Maternal overnutrition impacts offspring adiposity and brain appetite markers-modulation by postweaning diet

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Abstract

The maternal nutritional state has long term metabolic consequences in offspring. We examined the relationship between different degrees of maternal overfeeding and adiposity on lipid and glucose homeostasis and central appetite regulators in offspring. F0 founders were fed chow (C) or high fat diet (HFD). F1 females from chow fed F0 were raised in normal (CN) or small (CS) litters (12 vs 3 pups/dam) and fed chow; a third group from HFD fed F0 was raised in normal litters and fed HFD (HN). Thus two degrees of overnutrition at F1 (CS, HN) were compared to control, CN. F2 pups were weaned onto chow (CN_C, CS_C, HN_C) or HFD (CN_H, CS_H, HN_H). At 21 weeks, CS_C were heavier with 50% increased fat mass (vs CN_C; $P<0.01$), while HN_C and HN_H offspring were heavier with 100% and 62% increases in fat mass (vs CN_C and CN_H; $P<0.01$). Both degrees of maternal overnutrition caused glucose intolerance in offspring consuming HFD. Maternal and postweaning HFD consumption was linked to increased leptin, insulin and triglycerides in offspring, with no maternal litter size effect. Hypothalamic signal transducer and activator of transcription-3 (STAT-3) and suppressor of cytokine signaling-3 (SOCS-3) mRNA were significantly elevated in HN_H (vs CN_H; $P<0.05$). Skeletal muscle uncoupling protein3 (UCP-3) mRNA and adipose triglyceride lipase mRNA were downregulated only by postweaning HFD. Our data suggest that any level of F1 overnutrition increases adiposity in F2, with a greater impact of HFD induced maternal obesity. Additive effects were observed when F2 animals consumed HFD.

Key words: maternal overnutrition, leptin, energy regulation, high fat diet, glucose homeostasis.

Abbreviations:

AgRP	Agouti-gene-related protein
ANOVA	Analysis of Variance
ATGL	Adipose triglyceride lipase
BMI	Body mass index
CART	Cocaine and amphetamine-regulated transcript
CN	Chow normal litter
CPT-1	Carnitine palmitoyltransferase-1
CS	Chow small litter
FA	Fatty acid
HE	Hemotoxylin and eosin
HFD	High fat diet
HN	High fat diet normal litter
HOMA	Homeostatic model assessment
IPGTT	Intraperitoneal glucose tolerance test
NPY	Neuropeptide Y
PCR	Polymerase Chain Reaction
POMC	Pro-opiomelanocortin
rRP	Right retroperitoneal
SOCS-3	Suppressor of cytokine signaling-3
SPSS	Statistical Package for Social Sciences
STAT-3	Signal transducer and activator of transcription-3
UCP-3	Uncoupling protein3
UCPs	Uncoupling proteins
WAT	White adipose tissue pads

Introduction

Obesity has become a worldwide epidemic both in adults and in children. In many societies, maternal obesity is becoming the most common perturbation in pregnancy (Sebire *et al.*, 2001). This increases the likelihood of a cluster of disorders in offspring, including impaired glucose tolerance, insulin resistance, dyslipidemia, hypertension, and obesity (Boney *et al.*, 2005). Heavier mothers tend to have heavier babies and these babies go on to have a higher BMI in later life (Parsons *et al.*, 2001). Obesity is also exacerbated by a variety of postnatal factors including lack of physical activity, unhealthy food choices, eg. calorie dense food (Serour *et al.*, 2007; Mietus-Snyder & Lustig, 2008). As a result, a child's current diet along with the prenatal environment, are critical determinants of the biochemical alterations that may predispose to metabolic disorders later in life.

Food intake is regulated via a complex process comprising peripheral and central mechanisms. Animal studies indicate that maternal overnutrition exerts an important influence on the development of these appetite regulatory neural networks in offspring (Muhlhausler, 2007). The adipocyte-derived hormone leptin, a long term adiposity signal, acts centrally on Ob-Rb receptors predominantly in the hypothalamus (Tartaglia *et al.*, 1995). It leads to phosphorylation of signal transduction and activator of transcription (STAT-3) to inhibit the expression of orexigenic neuropeptides, neuropeptide Y (NPY) and agouti-gene-related protein (AgRP) while anorexigenic neuropeptides pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) are stimulated to inhibit food intake (Schwartz *et al.*, 2000), thus regulating body weight and fuel metabolism (Friedman & Halaas, 1998). However, high leptin levels are found in obese subjects, correlated with adipose tissue mass, which fail to bring about weight loss due to central leptin resistance (Scarpace & Zhang, 2009). This leptin resistance hypothesis was first suggested by Considine

et al. (1996) and in particular, SOCS-3 has been suggested as a potential mediator of central leptin resistance in obesity (Bjørbæk *et al.*, 1998). Leptin has also been reported to increase sympathetic nerve activity resulting in hypertension (Mark *et al.*, 1999).

Skeletal muscle is thought to be a principal site for glucose metabolism and fatty acid (FA) oxidation thus contributing to insulin resistance (Petersen *et al.*, 2007). Several factors may contribute to increased lipid deposition and insulin resistance. An increase in FA uptake may lead to cytosolic lipid accumulation that is associated with the development of insulin resistance (Hegarty *et al.*, 2002). FA oxidation occurs in mitochondria through the process of β-oxidation and carnitine palmitoyltransferase-1 (CPT-1), a mitochondrial transmembrane enzyme, transports FA into mitochondria for β-oxidation and thus is a rate-limiting step in this process (McGarry & Brown, 1997). An increase in CPT-1 activity is sufficient to improve insulin resistance caused by high fat diet (HFD) (Bruce *et al.*, 2009). Uncoupling proteins (UCPs), members of mitochondrial carrier family, uncouple oxidative metabolism from ATP synthesis, resulting in the production of heat instead of energy storage (Stuart *et al.*, 1999). Adipose triglyceride lipase (ATGL) was recently cloned and known to hydrolyze triglycerides (TG) in adipose tissue (Jenkins *et al.*, 2004). Loss of ATGL activity accumulates TG in several tissues (Haemmerle *et al.*, 2006).

The process by which prenatal and early postnatal environmental factors have long-term effects in adulthood is called programming and this is now considered a potential mechanism underlying the development of obesity, hyperphagia, cardiovascular and metabolic disorders later in life (Lucas, 1998; Armitage *et al.*, 2004; McMillen & Robinson, 2005). The pathogenesis of programming is based on altered gene expression as a consequence of an adaptation to environmental changes (Breier *et al.*, 2001). The plasticity of the hypothalamus

in late gestational age and early postnatal life (Grove & Smith, 2003) plays a key role in programming where adipose tissue secreted cytokines such as leptin are believed to act as key neurotrophic factors during brain development which may permanently influence appetite and metabolism (Bouret *et al.*, 2004; Muhlhausler, 2007; Taylor & Poston, 2007; Cripps *et al.*, 2009). The impact of maternal undernutrition on programming of obesity in offspring has been widely studied across species (Budge *et al.*, 2004; Ozanne *et al.*, 2004; Bispham *et al.*, 2005; Stocker *et al.*, 2005); whereas relatively fewer studies have investigated the effect of maternal overnutrition (Armitage *et al*., 2005; Bayol *et al*., 2007; Samuelsson *et al*., 2008; Shankar *et al*., 2008), which is a more prominent health problem worldwide.

Here we investigated the relationship between different degrees of maternal overnutrition and adiposity, hypothalamic appetite regulators, as well as glucose and lipid metabolism in the next generation. We generated two degrees of maternal overnutrition at F1, one group overfed during suckling and another rendered obese by postweaning HFD. To examine the impact of offspring's current diet, half of the offspring from each mother group were exposed to HFD. Measurements on F2 offspring included food intake, blood pressure, glucose tolerance and plasma leptin, insulin and triglyceride levels. As our focus was the alteration in hypothalamic appetite regulators as well as glucose and lipid homeostasis, we also examined mRNA expression of hypothalamic appetite regulators (NPY, POMC, STAT-3, SOCS-3), CPT-1 and UCP-3 in muscle and ATGL in adipose tissue.

