# Sex-specific effects of prenatal stress on glucose homoeostasis and peripheral metabolism in rats

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## Abstract

Glucocorticoid overexposure during pregnancy programmes offspring physiology and predisposes to later disease. However, any impact of ethologically relevant maternal stress is less clear, yet of physiological importance. Here, we investigated in rats the short- and long-term effects in adult offspring of repeated social stress (exposure to an aggressive lactating female) during late pregnancy on glucose regulation following stress, glucose–insulin homoeostasis and peripheral expression of genes important in regulating glucose and lipid metabolism and glucocorticoid action. Prenatal stress (PNS) was associated with reduced birth weight in female, but not male, offspring. The increase in blood glucose with restraint was exaggerated in adult PNS males compared with controls, but not in females. Oral glucose tolerance testing showed no effects on plasma glucose or insulin concentrations in either sex at 3 months; however, at 6 months, PNS females were hyperinsulinaemic following an oral glucose load. In PNS males, plasma triglyceride concentrations were increased, with reduced hepatic mRNA expression of 5$\alpha$-reductase and peroxisome proliferator-activated receptor $\alpha$ (*Ppar*$\alpha$ (*Ppara*)) and a strong trend towards reduced peroxisome proliferator-activated receptor gamma coactivator 1$\alpha$ (*Pgc1*$\alpha$ (*Ppargc1a*)) and *Ppar*$\gamma$ (*Pparg*) expression, whereas only *Pgc1*$\alpha$ mRNA was affected in PNS females. Conversely, in subcutaneous fat, PNS reduced mRNA expression of 11$\beta$-hydroxysteroid dehydrogenase type 1 (*11*$\beta$*hsd1*), phosphoenolpyruvate carboxykinase (*Pepck* (*Pck1*)), adipose triglyceride lipase (*Atgl*) and diglyceride acyltransferase 2 (*Dgat2*) in females, but only *Pepck* mRNA expression was reduced in PNS males. Thus, prenatal social stress differentially programmes glucose homoeostasis and peripheral metabolism in male and female offspring. These long-term alterations in physiology may increase susceptibility to metabolic disease.

**Key Words**
- 5$\alpha$-reductase
- adipose
- corticosterone metabolism
- early life stress
- liver
- sex difference

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## Introduction

Exposure to an adverse environment *in utero*, manifesting in a reduction in birth weight, is associated with a substantially increased risk of cardiometabolic disorders, neuroendocrine dysfunction and psychiatric disease in

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childhood and adulthood (Seckl & Holmes 2007). Moreover, evidence from human studies indicate that prenatal stress (PNS) is associated with a greater risk for developing insulin resistance and type 2 diabetes later in life (Entringer *et al*. 2008, Li *et al*. 2012). One of the major mechanisms proposed to explain this link is prenatal glucocorticoid overexposure (Edwards *et al*. 1993). In rats, maternal stress, synthetic glucocorticoid administration or inhibition of 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2; the placental 'barrier' to maternal glucocorticoids that deactivates endogenous glucocorticoids) during late pregnancy results in offspring of low birth weight (Lindsay *et al*. 1996*a*, Benediktsson *et al*. 1997, Nyirenda *et al*. 1998, Welberg *et al*. 2000, Lesage *et al*. 2004, Brunton & Russell 2010) that later develop glucose intolerance (Lindsay *et al*. 1996*b*, Nyirenda *et al*. 1998, Lesage *et al*. 2004), hypertension (Benediktsson *et al*. 1993, Lindsay *et al*. 1996*a*, Nyirenda *et al*. 1998), hyperactive hypothalamic–pituitary–adrenal (HPA) axis responses to stress and/or increased anxiety (Welberg *et al*. 2001, Maccari & Morley-Fletcher 2007, Brunton & Russell 2010). Antenatal glucocorticoid exposure also has similar 'programming effects' in other species including non-human primates (de Vries *et al*. 2007).

Programming of altered offspring HPA axis activity (and hence circulating glucocorticoid levels) and/or peripheral glucocorticoid metabolism may be a key mechanism underpinning programming effects on both the brain and metabolism (Friedman *et al*. 1993, Huang *et al*. 2002). Prenatal dexamethasone administration in the last week of pregnancy is associated with increased expression of the glucocorticoid receptor (GR) in both liver and adipose tissue (Nyirenda *et al*. 1998, Cleasby *et al*. 2003) and with increased hepatic expression of genes known to be important in regulating gluconeogenesis, such as phosphoenolpyruvate carboxykinase (*Pepck (Pck1)*; Nyirenda *et al*. 1998). These changes may be important in driving the observed alterations in glucose–insulin homoeostasis (Nyirenda *et al*. 1998). We have recently shown that, in rats, exposure to social stress in late pregnancy is associated with increased HPA axis responses to stress in both the male and female offspring (Brunton & Russell 2010). This appears to be a result of increased drive of the stress axis by corticotrophin-releasing hormone neurones, concomitant with impaired central glucocorticoid feedback mechanisms (Brunton & Russell 2010). However, it is not clear whether maternal stress during pregnancy results in changes in *GR* expression in the periphery or alters the expression of other genes involved in glucocorticoid, carbohydrate or fatty acid metabolism.

A further level of control of glucocorticoid action occurs at a tissue level as a consequence of intracellular metabolism in target cells. Glucocorticoids are metabolised by the 11βHSDs and the A-ring reductase enzymes 5α- and 5β-reductase. Of these enzymes, 11βHSD1, which reactivates glucocorticoids from their inert 11-keto metabolites, and the A-ring reductase enzymes which are involved in glucocorticoid clearance, are highly expressed in liver and may contribute to bulk glucocorticoid metabolism. Altered steroid clearance as a consequence of alterations in the expression and activity of these enzymes can impact on HPA axis activity and maintain circulating glucocorticoid levels (Kotelevtsev *et al*. 1997, Harris *et al*. 2001). Programming of altered peripheral glucocorticoid metabolism has been reported in models of prenatal glucocorticoid overexposure with documented changes in hepatic and renal 11βHSD1 expression (Nyirenda *et al*. 2009, Tang *et al*. 2011). Additionally, reduced 5α-reductase activity has been proposed as an important contributor to the 'programmed' phenotype seen in individuals exposed to the severe physical, nutritional and psychological challenges of the World War II Holocaust early in life who subsequently developed post-traumatic stress disorder (PTSD; Yehuda *et al*. 2009*a*).

