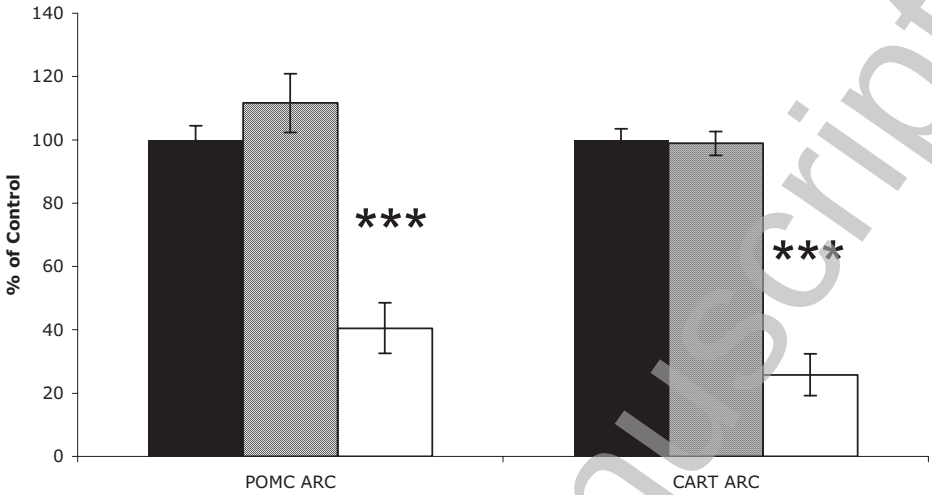
Figure 4

A



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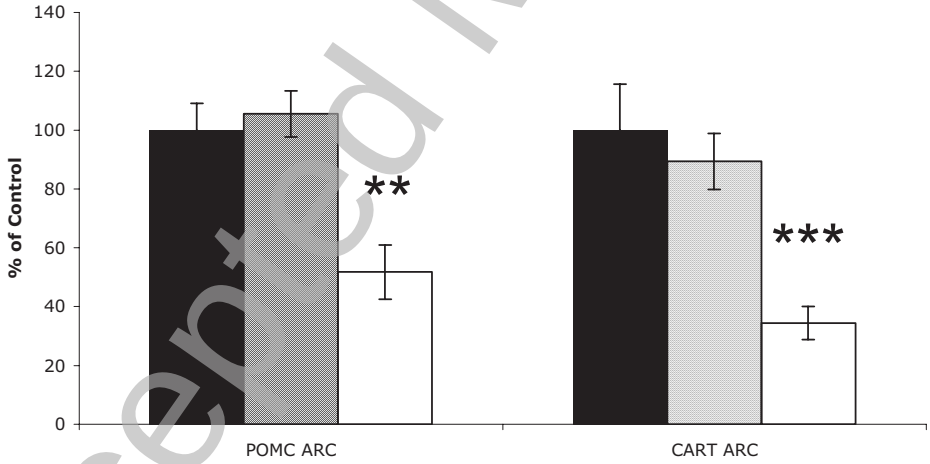

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