Materials and methods

Animals and diet

All animal experiments were approved by the Animal Experimentation Ethics Committee of the University of New South Wales. F0 female Sprague-Dawley rats were housed at 20 ± 2 °C and maintained on a 12 h light and 12 h dark cycle. They were fed normal chow (14% fat by energy) or cafeteria HFD (34% fat by energy) for 6 weeks to produce two groups: Chow and HFD. At 13 weeks they were mated with chow fed male Sprague-Dawley rats. On day 1 of life some F1 female pups from chow fed F0 mothers were adjusted in normal litters (12 pups per mother) and fed chow yielding chow normal litter (CN) while others were maintained in small litters (3 pups per mother) and fed chow to produce chow small litter (CS). Female pups from HFD fed F0 mothers were maintained in normal litters (12 pups per mother) and after weaning they were offered HFD to produce HFD normal litter (HN). Thus at F1 two degrees of overnutrition had been achieved, by either early overfeeding during suckling (CS) or by offering HFD after weaning (HN). At 14 weeks of age these three groups CN, CS, HN were mated with chow fed males and were killed at approximately 22 weeks. After delivery the F2 litters were adjusted to 11 pups per mother and at weaning male rats were divided into two diets, HFD and chow which produced 6 groups of rats with different nutrition: CN-chow (CN_C), CN-HFD (CN_H), CS-chow (CS_C), CS-HFD (CS_H), HN-chow (HN_C), HN-HFD (HN_H) (Across all the treatments n = 12-17; Table 1). At the end point animals were killed at 21 weeks.

Across all generations the chow diet groups were fed standard rodent chow (11 kJ/g, 14% fat, 21% protein, 65% carbohydrates by energy) *ad libitum* and the HFD groups were presented daily with fresh and highly palatable, cafeteria-style high-fat foods along with normal chow (15.33 kJ/g, 34% fat, 19% protein, 47% carbohydrates by energy), consisting of meat pie,

dim sim, biscuits, cakes, pasta, chips and chow supplemented with condensed milk, milk powder and lard *ad libitum*.

Body weight and energy intake measurement

Body weights of F2 offspring were monitored every three days till weaning (20 days). F1 mothers and postweaning F2 offspring were weighed weekly. Average 24 hour food intake (g) was calculated weekly from 5 to 15 weeks by carefully collecting and weighing the food remaining in the cage, subtracting this from the known amount given. The total energy (kJ) consumed by each group was calculated from the energy information supplied on the packet.

Blood pressure measurement

Blood pressure was measured at 12 weeks of age in conscious animals using tail-cuff pulse plethysmography (CODA-6, Kent Scientific Corporation, USA). Rats were pre-warmed by placing at 37 °C for 5 to 10 minutes to facilitate tail blood flow before blood pressure was measured. After 5 acclimation readings the average of 3-7 readings was used for each rat.

Glucose tolerance test

At 16 weeks an intraperitoneal glucose tolerance test (IPGTT) was performed on conscious animals after 6 hours fasting. A blood sample of 15µL was collected from the tail and baseline glucose was determined using a handheld glucometer (Accu-Chek Advantage Glucometer; Roche Diagnostics, NSW, AUS). The rats were then administered 2 g glucose/kg body weight (30% w/v) by intraperitoneal injection. Further blood samples were taken at 15, 30, 45, 60, 90 and 120 minutes for glucose determination.

Sample collection

Blood samples (0.30 mL) were obtained from the tail vein of warmed conscious rats using a heparinised syringe (Pfizer, ON, USA; 1000 IU/mL saline) at 14 weeks of age to analyze the fasting glucose and insulin level. The insulin resistance index was estimated by homeostasis model assessment (HOMA): plasma insulin (ng/mL) × fasting plasma glucose (mmol/L)/(22.5 × 0.0417): the greater the HOMA value, the greater the level of insulin resistance {Matthews, 1985 #271}{Schaalan, #272}

At day 20, when the pups were weaned into two diets, their mothers (F1) were deeply anesthetized with xylazine/ketamine (20/80 mg/kg). Body length (naso-anal) and girth (measured at base of ribcage) were taken before blood was removed by cardiac puncture. Then animals were decapitated to allow the rapid dissection and weighing of fat depots. At the endpoint (21 weeks), non-fasted adult F2 rats were killed by decapitation and the brain was placed ventral side up. Coronal cuts were made at the optic chiasm and the rostral border of the hypothalamus. The hypothalamus (minus preoptic area) was separated by making incisions at the lateral edges of the hypothalamic sulcus and above the third ventricle. The ventral hypothalamus containing the arcuate nucleus (ARC) was snap frozen. White adipose tissue (WAT: rRP, right retroperitoneal, gonadal and mesenteric), left kidney, heart, muscle and liver were collected and weighed. Hypothalamus, soleus muscle and rRP WAT were snap frozen in liquid nitrogen and then stored at -80 °C for later measurement of mRNA expression. Tibia length was recorded. Body mass index (BMI) was calculated as kg/m^2. A section of liver was fixed in 4% formaldehyde for hemotoxylin and eosin (HE) staining.

Plasma biochemistry

Glucose was measured (Accu-Check Advantage Glucometer; Roche Diagnostics, NSW, AUS) before the blood was centrifuged at 10,000 rpm for 8 minutes (Eppendorf Minispin;

Crown Scientific, NSW, AUS). Separated plasma was stored at -20 °C. Plasma leptin and insulin concentrations were analyzed using commercially available radioimmunoassay kits according to manufacturer's instructions (Linco, MO, USA). Plasma TG was analyzed colorimetrically (490 nm; Bio-Rad 680XR, NSW, AUS) using a commercially available triglyceride reagent (Roche, NSW, AUS) and standard (Sigma, VIC, AUS).

HE staining of liver

A section of the middle liver lobe from each rat was placed in 4% formaldehyde overnight, then transferred to 70% ethanol till embedded in paraffin wax. Mounted tissues were then deparaffinized and rehydrated. Nuclei were stained with hematoxylin and cell cytoplasm was stained with eosin. Fatty change in liver was graded by an observer blinded to the treatment groups by assessing the amount and size of white vacuoles present throughout the stained section (0 = No fat vacuoles, 1 = few small lipid vacuoles, 2 = increased number of small fat vacuoles and some big vacuoles, 3 = hepatocytes filled by big fat vacuoles). The average of the grades within each treatment group was then calculated.

Real – time PCR:

Quantitative real time polymerase chain reaction (PCR) was used to determine mRNA expression of NPY, POMC, SOCS-3, STAT-3, and leptin receptor (Ob-Rb) in ventral hypothalamus, CPT-1 and UCP-3 in soleus and ATGL in rRP WAT. Total RNA was extracted by homogenizing tissue with Tri reagent (SIGMA, Missouri, USA), separated by chloroform, precipitated with isopropyl alcohol and then washed with 75% ethanol. The purified total RNA was used as a template to generate first-strand cDNA using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant Kit, (Promega Corporation, WI, USA). Pre-optimized probe/primers (Applied biosystem) were used for quantitative PCR (Realplex

software). Ob-Rb, STAT-3, SOCS-3, NPY and POMC mRNA levels in hypothalamus and UCP3 in muscle were compared to housekeeping gene ribosomal 18s RNA. ATGL mRNA levels in rRP were compared to housekeeping gene beta actin. The housekeeping gene was labeled by VIC and target gene by FAM where our gene of interest was standardized to control. An individual sample from the control CN-chow group was then arbitrarily assigned as a calibrator against which all other samples are expressed as fold difference.

Statistics

Results are expressed as mean ± SEM. Maternal characteristics and pre-weaning data were analyzed by one way ANOVA followed by least significant difference using SPSS (version 15). Post weaning weekly body weights and blood glucose concentrations in IPGTT were analyzed by one-way ANOVA with repeated measures, followed by LSD. Food intake, blood pressure and endpoint organ weights, body weight, fat masses, plasma markers, mRNA levels were analyzed by two-way ANOVA followed by least significant difference test using SPSS. If two-way ANOVA showed significant overall maternal effects, then it was followed by one way ANOVA to look at maternal effects separately in chow and HFD groups. Correlation between the body weight and fat masses was assessed by Pearson's correlation.

Results

Maternal characteristics (F1)

Consumption of HFD led to marked increases in body weight in F1 females with modest effects of litter size adjustment (Fig 1). At 22 weeks HN rats were 17% heavier than CN rats, with a marked increase in sampled WATS ($P < 0.001$) and a doubling of plasma leptin and triglyceride concentrations ($P < 0.01$ and 0.05 respectively; Table 2). Although CS F1 females were 6% heavier than CN rats, one way ANOVA showed no overall effect of litter

size (CS vs CN) on body weight, fat masses, plasma leptin and TG. Plasma insulin remained unchanged by HFD feeding and litter size adjustment (HN, CS vs CN; $P > 0.05$; Table 2).