In this study, we exposed pregnant rats to an ethologically relevant social stress paradigm (to more accurately model the type of stress pregnant women may experience) with the aim of establishing whether PNS leads to alterations in glucose tolerance, insulin resistance and the expression of genes known to be important in regulating glucose and lipid metabolism and glucocorticoid action in liver, adipose and muscle in the adult offspring.

## Materials and methods

### Animals

Sprague Dawley rats weighing 270–295 g were purchased from Charles River Laboratories (Margate, Kent, UK). Rats were housed in a SPF rodent facility in open top cages, initially in groups of four to six, with food (standard maintenance rat chow, 14% protein and 4% fat) and water available *ad libitum*, under standard conditions of temperature (20–21 °C), humidity (50–60%) and lighting (12 h light:12 h darkness cycle, lights on at 0700 h GMT). Pregnant rats were obtained by overnight mating with a sexually experienced male and pregnancy was confirmed by the presence of a semen plug in the breeding cage the following morning, designated day 1 of pregnancy (parturition expected on day 22). All pregnant rats had



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their standard chow supplemented with breeding diet (19% protein and 9% fat) throughout gestation and lactation. Pregnant rats were caged singly from day 14. The numbers of rats in each group are given in the relevant figure legends. All procedures were performed with approval from the University of Edinburgh Ethical Committee and in accordance with the UK Home Office Animals (Scientific Procedures) Act, 1986 legislation.

### PNS model

Pregnant rats were exposed to stress during late gestation at a time when the fetal neuroendocrine and endocrine systems are differentiated and functional. Pregnant 'intruder' rats were placed in the cage of an unfamiliar aggressive lactating 'resident' rat (days 2–8 of lactation; day 1, day of parturition) for 10 min between 0900 and 1200 h on five consecutive days starting on day 16 of gestation (Brunton & Russell 2010). Intruder rats were paired with a different lactating resident for each social defeat exposure. Following the final defeat on day 20, pregnant rats remained undisturbed, except for routine husbandry, in their home cage through parturition and lactation. Pregnant control rats remained in their home cages throughout. All rats were weighed daily during the stress exposure period. Soon after parturition, litter size, pup weights and male:female ratio of each litter was recorded. Litter sizes were not adjusted and dams remained with their litters until weaning on postnatal day 22. After weaning, the offspring were housed in groups by litter and sex under standard conditions (described earlier). For each experiment, groups were selected across litters, i.e. one male and one female (selected at random stages of the oestrous cycle) from each litter were used to directly compare differences between control and PNS offspring. The number of rats per group was 10, unless otherwise stated in the relevant figure legend.

### Jugular vein cannulation, blood sampling and blood glucose measurements

Rats were fitted with a silastic (bore, 0.5 mm; wall, 0.25 mm) jugular vein cannula filled with sterile heparinised (50 U/ml) saline (0.9%) under halothane anaesthesia (2–3% in 1200 ml/min oxygen). Following surgery, rats were caged singly. Three or four days later, between 0800 and 0900 h, silastic cannulae were connected to PVC tubing filled with sterile heparinised saline and connected to a 1 ml syringe. Rats were left undisturbed in their home cage for 90 min and then two basal blood samples (ca. 30 μl) were collected 30 min apart from male (aged 11–12 weeks) and female (aged 12–13 weeks) control and PNS offspring. Next, rats were restrained in a ventilated Perspex tube (internal diameter, 65 mm; length adjusted for each rat) for 30 min. Further blood samples were collected 15, 30 and 60 min after the onset of restraint. Non-stressed controls remained in their home cages throughout. Withdrawn blood was replaced with sterile saline (0.9%). Blood glucose concentrations were determined immediately using Accu-chek Active test strips (Roche) and an Accu-check blood glucose meter (Roche). The principle of the test is based on the glucose oxidase/peroxidase reaction and the manufacturers have determined that it is specific for glucose. The detection range of the test strips used was 0.5–33.3 mmol/l. Plasma corticosterone concentrations were also determined and are published elsewhere (Brunton & Russell 2010).

### Glucose tolerance tests, plasma and tissue lipid parameters

Oral glucose tolerance tests (OGTTs) were performed in rats at 3 months and again at 6 months of age as described previously (Drake *et al.* 2005). Briefly, rats were fasted overnight (from 1600 h) before administration of 2 g/kg glucose solution (0.5 g/ml) by oral gavage the following morning (between 0830 and 0915 h). Tail-nick blood samples were collected at 0, 30 and 120 min. Plasma was separated and stored at −20 °C. Glucose was determined by the hexokinase method (Sigma) and plasma insulin by ELISA (Crystal Chem, Downers Grove, IL, USA). Plasma triglycerides were measured on the fasted sample, using Infinity Triglyceride Liquid Stable Reagent and plasma cholesterol was quantified using the Infinity Cholesterol Liquid Stable Reagent (both from Thermo Electron, Pittsburgh, PA, USA).