Effects of maternal (F1) overnutrition and postweaning HFD (F2) on F2 offspring

Preweaning

During early development F2 offspring maintained the body weight pattern of their mothers (Fig 2A). By 4 days the offspring of HN dams (11.04 ± 0.18) were significantly heavier than those of CN dams (10.34 ± 0.17g; $P < 0.01$). After 7 days both HN (17.48 ± 0.28) and CS (15.45 ± 0.28g) offspring were significantly heavier than CN offspring (14.61 ± 0.26g; $P < 0.05$). At 19 days of age, maternal HFD had caused a 36% increase (HN vs CN offspring, 46.74 ± 0.51 vs 34.23 ± 0.47g; $P < 0.001$) and maternal litter size a 7% increase (CS offspring 36.89 ± 0.70g; $P < 0.01$) in body weight over CN offspring (Fig 2A).

Postweaning

Body weight. Overnutrition of F1 mothers due to both HFD related maternal obesity and reduced litter size led to significantly greater body weights in postweaning F2 offspring. Maternal HFD effects were significant from 5 weeks ($P < 0.001$; Fig 2B) and maternal litter size effects from 9 weeks ($P < 0.05$; Fig 2B). This pattern was observed in both diet groups, with the greatest impact in offspring of HN mothers consuming HFD. At 20 weeks the maternal litter size effect on body weight was abolished (Fig 2B). At the end of the study (21 weeks), body weight of F2 offspring of HN and CS dams were 16% and 11% greater than that of CN dams consuming chow (HN_C vs CN_C, CS_C vs CN_C; $P < 0.001$, < 0.01 respectively; Table 3). In HFD animals, the impact of maternal liter size was not evident; while offspring from HN dams were 19% heavier than CN offspring (HN_H vs CN_H; $P < 0.01$; Table 3). No significant difference was observed in body length (data not shown) while BMI was

significantly increased by both degrees of maternal overnutrition across both diet groups (HNc, CS_C vs CN_C; HN_H, CN_H vs CN_H; $P < 0.001$; Table 3). As expected, postweaning HFD led to significant increases in body weight from 8 weeks across all maternal groups ($P < 0.001$; Fig 2B) and continued till the end point. At that time consumption of postweaning HFD also led to increases in BMI across all mother groups (HFD vs chow offspring; $P < 0.001$; Table 3).

Glucose homeostasis. During the 16 week IPGTT, baseline glucose levels were not affected by maternal groups. However glucose concentrations were increased by both degrees of maternal overnutrition at 30 minutes in HFD fed groups (HN_H, CS_H vs CN_H; $P < 0.05$, 0.01 respectively; Fig 3A). Only maternal HFD effect continued at 45, 60 and 90 minutes (HN_H vs CN_H; $P < 0.01$). Current HFD effect was observed in offspring of CN dams at 15 and 30 minutes (CN_H vs CN_C), in CS offspring from 0 to 60 minutes (CS_H vs CS_C) and in HN offspring from 30 to 120 minutes (HN_H vs HN_C; Fig 3A).

The area under the glucose tolerance test is shown in Fig 3B. No significant maternal effect was observed in animals consuming chow, however when rats consumed HFD, AUC was increased in offspring by both degrees of maternal overnutrition (HN_H, CS_H vs CN_H; $P < 0.05$). AUC were also higher in rats currently consuming HFD across all mother groups ($P < 0.001$).

At 14 weeks insulin concentration (Fig 3C) and HOMA (Fig 3D) were significantly higher in the offspring of HN dams relative to those of CN dams in both diet groups (HN_H vs CN_H; $P < 0.001$ for all). No significant maternal litter size effect was observed on fasting plasma insulin and HOMA (CS vs CN offspring, both diet groups). Consumption of HFD postweaning

caused significant increases in insulin concentration and HOMA in F2 offspring (HFD vs chow fed offspring; $P < 0.001$, Fig 3B, 3C, 3D).

Adiposity. In line with the increased body weight, in both diet groups a significant maternal HFD effect was observed on offspring rRP, gonadal and mesenteric WAT ($P < 0.001$, Table 3). A similar pattern is apparent in total sampled fat mass expressed as net weight or percent body weight (HNc vs $CN_{C,}$ HN_H vs CN_H; $P < 0.001$, Fig 4A, 4B). A significant maternal litter size effect on WAT was only observed in the chow fed offspring (CS_C vs CN_C $P < 0.01$; Fig 4A, 4B; Table 3). When both diet groups were combined, there was a significant positive correlation between body weight and % total fat in offspring across all mother groups (CN offspring r = 0.833, $P < 0.001$; CS offspring r = 0.882, $P < 0.001$; HN offspring r = 0.870, $P < 0.001$). Postweaning HFD consumption in F2 offspring led to significant increases in fat masses both when expressed as net or percent body weight (HFD vs chow fed offspring; $P < 0.001$; Fig 4A, 4B; Table 3).

Leptin was increased in offspring of HN dams compared to CN offspring in both diet groups (HNc vs $CN_{C,}$ HN_H vs CN_H; Fig 4C; $P < 0.001$). No significant effect of maternal litter size was observed. As expected offspring postweaning HFD led to marked increases in leptin ($P < 0.001$; Fig 4C). Leptin concentration and percent total fat showed a highly positive correlation (r = 0.853, $P < 0.001$) when offspring from the three mother groups and both diets were combined.

Lipid regulators. Significant effects of maternal HFD (HNc vs $CN_{C,}$ HN_H vs CN_H; $P < 0.001$) and offspring postweaning HFD (HFD vs chow fed offspring across all mother groups; $P <$

0.01) were observed on plasma TG (Fig 4D). No effect of maternal litter size was observed on plasma TG (CS vs CN offspring; Fig 4D).

In rRP WAT, mRNA expression of lipase ATGL were significantly decreased by postweaning HFD in offspring across all mother groups (HFD vs chow fed offspring; $P < 0.05$, Fig 5A). In skeletal muscle there was also overall postweaning HFD effect in UCP-3 mRNA expression (HFD vs chow fed offspring; $P < 0.05$, Fig 5B). UCP-3 expression was increased in offspring of HN dams fed HFD (HN_H vs CN_H; $P < 0.05$; Fig 5B). We did not see any significant result in CPT-1 expression (data not shown).

Systolic blood pressure. Within each diet group, no significant maternal effect was observed on systolic blood pressure, diastolic blood pressure and heart rate (Table 3). Obesity induced by consumption of HFD in F2 offspring was associated with significant increases in systolic and diastolic pressures (HFD vs chow fed offspring; $P < 0.01$; Table 3).

Food intake and central appetite regulators. The total kJ consumed by the offspring of HN dams from 5 to 15 weeks was 11% higher than that of offspring of CN dams in both chow and HFD groups (HNc vs CN_C, HN_H vs CN_H; $P < 0.05$, 0.01 respectively; Table 3). A similar pattern was observed when gram of food consumed was examined (data not shown). However, when total kJ was adjusted by body weight, no significant effect on food intake was observed in HN offspring. CS offspring ate similar amounts to those of CN mothers. Across all maternal groups, consumption of HFD led to a doubling of kJ intake compared to chow fed offspring ($P < 0.001$; Table 3).

Hypothalamic Ob-Rb mRNA expression was significantly increased in the offspring of HN dams compared to CN offspring consuming HFD (HN_H vs CN_H; $P < 0.01$; Fig 6A). Within the chow diet group CS offspring showed a significant decrease in Ob-Rb mRNA expression compared to CN offspring (CSc vs CNc; $P < 0.05$; Fig 6A). STAT-3 mRNA expression showed a similar pattern to that of Ob-Rb (Fig 6B). The analysis also showed significant increase in SOCS-3 mRNA expression in offspring of HN dams consuming HFD, compared to CN offspring (HN_H vs CN_H; $P < 0.05$) $P < 0.05$; Fig 6C). Postweaning HFD also caused an overall increase in SOCS-3 expression (HFD vs chow offspring, $P < 0.01$; Fig 6C). No significant differences were observed in hypothalamic NPY and POMC mRNA expression (6D, 6E).

Discussion

A number of animal studies indicate that obesity may have a "programmed origin" (Lucas, 1998; Armitage *et al.*, 2005; Muhlhausler, 2007; Taylor & Poston, 2007). The current study shows that any degree of maternal obesity, either maternal obesity due to HFD feeding prior to and during pregnancy or early postnatal overnutrition of the mother due to small litter size, increased adiposity and glucose intolerance in F2 offspring. Overall, maternal obesity exerted greater effects on offspring adiposity, and glucose and lipid homeostasis than maternal early postnatal overfeeding. The current diet also had an important impact. While effects of maternal early overnutrition were only evident in offspring consuming chow, those of maternal obesity appeared to be amplified when offspring consumed HFD.

Maternal HFD consumption during pregnancy and lactation is an important contributing factor to the development of obesity in adulthood (Bayol *et al.*, 2007). An enriched milk

content of obese mothers (Gorski *et al.*, 2006) as well as increased quantity of milk consumed by pups of obese mothers (Chen *et al.*, 2008), may have contributed to their obesity. As a result, in our study offspring from HN dams showed significant increases in body weight gain during development and at the endpoint they were heavier with elevated BMI in both diet groups. In this study overnutrition imposed by reduced competition for milk during suckling did not result in marked changes in adiposity or body weight in mothers. Insulin was increased albeit non significantly. Previous work from this and other laboratories showed litter size reduction resulted in time-dependent increases in body weight, fat mass and fat cell number (Bassett & Craig, 1988; Velkoska *et al.*, 2005). This may be related to the duration of the intervention, as previously, significant effects of litter size adjustment were observed early in life (up to 10 weeks) and abated over time (Velkoska *et al.*, 2005). Further, our number of animals (6-7) may have influenced our capacity to observe significant effects. To our knowledge, this is the first study documenting an impact of maternal overnutrition induced by litter size adjustment on the next generation. A late onset impact on body weight was observed in offspring from CS dams, which were heavier than those from CN from 9 weeks of age. At 21 weeks, chow fed offspring from CS dams showed significant increases in body weight compared to offspring of CN mothers, but this effect was absent in rats consuming HFD. This suggests that the effect of postweaning HFD is so strong that it can override the impact of maternal early-life overnutrition on offspring.

In our study maternal obesity induced by HFD caused hyperleptinemia in offspring, which would be predicted for their increased fat content, in line with other studies (Parente *et al.*, 2008; Samuelsson *et al.*, 2008). Earlier reports of overnutrition due to litter size adjustment showed a time dependent elevation in plasma leptin at 8 weeks, but by 16 weeks it disappeared (Morris *et al.*, 2005). In this study there was no significant litter size effect on

plasma leptin concentration in the F1 mother, so it is perhaps not surprising that leptin was normal in her offspring. The time points chosen may limit our ability to see any significant changes in plasma leptin concentration. However CS offspring showed increased fat mass only in the chow group, suggesting maternal overfeeding due to small litter size may still play a small but important role in the offspring.

In agreement with other studies examining adult offspring maternal HFD consumption led to hyperinsulinemia with significantly higher HOMA in offspring, suggesting maternal obesity is strongly linked to insulin resistance in offspring (Samuelsson *et al.*, 2008; Nivoit *et al.*, 2009). Postweaning HFD also resulted in a greater HOMA value, indicating insulin resistance. Maternal litter size did not have any effect on HOMA, however it impaired glucose handling during IPGTT. The offspring of small litter dams showed delayed glucose clearance at 15 and 30 min, with more prolonged effects in HFD fed rats from HN dams. Higher glucose levels resulting from lower glucose utilization in peripheral tissues is a typical feature in obesity. Increased area under the IPGTT curve was seen in offspring from both degrees of maternal overnutrition, but this was only unmasked when offspring consumed HFD, suggesting an interaction between maternal phenotype and current diet increased the risk of glucose intolerance.

Maternal obesity due to a fat rich diet during pregnancy and lactation (Chang *et al.*, 2008) as well as a lifetime of HFD consumption (Howie *et al.*, 2009) might reflect a "malprogramming" of hypothalamic neuropeptidergic systems possibly related to defective leptin signaling in the hypothalamus, leading to permanently altered appetite regulatory functions in offspring. Maternal junk food during gestation and lactation may lead pups to develop an exacerbated preference for fatty, sugary and salty foods (Bayol *et al.*, 2007). In

this study central Ob-Rb was upregulated by maternal obesity in offspring consuming HFD. Changes in its downstream mediator STAT-3 mRNA expression mirrored those in Ob-Rb receptor. But the simultaneous upregulation of the suppressor of cytokine signaling SOCS-3 suggests central leptin resistance (Bjørbæk *et al.*, 1998). Although cafeteria diet and leptin are known to modulate the hypothalamic appetite regulators NPY and POMC (Korner *et al.*, 1999; Hansen *et al.*, 2004), central leptin resistance due to upregulated SOCS-3, may help to explain why in our study no significant changes in hypothalamic NPY and POMC expression were observed in response to elevated leptin induced by maternal HFD. Hyperphagia would be consistent with SOCS-3 overexpression by maternal HFD, and offspring of HN dams tended to eat more than the other groups, especially when consuming HFD. This suggests that the altered hypothalamic neuronal changes due to maternal obesity led to transmission of increased susceptibility to hyperphagia and obesity in offspring. Upregulation of SOCS-3 was also found with prolonged postweaning HFD, may contribute to central leptin resistance and thus alter normal control of food intake. Although maternal litter size caused downstream regulation of Ob-Rb and STAT-3 in chow fed offspring, they appeared to consume slightly more kJ, which was entirely accounted for their heavier body weight. Thus, different degrees of maternal obesity at F1 can alter offspring appetite control differently.

Postweaning HFD caused increased fat mass with elevated leptin and downregulation of UCP-3 in muscle and ATGL in rRP WAT. It has been suggested that UCP-3 acts as a FA anion exporter, protecting mitochondria against peroxidation and allowing continued rapid FA oxidation (Echtay, 2007). However the role of UCP-3 in energy expenditure is controversial. Some studies showed significantly elevated UCP-3 in HFD animals (Chou *et al.*, 2001; Turner *et al.*, 2007) while there was also evidence of downregulation of UCP-3 by high fat feeding (Corbalan *et al.*, 1999). The fasting state can also cause the upregulation of

UCP-3 to oxidize FA as energy supply (Boss *et al.*, 1998; Weigle *et al.*, 1998). In our study the animals were not fasted and downregulation of UCP-3 expression across all mother groups may be linked to reduced capacity of FA oxidation leading to the accumulation of TG. On the other hand, fat ATGL mRNA expression, which has strong negative correlations with obesity and insulin resistance (Jocken *et al.*, 2007), was also downregulated in insulin resistant HFD-fed offspring across all maternal groups in our study, suggesting a slower rate of lipolysis in HFD rats. Concomitant increases in plasma TG and body fat mass may be due to this reduced lipolysis, behind which ATGL and UCP-3 plays a vital role.

Other studies in mice showed increased blood pressure in offspring from obese mothers (Samuelsson *et al.*, 2008); however this was not observed in this study possibly due to differences in methodology. Postweaning HFD caused an elevation in systolic and diastolic blood pressure. Hyperleptinemia and hyperinsulinemia have both been linked to sympathetic activation associated with obesity related hypertension (Rahmouni *et al.*, 2005; Ferrannini, 2006). This may explain our finding of an overall HFD effect on blood pressure with increased leptin and insulin across all mother groups. The HFD rats were also hyperphagic with leptin resistance related to SOCS-3 overexpression. Selective leptin resistance may contribute to the inability of leptin to activate downstream signaling pathways in the arcuate nucleus but preservation of leptin action in other cardiovascular related hypothalamic areas (Rahmouni *et al.*, 2005). Further investigation is needed to examine the cardiovascular consequences of maternal overnutrition and postweaning HFD.

Conclusion

Our data indicate that any level of maternal overnutrition increases offspring adiposity at adulthood, with a greater impact of HFD induced maternal obesity than early overfeeding due to litter size reduction. Thus offspring from HFD-fed mothers were at the highest risk of a cluster of disorders including impaired glucose tolerance, insulin resistance, dyslipidemia, and obesity. Additive effects were observed with offspring's own HFD. Modest maternal overweight due to early life overnutrition influenced offspring body weight more in those consuming a low fat diet post weaning. Further study is required to investigate the reason for the elevation of blood pressure induced by HFD across all mother groups.

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Author contributions

S Rajia; animal work, molecular biology and hormone analysis, statistical analysis, interpretation of data, drafting the article. **H Chen;** project design, molecular biology and hormone analysis, interpretation of data. **MJ Morris;** project design and oversight, data analysis and interpretation; critical revision of the manuscript. All authors provided intellectual input and contributed to the writing of the manuscript. All of the experiments described in this manuscript were performed at University of New South Wales, Sydney, Australia.

References

Armitage JA, Khan IY, Taylor PD, Nathanielsz PW & Poston L (2004). Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? *J Physiol* **561,** 355-377.