The day after the 6-month OGTT (ca. 27 h after OGTT), rats were killed by an overdose of carbon dioxide. Epididymal, mesenteric and retroperitoneal fat depots and the liver were rapidly removed and weighed before being frozen on dry ice. Samples of subcutaneous fat and skeletal muscle (quadriceps) were also removed and frozen on dry ice. Tissue was stored at −80 °C until subsequent assay. For analysis of tissue triglyceride contents, liver and quadriceps tissue (~100 mg) were digested at 55 °C overnight in 10% (w/v) potassium hydroxide in ethanol; undigested tissue was removed by centrifugation at 15 000 ***g*** for 5 min. The supernatant was removed, mixed with 1 M magnesium chloride (1:1), vortexed, incubated on ice for 10 min and centrifuged at 15 000 ***g*** for 5 min. The supernatant was used to measure triglyceride using Infinity Triglyceride Liquid Stable Reagent (Thermo Electron).



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**Table 1** Details of primers used for real time PCR assays

| Gene | Abbreviation | Forward primer | Reverse primer | UPL | ABI assay ID |
|---|---|---|---|---|---|
| AMP-activated protein kinase 2 | *Ampk2* | ccggaggtcatctcagga | agggcatacaggataacacca | 50 | |
| Adiponectin | *AdipoQ* | tggtcacaatgggataccg | cccttaggaccaagaacacct | 80 | |
| Adipose triglyceride lipase | *Atgl* | | | | Rn01479969_m1 |
| Cyclophilin A | | | | | Rn00690933_m1 |
| Diacylglycerol acyltransferase 1 | *Dgat1* | ccgtggtatcctgaattggt | aaaaataaccttgcattactcagga | 9 | |
| Diacylglycerol acyltransferase 2 | *Dgat2* | gtgtggcgctattttcgag | ggtcagcaggttgtgtgtctt | 42 | |
| Glucocorticoid receptor | *Gr* | | | | Rn01405584_m1 |
| 11β-Hydroxysteroid dehydrogenase type 1 | *11βhsd1* | tctacaaatgaagagttca-gaccag | gccccagtgacaatcacttt | 1 | |
| Leptin | | | | | Rn00565158_m1 |
| Lipoprotein lipase | *Lpl* | | | | Rn00561482_m1 |
| Phosphoenolpyruvate carboxykinase | *Pepck* | | | | Rn01529014_m1 |
| Peroxisome proliferator-activated receptor coactivator 1α | *Pgc1α* | | | | Rn00580241_m1 |
| Peroxisome proliferator-activated receptor α | *Pparα* | | | | Rn00566193_m1 |
| Peroxisome proliferator-activated receptor γ | *Pparγ* | | | | Rn00440945_m1 |
| 5α-Reductase | *5αR* | ctgggcaacctccctaac | gggaaaaccagcgtgcct | 18 | |
| 5β-Reductase | *5βR* | gagatgcccatggccttta | cactcggccattctcatctt | 80 | |
| Uncoupling protein 2 | *Ucp2* | gactctgtaaagcagttcta-caccaa | gggcacctgtggtgctac | 79 | |

UPL, Universal Probe Library

### Corticosterone RIA

Plasma corticosterone concentrations were determined using a commercially available RIA kit (MP Biomedical, Cambridge, UK). The sensitivity of the assay was 25 ng/ml and intra-assay variation was 4–5%. All samples were measured in a single RIA.

### Quantification of mRNA by real-time PCR

Total RNA was extracted from snap-frozen liver, adipose and quadriceps muscle using the Qiagen RNeasy system (Qiagen Ltd.) and reverse transcribed using the Promega RT kit (Promega UK Ltd.). cDNA (equivalent to 1 ng total RNA) was incubated in triplicate with gene-specific primers and fluorescent probes (either using the UPL system from Roche Diagnostics Ltd., or predesigned assays from Applied Biosystems) in 1× Roche LightCycler 480 Probes mastermix (Table 1). PCR cycling and detection of fluorescent signal were carried out using a Roche LightCycler 480. A standard curve was constructed for each primer–probe set using a serial dilution of cDNA pooled from all samples. Results were corrected for the expression of cyclophilin A, which was unchanged among groups. In this study, we set out to characterise the expression of genes important in glucocorticoid signalling and metabolism whose expression is altered in other models of programming and in humans (GR, 11βHSD1 and 5α- and 5β-reductase). We also studied the expression of genes that we have previously shown to be altered as a consequence of prenatal dexamethasone overexposure (Nyirenda *et al.* 1998, 2009, Drake *et al.* 2010) and are known to be important in glucose–insulin homoeostasis and in lipid metabolism in liver, subcutaneous fat and muscle including *Pepck*, peroxisome proliferator-activated receptor α and γ (*Pparα* (*Ppara*) and *Pparγ* (*Pparg*)), peroxisome proliferator-activated receptor gamma coactivator 1α (*Pgc1α* (*Ppargc1a*)), adipose triglyceride lipase (*Atgl*), diglyceride acyltransferase 1 and 2 (*Dgat1* and *2*), leptin, adiponectin (*AdipoQ*), AMP-activated protein kinase (*Ampk (PRKAA1)*) and uncoupling protein 2 (*Ucp2*).

**Table 2** Body weight in control and PNS offspring. Body weight data are expressed in grams ± S.E.M.

| Postnatal days | Control ♂ | PNS ♂ | Control ♀ | PNS ♀ |
|---|---|---|---|---|
| 1 | 6.4±0.2 | 6.0±0.2 | 6.1±0.2 | 5.6±0.1† |
| 10 | 18.0±0.7 | 17.2±0.8 | 17.2±0.8 | 16.5±0.6 |
| 22 | 44.5±1.6 | 39.8±0.9 | 41.1±1.5 | 38.9±1.4 |
| 70 | 315.8±6.7 | 295.9±8.3* | 219.7±2.9 | 214.2±6.0 |
| 91 | 400.1±7.0 | 359.8±10.9† | 255.6±4.3 | 241±7.0* |
| 189 | 518.7±7.9 | 478.1±16.0* | 303.4±6.0 | 290±7.9 |

*$P<0.05$ and †$P<0.005$ vs control. Student's *t*-test, $n=16$–17 litters/group for postnatal days 1–22 and $n=10$ rats/group post-weaning. PNS, prenatal stress.