Armitage JA, Lakasing L, Taylor PD, Balachandran AA, Jensen RI, Dekou V, Ashton N, Nyengaard JR & Poston L (2005). Developmental programming of aortic and renal structure in offspring of rats fed fat-rich diets in pregnancy. *J Physiol* **565,** 171-184.

Bassett DR & Craig BW (1988). Influence of early nutrition on growth and adipose tissue characteristics in male and female rats. *J Appl Physiol* **64,** 1249-1256.

Bayol SA, Farrington SJ & Stickland NC (2007). A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. *Br J Nutr* **98,** 843-851.

Bispham J, Gardner DS, Gnanalingham MG, Stephenson T, Symonds ME & Budge H (2005). Maternal nutritional programming of fetal adipose tissue development: differential effects on messenger ribonucleic acid abundance for uncoupling proteins and peroxisome proliferator-activated and prolactin receptors. *Endocrinology* **146,** 3943-3949.

Bjørbæk C, Elmquist JK, Frantz JD, Shoelson SE & Flier JS (1998). Identification of SOCS-3 as a potential mediator of central leptin resistance. *Mol Cell* **1,** 619-625.

Boney CM, Verma A, Tucker R & Vohr BR (2005). Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. *Pediatrics* **115,** e290-296.

Boss O, Samec S, Kuhne F, Bijlenga P, Assimacopoulos-Jeannet F, Seydoux J, Giacobino J-P & Muzzin P (1998). Uncoupling protein-3 expression in rodent skeletal muscle is modulated by food intake but not by changes in environmental temperature. *J Biol Chem* **273,** 5-8.

Bouret SG, Draper SJ & Simerly RB (2004). Trophic action of leptin on hypothalamic neurons that regulate feeding. *Science* **304,** 108-110.

Breier BH, Vickers MH, Ikenasio BA, Chan KY & Wong WP (2001). Fetal programming of appetite and obesity. *Mol Cell Endocrinol* **185,** 73-79.

Bruce CR, Hoy AJ, Turner N, Watt MJ, Allen TL, Carpenter K, Cooney GJ, Febbraio MA & Kraegen EW (2009). Overexpression of carnitine palmitoyltransferase-1 in skeletal muscle is sufficient to enhance fatty acid oxidation and improve high-fat diet-induced insulin resistance. *Diabetes* **58,** 550-558.

Budge H, Edwards LJ, McMillen IC, Bryce A, Warnes K, Pearce S, Stephenson T & Symonds ME (2004). Nutritional manipulation of fetal adipose tissue deposition and

uncoupling protein 1 messenger RNA abundance in the sheep: differential effects of timing and duration. *Biol Reprod* **71,** 359-365.

Chang GQ, Gaysinskaya V, Karatayev O & Leibowitz SF (2008). Maternal high-fat diet and fetal programming: increased proliferation of hypothalamic peptide-producing neurons that increase risk for overeating and obesity. *J Neurosci* **28,** 12107-12119.

Chen H, Simar D, Lambert K, Mercier J & Morris MJ (2008). Maternal and postnatal overnutrition differentially impact appetite regulators and fuel metabolism. *Endocrinology* **149,** 5348-5356.

Chou CJ, Cha MC, Jung DW, Boozer CN, Hashim SA & Pi-Sunyer FX (2001). High-fat diet feeding elevates skeletal muscle uncoupling protein 3 levels but not its activity in rats. *Obesity* **9,** 313-319.

Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL & Caro JF (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *New Engl J Med* **334,** 292-295.

Corbalan MS, Margareto J, Martinez JA & Marti A (1999). High-fat feeding reduced muscle uncoupling protein 3 expression in rats. *J Physiol Biochem* **55,** 67-72.

Cripps RL, Martin-Gronert MS, Archer ZA, Hales CN, Mercer JG & Ozanne SE (2009). Programming of hypothalamic energy balance gene expression in rats by maternal diet during pregnancy and lactation. *Clin Sci (Lond)*; doi:10.1042/CS20080393

Echtay KS (2007). Mitochondrial uncoupling proteins-What is their physiological role? *Free Radic Biol Med* **43,** 1351-1371.

Ferrannini E (2006). Is insulin resistance the cause of the metabolic syndrome? *Ann Med* **38,** 42-51.

Friedman JM & Halaas JL (1998). Leptin and the regulation of body weight in mammals. *Nature* **395,** 763-770.

Gorski JN, Dunn-Meynell AA, Hartman TG & Levin BE (2006). Postnatal environment overrides genetic and prenatal factors influencing offspring obesity and insulin resistance. *Am J Physiol Regul Integr Comp Physiol* **291,** R768-R778.

Grove KL & Smith MS (2003). Ontogeny of the hypothalamic neuropeptide Y system. *Physiol Behav* **79,** 47-63.

Haemmerle G, Lass A, Zimmermann R, Gorkiewicz G, Meyer C, Rozman J, Heldmaier G, Maier R, Theussl C, Eder S, Kratky D, Wagner EF, Klingenspor M, Hoefler G & Zechner R (2006). Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. *Science* **312,** 734-737.

Hansen MJ, Jovanovska V & Morris MJ (2004). Adaptive responses in hypothalamic neuropeptide Y in the face of prolonged high-fat feeding in the rat. *J Neurochem* **88,** 909-916.

Hegarty BD, Cooney GJ, Kraegen EW & Furler SM (2002). Increased efficiency of fatty acid uptake contributes to lipid accumulation in skeletal muscle of high fat-fed insulin-resistant rats. *Diabetes* **51,** 1477-1484.

Howie GJ, Sloboda DM, Kamal T & Vickers MH (2009). Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet. *J Physiol* **587**, 905-915.

Jenkins CM, Mancuso DJ, Yan W, Sims HF, Gibson B & Gross RW (2004). Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. *J Biol Chem* **279,** 48968-48975.

Jocken JWE, Langin D, Smit E, Saris WHM, Valle C, Hul GB, Holm C, Arner P & Blaak EE (2007). Adipose triglyceride lipase and hormone-sensitive lipase protein expression is decreased in the obese insulin-resistant state. *J Clin Endocrinol Metab* **92,** 2292-2299.

Korner J, Chua SC, Jr., Williams JA, Leibel RL & Wardlaw SL (1999). Regulation of hypothalamic proopiomelanocortin by leptin in lean and obese rats. *Neuroendocrinology* **70,** 377-383.

Lucas A (1998). Programming by early nutrition: an experimental approach. *J Nutr* **128,** 401S-406S.

Mark AL, Correia M, Morgan DA, Shaffer RA & Haynes WG (1999). Obesity-induced hypertension: new concepts from the emerging biology of obesity. *Hypertension* **33,** 537-541.

McGarry JD & Brown NF (1997). The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur J Biochem* **244,** 1-14.

McMillen IC & Robinson JS (2005). Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol Rev* **85,** 571-633.

Mietus-Snyder ML & Lustig RH (2008). Childhood obesity: adrift in the "limbic triangle". *Annu Rev Med* **59,** 147-162.

Morris MJ, Velkoska E & Cole TJ (2005). Central and peripheral contributions to obesity-associated hypertension: impact of early overnourishment. *Exp Physiol* **90,** 697-702.

Muhlhausler BS (2007). Programming of the appetite-regulating neural network: a link between maternal overnutrition and the programming of obesity? *J Neuroendocrinol* **19,** 67-72.

Nivoit P, Morens C, Van Assche FA, Jansen E, Poston L, Remacle C & Reusens B (2009). Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. *Diabetologia* **52,** 1133-1142.

Ozanne SE, Lewis R, Jennings BJ & Hales CN (2004). Early programming of weight gain in mice prevents the induction of obesity by a highly palatable diet. *Clin Sci (Lond)* **106,** 141-145.

Parente LB, Aguila MB & Mandarim-de-Lacerda CA (2008). Deleterious effects of high-fat diet on perinatal and postweaning periods in adult rat offspring. *Clin Nutr* **27,** 623-634.

Parsons TJ, Power C & Manor O (2001). Fetal and early life growth and body mass index from birth to early adulthood in 1958 British cohort: longitudinal study. *Br Med J* **323,** 1331-1335.

Petersen KF, Dufour S, Savage DB, Bilz S, Solomon G, Yonemitsu S, Cline GW, Befroy D, Zemany L, Kahn BB, Papademetris X, Rothman DL & Shulman GI (2007). The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. *Proc Natl Acad Sci U S A* **104,** 12587-12594.

Rahmouni K, Correia ML, Haynes WG & Mark AL (2005). Obesity-associated hypertension: new insights into mechanisms. *Hypertension* **45,** 9-14.

Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EH, Piersma AH, Ozanne SE, Twinn DF, Remacle C, Rowlerson A, Poston L & Taylor PD (2008). Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. *Hypertension* **51**, 383-392.

Scarpace PJ & Zhang Y (2009). Leptin resistance: a prediposing factor for diet-induced obesity. *Am J Physiol Regul Integr Comp Physiol* **296,** R493-R500.

Schwartz MW, Woods SC, Porte D, Seeley RJ & Baskin DG (2000). Central nervous system control of food intake. *Nature* **404,** 661-671.

Sebire NJ, Jolly M, Harris JP, Wadsworth J, Joffe M, Beard RW, Regan L & Robinson S (2001). Maternal obesity and pregnancy outcome: a study of 287,213 pregnancies in London. *Int J Obes Relat Metab Disord* **25,** 1175-1182.

Serour M, Alqhenaei H, Al-Saqabi S, Mustafa AR & Ben-Nakhi A (2007). Cultural factors and patients' adherence to lifestyle measures. *Br J Gen Pract* **57,** 291-295.

Shankar K, Harrell A, Liu X, Gilchrist JM, Ronis MJJ & Badger TM (2008). Maternal obesity at conception programs obesity in the offspring. *Am J Physiol Regul Integr Comp Physiol* **294,** R528-R538.

Stocker CJ, Arch JR & Cawthorne MA (2005). Fetal origins of insulin resistance and obesity. *Proc Nutr Soc* **64,** 143-151.

Stuart JA, Brindle KM, Harper JA & Brand MD (1999). Mitochondrial proton leak and the uncoupling proteins. *J Bioenerg Biomembr* **31,** 517-525.

Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA & Tepper RI (1995). Identification and expression cloning of a leptin receptor, OB-R. *Cell* **83,** 1263-1271.

Taylor PD & Poston L (2007). Developmental programming of obesity in mammals. *Exp Physiol* **92,** 287-298.

Turner N, Bruce CR, Beale SM, Hoehn KL, So T, Rolph MS & Cooney GJ (2007). Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents. *Diabetes* **56,** 2085-2092.

Velkoska E, Cole TJ & Morris MJ (2005). Early dietary intervention: long-term effects on blood pressure, brain neuropeptide Y, and adiposity markers. *Am J Physiol Endocrinol Metab* **288,** E1236-E1243.

Weigle DS, Selfridge LE, Schwartz MW, Seeley RJ, Cummings DE, Havel PJ, Kuijper JL & BeltrandelRio H (1998). Elevated free fatty acids induce uncoupling protein 3 expression in muscle: a potential explanation for the effect of fasting. *Diabetes* **47,** 298-302.

Figure legends

Fig 1 Mother's (F1) body weight in rats raised in normal sized litters (CN) or small sized litters (CS) and consuming chow or raised in normal sized litters and consuming HFD (HN). Results are expressed as mean ± SEM (n = 6-7).

††, ††† HFD effect $P < 0.01, 0.001$ (HN vs CN).

Fig 2 F2 offspring body weight from day 1 to day 19 **(A)** and postweaning from 5 to 20 weeks **(B)** from CN (circle), CS (triangle) and HN (square) mothers. Results are expressed as mean ± SEM; n = 34-44 (A) or n = 10-11 (B). In panel B, solid symbols represent mothers ID and the subscript refer to the offspring's current diet (C, chow; H, HFD).

††, ††† Maternal (F1) HFD effect $P < 0.01, 0.001$ (HN vs CN offspring)

#, ## Maternal litter size (F1) effect $P < 0.05, 0.01$ (CS vs CN offspring)

*** Current (F2) HFD effect $P < 0.001$ (HFD vs chow fed offspring across all mother groups).

Fig 3 (A) Blood glucose concentrations during GTT in offspring from CN mother fed chow (○), CN mother fed HFD (●), CS mother fed chow (Δ), CS mother fed HFD (▲), HN mother fed Chow (□), HN mother fed HFD (■). **(B)** AUC of GTT **(C)** Fasting insulin concentrations **(D)** HOMA of CN-offspring fed chow (CN_C) and HFD (CN_H), CS-offspring fed chow (CS_C) and HFD (CS_H) and HN-offspring fed chow (HN_C) and HFD (HN_H). Results are expressed as mean ± SEM (n = 8-12).

†, ††† Maternal (F1) HFD effect $P < 0.05, 0.001$ (HN_C vs CN_C / HN_H vs CN_H)

Maternal (F1) litter size effect $P < 0.05$ (CS_H vs CN_H)

*** Current (F2) HFD effect $P < 0.001$ (HFD vs chow fed offspring across all mother groups).

Fig 4 Total WAT sampled expressed as g **(A)** and **(B)** percent body weight. **(C)** plasma leptin and **(D)** triglycerides at 21 weeks in F2 offspring of CN mother fed chow (CN_C) and HFD (CN_H), CS mother fed chow (CS_C) and HFD (CS_H) and HN mother fed chow (HN_C) and HFD (HN_H). Results are expressed as mean ± SEM (n = 12-17).

††† Maternal HFD effect $P < 0.001$ (HN_C vs CN_C / HN_H vs CN_H)

Maternal litter size effect $P < 0.01$ (CS_C vs CN_C)

, * Current HFD effect $P < 0.01$, 0.001 (HFD vs chow fed offspring across all mother groups).

Fig 5 Lipid regulators **(A)** Adipose triglyceride lipase (ATGL) in retroperitoneal WAT (rRP) **(B)** Uncoupling proteins (UCP-3) in skeletal muscle at 21 weeks in F2 offspring of CN mother fed chow (CN_C) and HFD (CN_H), CS mother fed chow (CS_C) and HFD (CS_H) and HN mother fed chow (HN_C) and HFD (HN_H). Results are expressed as mean ± SEM (n = 6-8).

† Maternal HFD effect $P < 0.05$ (HN_H vs CN_H)

* Current HFD effect $P < 0.05$ (HFD vs chow fed offspring across all mother groups).

Fig 6 mRNA expression of **(A)** Ob-Rb receptor **(B)** signal transducer and activator of transcription, STAT-3 **(C)** suppressor of cytokine signaling, SOCS-3 **(D)** Pro-opiomelanocortin, POMC **(E)** Neuropeptide Y, NPY in ventral hypothalamus at 21 weeks in F2 offspring of CN mother fed chow (CN_C) and HFD (CN_H), CS mother fed chow (CS_C) and HFD (CS_H) and HN mother fed chow (HN_C) and HFD (HN_H). Results are expressed as mean ± SEM (n = 8).

†, †† Maternal HFD effect $P < 0.05$, 0.01 (HN_H vs CN_H)

Maternal litter size effect $P < 0.05$ (CS_C vs CN_C)

** Current HFD effect $P < 0.01$ (HFD vs chow fed offspring across all mother groups).

Table legends

Table 1. Experimental procedure.

Table 2. Maternal (F1) characteristics

Table 3. Body weight, fat mass, food intake, liver fatty changes and blood pressure in F2 rat.

Table 1. Experimental Procedure

F0 (Diet)	F1 (Intervention)	F2 (diet)	F2 ID	
Chow	Chow normal litter (CN); n = 7	Chow	CN-chow (CN_C);	n = 17
		HFD	CN-HFD (CN_H);	n = 14
	Chow small litter (CS); n = 6	Chow	CS-chow (CS_C);	n = 12
		HFD	CS-HFD (CS_H);	n = 12
HFD	HFD normal litter (HN); n = 7	Chow	HN-chow (HN_C);	n = 17
		HFD	HN-HFD (HN_H);	n = 15

Table 2. Maternal (F1) characteristics

Mothers	**CN (n = 7)**	**CS (n = 6)**	**HN (n =7)**
BW at kill (22 wks)	327.2 ± 14.5	345.6 ± 16.0	382.1 ± 8.0††
rRP (% BW)	0.24 ± 0.05	0.29 ± 0.05	1.93 ± 0.56††
Gonadal (% BW)	0.48 ± 0.08	0.65 ± 0.06	1.96 ± 0.19†††
Mesenteric (% BW)	0.40 ± 0.06	0.50 ± 0.04	1.45 ± 0.18†††
Total (% BW)	1.25 ± 0.19	1.64 ± 0.11	5.95 ± 0.44†††
Leptin (ng/mL)	1.59 ± 0.24	1.52 ± 0.11	3.23 ± 0.16†††
Insulin (ng/mL)	0.20 ± 0.03	0.44 ± 0.14	0.28 ± 0.07
Triglycerides (mmol/L)	0.16 ± 0.03	0.17 ± 0.02	0.28 ± 0.04†

Values expressed as mean ± SEM. †, ††, ††† HFD effect $P < 0.05, 0.01, 0.001$ (HN vs CN)

Table 3. Body weight, fat mass, food intake, liver fatty changes and blood pressure in F2 rat.