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**Table 3** Tissue weights at 6 months of age. Data are expressed as percentage (%) body weight ± S.E.M.

| Organ/fat depot | Control ♂ | PNS ♂ | Control ♀ | PNS ♀ |
|---|---|---|---|---|
| Liver | 3.44 ± 0.07 | 3.98 ± 0.10* | 3.56 ± 0.10 | 3.65 ± 0.10 |
| Retroperitoneal fat | 0.70 ± 0.01 | 0.60 ± 0.06 | 0.61 ± 0.06 | 0.53 ± 0.07 |
| Mesenteric fat | 0.62 ± 0.06 | 0.57 ± 0.04 | 0.75 ± 0.05 | 0.66 ± 0.08 |
| Epididymal fat | 0.64 ± 0.06 | 0.61 ± 0.04 | – | – |

*$P<0.001$ vs control. Student's *t*-test, $n=10$ rats/group. PNS, prenatal stress.

### Statistical analysis

Blood glucose in response to restraint and plasma glucose, insulin and corticosterone data from the OGTT studies were analysed using either a one-way or a two-way repeated measures (RM) ANOVA or Friedman RM ANOVA on ranks (Sigmaplot v11.0, Systat Software, Inc., Chicago, IL, USA). Enzyme mRNA expression in liver, adipose and muscle was analysed using Student's *t*-test. Plasma triglycerides, cholesterol, body weight and organ weights were also analysed by Student's *t*-test. In each case, the analyses conducted were stratified by sex, and $P<0.05$ was considered statistically significant. Data are presented as mean ± S.E.M.

## Results

### Litter size, body weight and organ weight

Social stress did not affect maternal weight gain. The increase in body weight between gestation days 16–20 was 42.0 ± 3.0 g in controls (11.7% increase) and 44.6 ± 2.1 g in the social stress group (12.6% increase).

PNS had no significant effect on litter size (controls: 13.0 ± 0.6, mode = 13; PNS: 13.4 ± 1.3, mode = 14; $n=17$ and 16 l respectively) or the proportion of male:female pups in the litters (1:1.1 and 1:0.9 in the control and PNS litters respectively). Birth weight was not significantly different between PNS and control males (Table 2). However, the female PNS offspring had significantly lower birth weights (Table 2), including when litter size was controlled for ($F_{(1,31)}=18.7$, $P<0.001$; ANCOVA). Catch up growth occurred postnatally such that there were no significant differences in weight between control and PNS females on postnatal day 10, at weaning on postnatal day 22 or at 10 weeks of age (Table 2). PNS females weighed less than control females at 3 months, but body weight had normalised by 6 months of age (Table 2). However, despite there being no difference in body weight between control and PNS males in the first 3 weeks of life while suckling (Table 2), PNS males weighed significantly less than controls at 10 weeks, 3 months and 6 months of age (Table 2).

There were no differences in the weight of mesenteric, retroperitoneal or epididymal fat depots corrected for body weight; however, PNS males had heavier livers (Table 3; $P<0.001$). There were no differences between PNS and control females in liver weight or fat depot weights (Table 3) at 6 months of age.

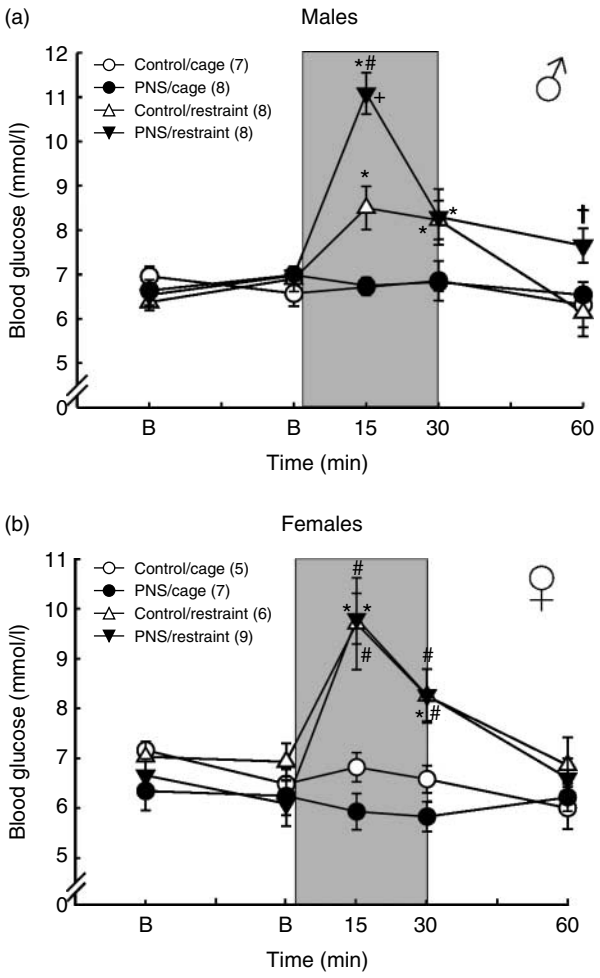


**Figure 1**
Blood glucose responses to acute restraint. Basal (B) blood samples were collected from conscious male (♂) and female (♀) control and prenatally stressed (PNS) rats before and after 30-min restraint (shaded bar) or not (cage). Blood glucose concentrations in (a) male and (b) female rats. Statistics: *$P<0.01$ vs B within same group; $^{+}P<0.001$ vs control/restraint group at same time point; $^{\#}P<0.001$ vs cage groups at same time point; two-way RM ANOVA. Data are group means ± S.E.M. Rat numbers per group are in parenthesis in the figure key.



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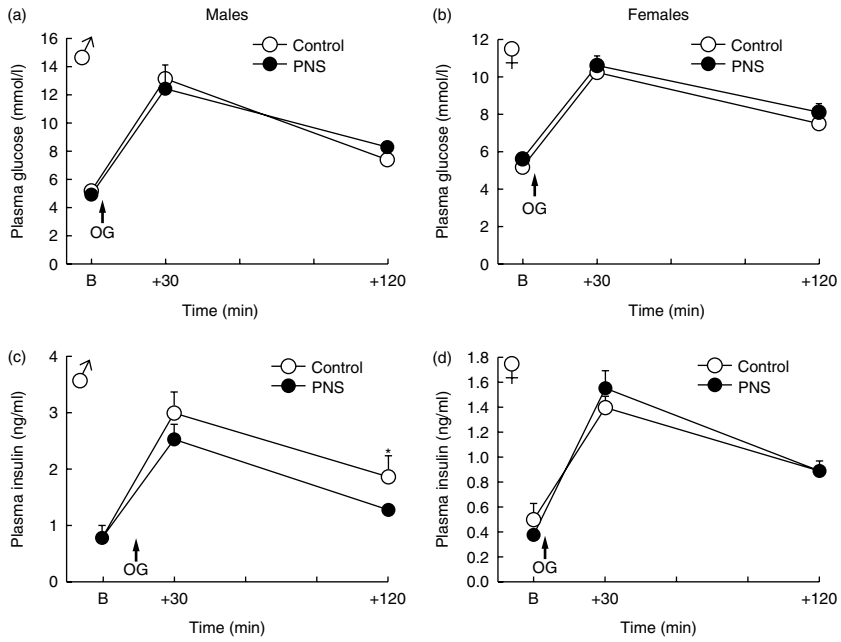


**Figure 2**
Oral glucose tolerance testing at 3 months. One basal (B) blood sample was collected from conscious 3-month-old male (♂) and female (♀) control and prenatally stressed (PNS) rats. Immediately after the basal sample, all rats were administered 2 g/kg glucose solution (0.5 g/ml) by oral gavage (OG). Further blood samples were collected 30 and 120 min after the glucose load. Plasma glucose concentrations in (a) male and (b) female rats and plasma insulin concentrations in (c) male and (d) female rats. Statistics: *$P<0.05$ vs PNS group at same time point; Friedman RM ANOVA on ranks. Data are group means+S.E.M. Rat numbers=10/group.

## Metabolism

**Blood glucose responses to acute restraint stress** **Males** At 3 months of age, basal blood glucose concentrations were not different between control and PNS males (Fig. 1a). Restraint stress significantly increased blood glucose concentrations ($F_{(3,106)}=15.3$; two-way RM ANOVA; Fig. 1a) in both control and PNS males; however, the response was significantly greater in the PNS males (1.3-fold greater response at 15 min, $P<0.001$; Fig. 1a).

**Females** Basal blood glucose concentrations were not different between 3-month-old control and PNS females (Fig. 1b). Restraint stress significantly increased blood glucose concentrations ($F_{(3,92)}=10.7$; two-way RM ANOVA, $P<0.001$; Fig. 1b) with no differences between the groups.

**Glucose tolerance testing: 3 months** At 3 months of age, there were no differences in basal plasma glucose concentrations between male (Fig. 2a) and female (Fig. 2b) control and PNS rats and no differences in the response to an oral glucose load between control and PNS rats in either sex (Fig. 2a and b). There was no difference in basal or peak plasma insulin concentrations between control and PNS male (Fig. 2c) or female rats (Fig. 2d); insulin concentration at 2 h was lower in PNS males than in controls (Fig. 2c; $P<0.05$, Friedman RM ANOVA on ranks). Females had significantly lower insulin concentrations than males under basal conditions ($F_{(1,36)}=7.0$, $P=0.012$; two-way ANOVA) and 30 min ($F_{(1,36)}=28.8$, $P<0.001$) after glucose administration, regardless of prenatal treatment.

**Glucose tolerance testing: 6 months** At 6 months of age, there were no differences in plasma glucose or insulin concentrations before or after an OGTT between control and PNS males (Fig. 3a and c). Plasma glucose concentrations were not different between PNS and control females (Fig. 3b), but plasma insulin concentrations were significantly greater in the PNS females than in the control females ($P=0.003$, one-way RM ANOVA) 30 min following oral glucose loading (Fig. 3d).

**Corticosterone** There was no difference in plasma corticosterone concentrations between male control and PNS rats (Fig. 3e) and female control and PNS rats (Fig. 3f) under basal conditions. The OGTT significantly increased circulating corticosterone in both the control ($P<0.001$) and PNS ($P<0.001$) males (Fig. 3e) and in the control