	Chow			HFD			Effects (P values)		
	CN offspring	*CS offspring*	*HN offspring*	*CN offspring*	*CS offspring*	*HN offspring*	*Mother's litter size*	*Mother's HFD*	Overall current diet
	(n = 17)	(n = 12)	(n = 16)	(n = 14)	(n = 14)	(n = 15)	CS_CvsCN_C/ CS_HvsCN_H	HN_CvsCN_C/ HN_HvsCN_H	HFD vs chow offspring
BW at 21 weeks (g)	454.9 ± 20.9	504.7 ± 14.3	528.8 ± 11.6	607.1 ± 8.9	627.5 ± 8.5	723.1 ± 17.0	<0.01 / NS	<0.001 / <0.001	<0.001
BMI (Kg/M^2)	6.85 ± 0.19	7.59 ± 0.28	7.93 ± 0.15	8.57 ± 0.15	9.21 ± 0.35	9.70 ± 0.21	<0.001 / 0.001	<0.001 / <0.001	<0.001
rRP WAT (g)	2.46 ± 0.21	3.95 ± 0.44	5.20 ± 0.32	12.47 ± 0.80	12.74 ± 0.87	22.77 ± 1.14	<0.01 / NS	<0.001 / <0.001	<0.001
Gonadal WAT (g)	5.14 ± 0.23	6.90 ± 0.70	8.87 ± 0.55	17.56 ± 1.08	19.49 ± 1.33	26.50 ± 1.41	<0.05 / NS	<0.001 / <0.001	<0.001
Mesenteric WAT (g)	3.49 ± 0.23	4.75 ± 0.48	5.93 ± 0.42	10.13 ± 0.49	11.98 ± 0.71	16.44 ± 1.21	<0.05 / NS	<0.001 / <0.001	<0.001
Liver (g)	12.9 ± 0.6	14.2 ± 0.7	14.4 ± 0.6	17.4 ± 0.9	17.2 ± 1.4	19.0 ± 0.7	NS / NS	<0.05 / NS	<0.001
rRP WAT (%BW)	0.54 ± 0.03	0.78 ± 0.07	0.98 ± 0.05	2.05 ± 0.13	2.02 ± 0.12	3.11 ± 0.13	<0.01 / NS	<0.001 / <0.001	<0.001
Gonadal WAT (%BW)	1.13 ± 0.05	1.35 ± 0.09	1.67 ± 0.08	2.88 ± 0.18	3.09 ± 0.17	3.64 ± 0.18	<0.05 / NS	<0.001 / <0.001	<0.001
Mesenteric WAT(%BW)	0.78 ± 0.04	0.86 ± 0.11	1.11 ± 0.06	1.66 ± 0.08	1.89 ± 0.09	2.23 ± 0.15	NS / NS	<0.001 / <0.001	<0.001
Liver (%BW)	2.85 ± 0.12	2.82 ± 0.09	2.72 ± 0.06	2.85 ± 0.15	2.73 ± 0.23	2.61 ± 0.07	NS / NS	NS / NS	NS
Food intake (kJ/rat)*10^3	25.2 ± 0.6	25.9 ± 0.8	28.0 ± 0.4	48.6 ± 0.3	51.7 ± 2.2	54.3 ± 0.9	NS / NS	<0.05 / <0.01	<0.001
Liver fatty changes	0.26 ± 0.12	0.33 ± 0.11	0.33 ± 0.06	1.30 ± 0.13	2.07 ± 0.21	1.92 ± 0.08	NS / <0.05	NS / <0.01	<0.001
Systolic BP (mmHg)	129.5 ± 4.4	125.6 ± 3.7	130.5 ± 5.5	143.9 ± 1.7	139.2 ± 7.4	151.9 ± 2.9	NS / NS	NS / NS	<0.001
Diastolic BP (mmHg)	84.4 ± 2.1	81.1 ± 2.8	83.5 ± 3.9	97.9 ± 1.7	91.3 ± 6.8	96.7 ± 2.9	NS / NS	NS / NS	<0.001
Heart rate (beats/min)	394.2 ± 12.2	403.6 ± 15.7	473.5 ± 18.4	428.4 ± 13.0	433.6 ± 25.6	377.0 ± 15.5	NS / NS	NS / NS	NS

Abbreviations: BW, body weight; BMI, body mass index; WAT, white adipose tissue; rRP, retroperitoneal. Time points: body weight, fat and organ weights in gram and % body weight at kill (21 weeks), food intake (5-15 weeks), Blood pressure (12 weeks). Values expressed as mean ± SEM.

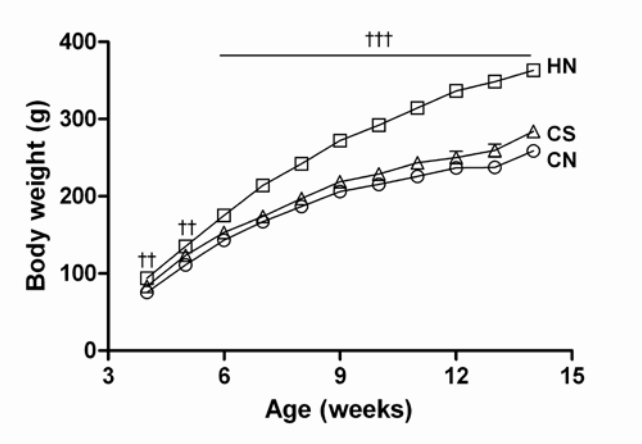
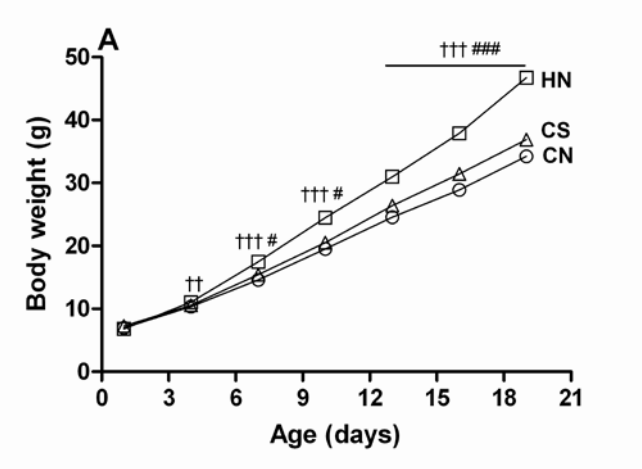