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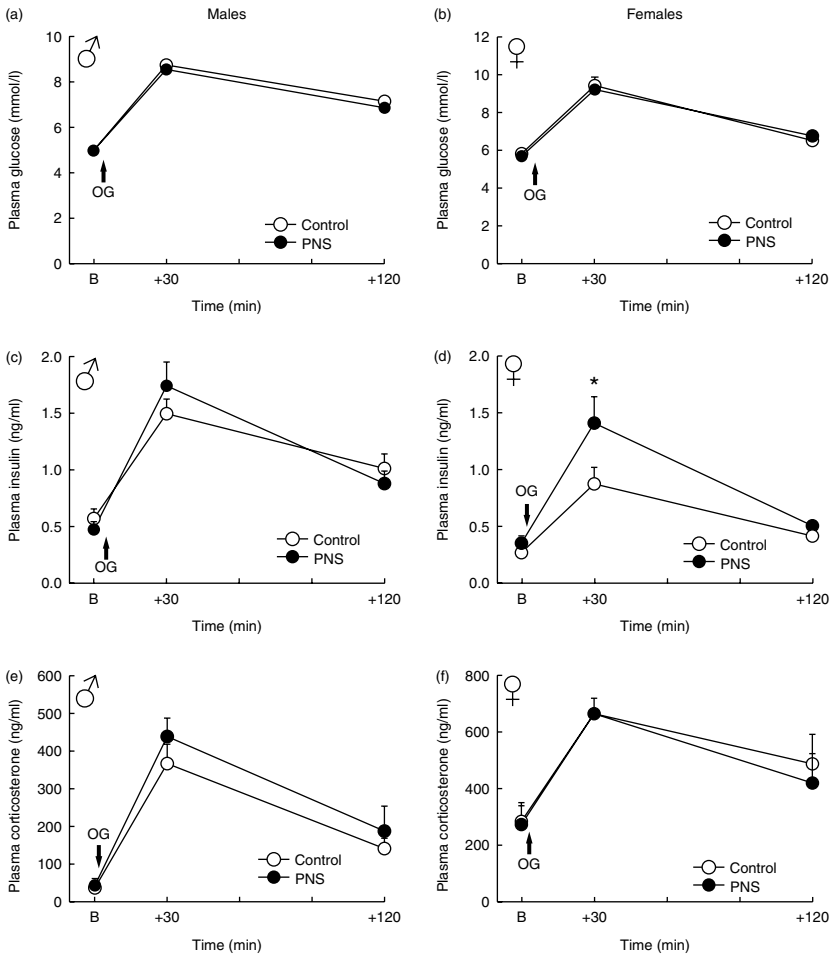


**Figure 3**
Oral glucose tolerance testing at 6 months. One basal (B) blood sample was collected from conscious 6-month-old male (♂) and female (♀) control and prenatally stressed (PNS) rats. Immediately after the basal sample, all rats were administered 2 g/kg glucose solution (0.5 g/ml) by oral gavage (OG). Further blood samples were collected 30 and 120 min after the glucose load. Plasma glucose concentrations in (a) male and (b) female rats; plasma insulin concentrations in (c) male and (d) female rats; and plasma corticosterone concentrations in (e) male and (f) female rats. Statistics: *$P=0.003$ vs control group at same time point; one-way RM ANOVA. Data are group means+S.E.M. Rat numbers=10/group.

($P<0.001$) and PNS ($P<0.001$) females (Fig. 3f), with no significant effects of prenatal treatment. However, at each time point, plasma corticosterone concentrations were significantly greater in females than in males regardless of prenatal experience ($F_{(1,36)}=25.9$, $P<0.001$; two-way ANOVA).

**Lipids** Plasma cholesterol concentrations were not different between control and PNS rats in either the males or females (Fig. 4a). PNS males had significantly greater circulating fasting concentrations of triglycerides than control males (Fig. 4b; *$P<0.05$, Student's *t*-test); however, there were no differences in females (Fig. 4b). There were no differences between any of the groups in hepatic (Fig. 4c) or muscle triglyceride (Fig. 4d) contents.

**Peripheral gene expression** **Males** PNS was associated with altered expression of genes important in glucocorticoid and lipid metabolism in liver and quadriceps muscle in the male rats. PNS reduced hepatic expression of mRNAs *Pparα* ($P=0.02$; Fig. 5a) and 5α-reductase ($P=0.03$, Student's *t*-test; Fig. 5a) and was associated with a trend towards a reduction in both *Pparγ* ($P=0.058$; Fig. 5a) and *Pgc1α* ($P=0.056$) mRNA levels. In subcutaneous fat, there was a significant reduction in *Pepck* mRNA level in the PNS males ($P=0.04$; Fig. 6a) and a trend towards a reduction in *Gr* mRNA level ($P=0.056$; Fig. 6a), with no changes in the expression of any of the other genes measured (Fig. 6a). In quadriceps, PNS significantly reduced *Gr* mRNA level ($P=0.04$), and there was a trend to reduced *Pparα* mRNA expression ($P=0.06$; Fig. 7a).

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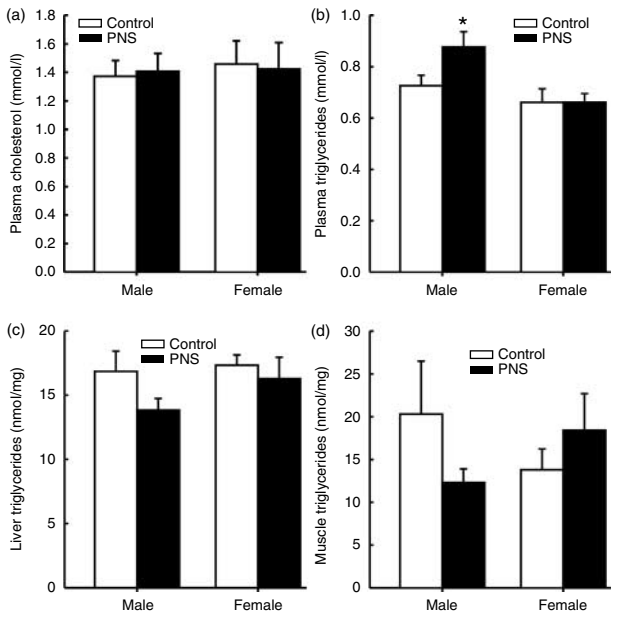


**Figure 4**
Plasma cholesterol and plasma, liver and muscle triglyceride concentrations. (a) There were no differences in plasma cholesterol levels between control and prenatal stress (PNS) males or females. (b) PNS was associated with increased plasma triglyceride concentrations in adult male but not female offspring. There were no differences in (c) liver or (d) muscle triglyceride concentrations in males or females. Statistics: $*P<0.05$ vs control group, Student's *t*-test. Data are group means + S.E.M. Rat numbers = 10/group.

**Females** There were fewer changes in gene expression in liver and muscle in females; PNS significantly reduced mRNA levels for *Pgc1α* in liver ($P=0.009$; Fig. 5b) and *Pparα* in quadriceps muscle ($P=0.03$; Fig. 7b). However, there were marked alterations in gene expression in subcutaneous fat in females (Fig. 6b), in which PNS reduced mRNA levels for *Atgl*, *Dgat2*, *11βhsd1*, leptin and *Pepck*, (Fig. 6b). PNS did not affect the expression of genes in mesenteric fat in either males (Fig. 6c) or females (Fig. 6d).

## Discussion

Here, we have shown that PNS exposure results in sex-specific effects on glucose–insulin and lipid homoeostasis in the adult offspring. Male, but not female, PNS offspring exhibited markedly increased glucose responses to acute restraint, whereas PNS females, but not males, were hyperinsulinaemic following oral glucose load. Moreover, the expression of genes important in glucocorticoid and lipid metabolism was altered by PNS predominantly in liver and skeletal muscle in the male rats, whereas in females the majority of changes were observed in subcutaneous fat. Importantly, these changes occurred in the absence of obesity, consistent with our previous reports in a rat model of prenatal programming by dexamethasone (Drake *et al.* 2010). In these studies, while prenatal dexamethasone exposure did not alter the severity of obesity or the accumulation of adipose tissue induced by high-fat feeding, there was evidence of altered fatty acid metabolism in liver and adipose tissue with altered expression of a number of relevant genes (Drake *et al.* 2010). Taken together, these studies suggest that prenatal glucocorticoid overexposure is associated with primary alterations in gene expression, which increase the risk of metabolic dysfunction in the absence of an increased risk of obesity.

Maternal stress in late pregnancy caused a marked stress-induced increase in blood glucose in male offspring, indicative of disrupted glucose homoeostasis. Here, pups remained with their mothers until weaning. Maternal behaviour was not monitored in this study; however, our unpublished observations indicate that this PNS paradigm does not affect subsequent maternal behaviour and others have reported similar findings using social stress exposure combined with restraint during pregnancy (Neumann *et al.* 2005). Nonetheless, we cannot exclude altered maternal behaviour as a contributing factor in the programming effects that we observed. As we have previously shown that both male and female PNS offspring display greater corticosterone responses to stress (Brunton & Russell 2010) and that male rats exposed to dexamethasone during the last week of gestation have increased glucose levels in response to exogenous corticosterone (Nyirenda *et al.* 1998), the stress-induced increase in plasma glucose concentrations indicates that PNS may programme increased sensitivity to glucocorticoids in males. The mechanism(s) are not known but may involve glucocorticoid-mediated increases in hepatic gluconeogenesis and/or reduced glucose uptake by muscle and fat (Pilkis & Granner 1992, Nyirenda *et al.* 1998, Cleasby *et al.* 2003), though this requires further investigation. Insulin homoeostasis was broadly unaltered in males; however, there was post-glucose hyperinsulinaemia in females indicating programming of increased insulin resistance and indicating gender-distinct mechanisms. Thus, PNS is associated with the programming of stress-induced hyperglycaemia in males and with hyperinsulinaemia in response to glucose in females.

The effects of PNS on hepatic gene expression were more marked in males than in females. In males, PNS decreased hepatic mRNA encoding *Pparα*, a key regulator of systemic and intrahepatic lipid homoeostasis (Leone



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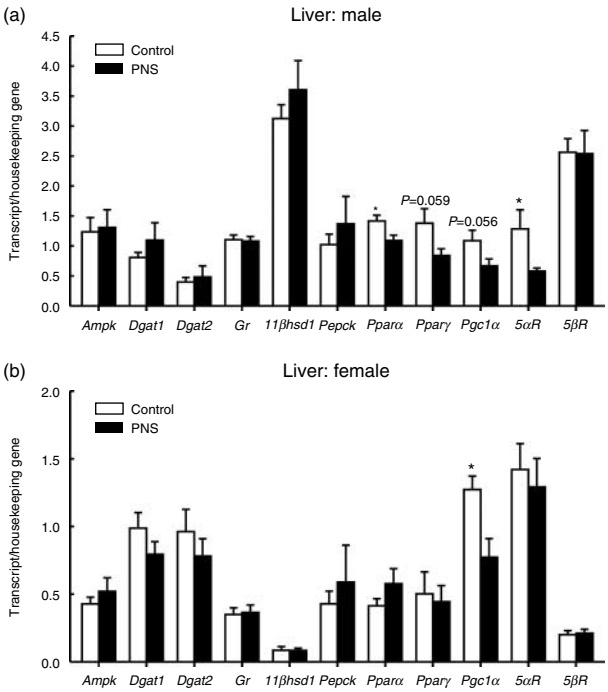


**Figure 5**
Hepatic gene expression at 6 months. (a) Prenatal stress (PNS) was associated with reduced expression of mRNAs for 5α-reductase (*5αR*) and peroxisome proliferator-activated receptor α (*Pparα*) in males. (b) In females, PNS was associated with reduced expression of peroxisome proliferator-activated receptor γ coactivator 1α(*Pgc1α*) mRNA. Statistics: **P*<0.04 vs control group; Student's *t*-test. Data are normalised to the expression of the housekeeping gene cyclophilin A. Data are group means+S.E.M. Rat numbers=10/group. *Ampk*, AMP-activated protein kinase; *Dgat*, diglyceride acyltransferase; *Gr*, glucocorticoid receptor; *11βhsd1*, 11β-hydroxysteroid dehydrogenase type 1; *Pepck*, phosphoenolpyruvate carboxykinase; *Pparγ*, peroxisome proliferator-activated receptor γ; *5βR*, 5β-reductase.