Fig 1


A
Body weight (g)
Age (days)
HN
CS
CN
†††###
†††#
†††#
††

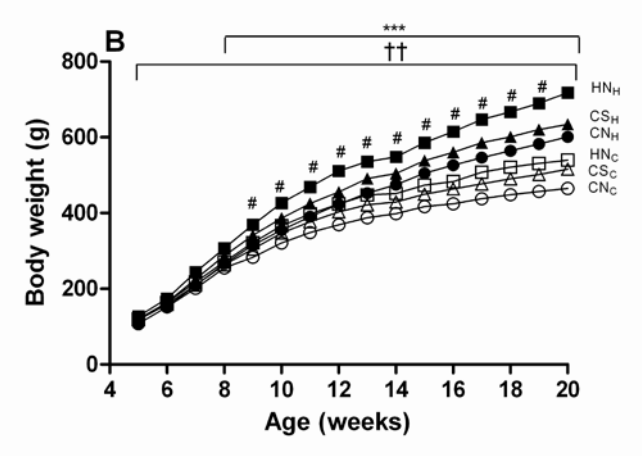

B
Body weight (g)
Age (weeks)
***
††
HNH
CSH
CNH
HNC
CSC
CNC

Fig 2

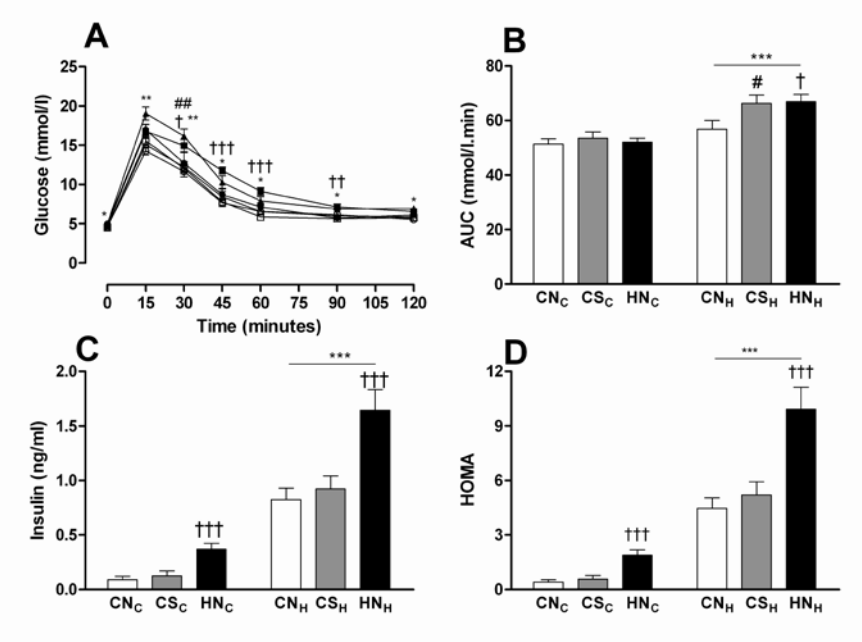
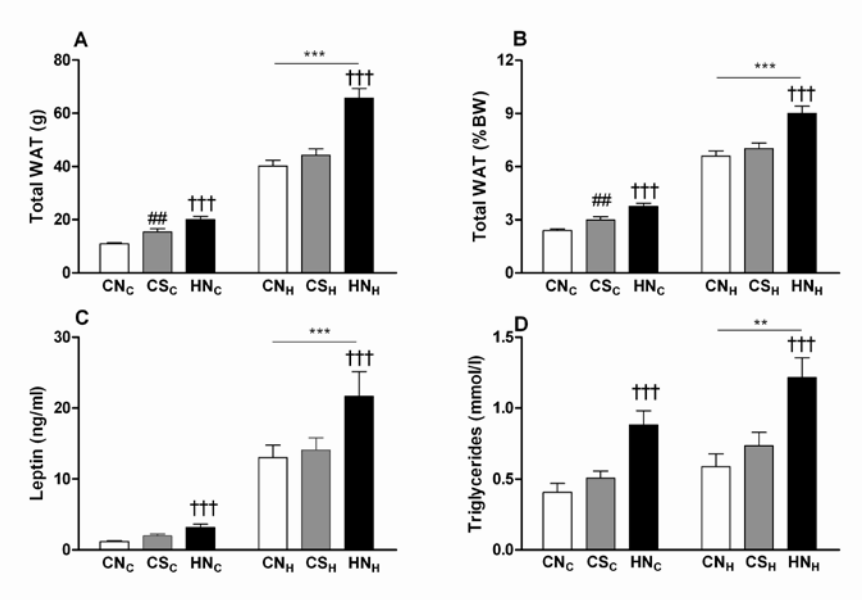

A
Glucose (mmol/l)
Time (minutes)
B
AUC (mmol/l.min)
C
Insulin (ng/ml)
D
HOMA
CNC CSC HNC CNH CSH HNH

Fig 3


A
Total WAT (g)
80
60
40
20
0
***
†††
##
†††
CN_C CS_C HN_C CN_H CS_H HN_H
B
Total WAT (% BW)
12
9
6
3
0
***
†††
##
†††
CN_C CS_C HN_C CN_H CS_H HN_H
C
Leptin (ng/ml)
30
20
10
0
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†††
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CN_C CS_C HN_C CN_H CS_H HN_H
D
Triglycerides (mmol/l)
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0.0
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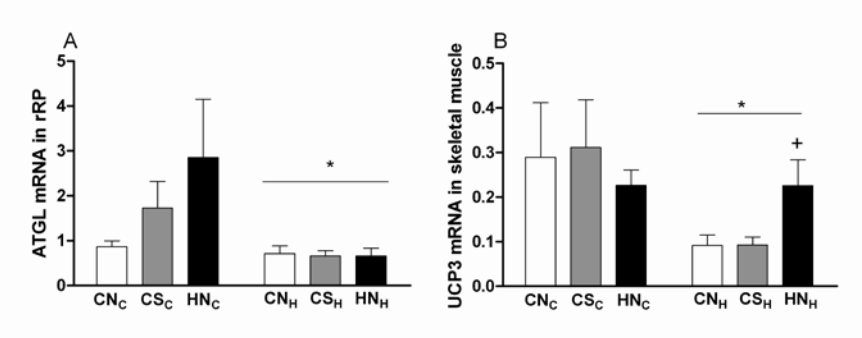
CN_C CS_C HN_C CN_H CS_H HN_H

Fig 4


A
ATGL mRNA in rRP
5
4
3
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1
0
*
CN$_C$ CS$_C$ HN$_C$ CN$_H$ CS$_H$ HN$_H$
B
UCP3 mRNA in skeletal muscle
0.5
0.4
0.3
0.2
0.1
0.0
*
+
CN$_C$ CS$_C$ HN$_C$ CN$_H$ CS$_H$ HN$_H$

Fig 5

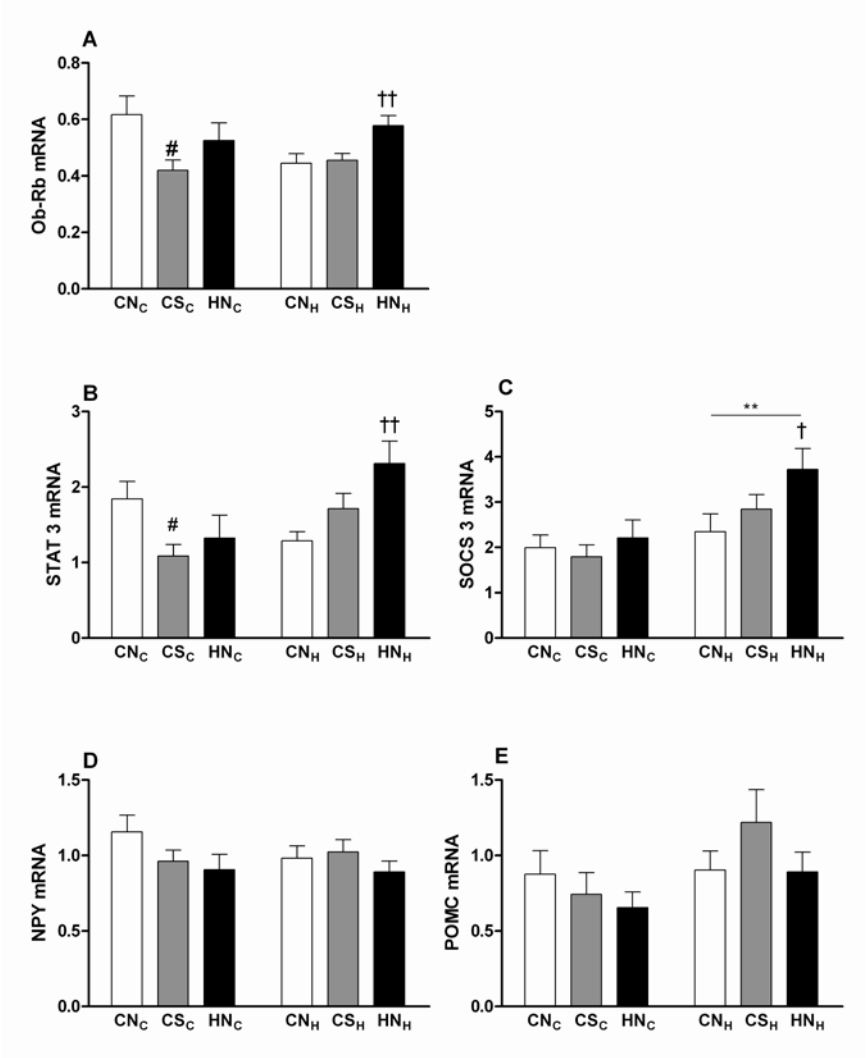
A
Ob-Rb mRNA
0.8
0.6
0.4
0.2
0.0
#
††
CN_C CS_C HN_C CN_H CS_H HN_H
B
STAT 3 mRNA
3
2
1
0
#
††
CN_C CS_C HN_C CN_H CS_H HN_H
C
SOCS 3 mRNA
5
4
3
2
1
0
**
†
CN_C CS_C HN_C CN_H CS_H HN_H
D
NPY mRNA
1.5
1.0
0.5
0.0
CN_C CS_C HN_C CN_H CS_H HN_H
E
POMC mRNA
1.5
1.0
0.5
0.0
CN_C CS_C HN_C CN_H CS_H HN_H

Fig 6