*et al.* 1999, Akiyama *et al.* 2001). Consistent with this, plasma triglyceride concentrations were increased in PNS males, and although hepatic triglyceride levels were not altered by PNS in rats here fed a normal diet, we have previously shown that exposure to a high-fat diet drives the development of hepatic steatosis in male rats exposed to dexamethasone prenatally (Drake *et al.* 2010). Reduced hepatic PPARγ occurs in offspring of dams exposed to dexamethasone (Drake *et al.* 2010) or dietary restriction (Magee *et al.* 2008); here, the trend for reduced *Pparγ* mRNA expression in PNS males indicates that PPARγ may be particularly sensitive to the prenatal environment. Hepatic *Pgc1α* mRNA expression was significantly decreased in PNS females, with a similar trend in males, consistent with the effects of prenatal dexamethasone exposure (Drake *et al.* 2010). *Pgc1α* has a key role in the regulation of multiple pathways involved in the control of energy metabolism; in animal models, hepatic *Pgc1α* deficiency causes the development of hepatic steatosis (Leone *et al.* 2005, Estall *et al.* 2009), and in humans, *PGC1α* expression is reduced in patients with non-alcoholic fatty liver disease (Westerbacka *et al.* 2007). Thus, these changes in the expression of key genes in fatty acid metabolism in liver indicate that programming of increased susceptibility to hepatic fat accumulation appears to be a common outcome of PNS/glucocorticoid overexposure.

Intriguingly, *Pparα* expression was also reduced in skeletal muscle in PNS females with a similar trend in PNS males. In addition to its role in hepatic lipid metabolism, PPARα may play a key role in muscle lipid metabolism (Muoio *et al.* 2002, Boyle *et al.* 2011) and reduced muscle lipid oxidation may precede lipid accumulation and insulin resistance (Kim *et al.* 2000, Muoio *et al.* 2002). Importantly, ageing exacerbates the phenotypic abnormalities associated with *Pparα* deficiency (Atherton *et al.* 2009), which might also exacerbate the phenotype associated with PNS.

The finding of down-regulated hepatic 5α-reductase mRNA in male PNS rats is complemented by findings from human studies. Elderly survivors of the World War II Holocaust display threefold lower 5α-reduction of cortisol, indicative of reduced hepatic 5α-reductase type 1 activity (Yehuda *et al.* 2009*a*). The greatest reductions in 5α-reductase activity were observed in those individuals who were the youngest at exposure to the Holocaust, indicating a potential 'programming' window in early life. Remarkably, in 9/11-exposed individuals with PTSD, lower 5α-reductase activity predicts resistance to psychotherapy (Yehuda *et al.* 2009*b*). While the mechanisms accounting for reduced 5α-reductase expression are unclear, 5α-reductase expression is 'programmed' in early life by androgens (Gustafsson & Stenberg 1974), and additionally, in adulthood, testosterone deficiency increases, while testosterone administration reduces hepatic 5α-reductase activity (Gustafsson & Stenberg 1974). Circulating testosterone levels are markedly elevated in PNS males (PJ Brunton & JA Russell, unpublished observations), so increased testosterone may contribute to the reduced hepatic 5α-reductase expression. The physiological significance of down-regulated hepatic 5α-reductase in PNS offspring is not fully understood. It may be an adaptive response to PNS exposure, where a shift in metabolism acts to prolong local glucocorticoid action in the liver (by down-regulating corticosterone inactivation in the liver by 5α-reductase), advantageous in generating fuels to meet metabolic demand. In accordance



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with the reduction in hepatic 5α-reductase expression in PNS male rats, 5α-reductase mRNA expression in the brains of fetal, prepubertal and adult PNS rats is down-regulated (Ordyan & Pivina 2005, Paris *et al*. 2011, PJ Brunton & JA Russell, unpublished observations). In the brain, 5α-reductase generates neurosteroids such as allopregnanolone (3α-hydroxy-5α-pregnan-20-one) and androstanediol (5α-androstane-3β,17β-diol), which have anxiolytic and stress-reducing actions (Bitran *et al*. 1995, Patchev *et al*. 1996, Edinger & Frye 2004, Brunton *et al*. 2009, Handa *et al*. 2009). Hence, reduced central 5α-reductase expression is consistent with the anxious and stress hyper-reactive phenotype of PNS rats (Brunton & Russell 2010). Evidently, equivalent changes in 5α-reductase expression in liver and in brain in response to PNS may be adaptive to promote metabolic efficiency and a cautious behavioural strategy. Additionally, consistent with changes predicting altered glucocorticoid metabolism in target tissues and in agreement with our previous studies in males exposed to dexamethasone prenatally (Cleasby *et al*. 2003), GR mRNA expression was reduced in skeletal muscle of PNS males.

In contrast to the findings in liver, alterations in the expression of genes in subcutaneous adipose tissue were more pronounced in PNS females. Decreased expression of *11βhsd1* mRNA in PNS females would predict decreased local corticosterone regeneration and lower tissue glucocorticoid levels, while decreased expression of *Atgl* and *Dgat2* mRNAs in PNS females indicates decreased lipid turnover. ATGL is regulated by glucocorticoids (Serr *et al*. 2011) and insulin (Yao-Borengasser *et al*. 2011), so altered *Atgl* mRNA expression may be a consequence of decreased tissue glucocorticoid concentrations and/or higher insulin levels. In adipose tissue, PEPCK is a key enzyme in glyceroneogenesis where it increases the incorporation of free fatty acids into triglycerides; thus, the reduction in subcutaneous fat *Pepck* mRNA expression may predict increased free fatty acid release into the circulation (Hanson & Reshef 2003, Olswang *et al*. 2003) and promote insulin resistance (Paolisso *et al*. 1995, Boden & Shulman 2002). It is not clear what triggers reduced *Pepck* gene expression; however, adipose *Pepck* expression is down-regulated by insulin and glucocorticoids (Granner *et al*. 1983, Nechushtan *et al*. 1987). Leptin mRNA expression

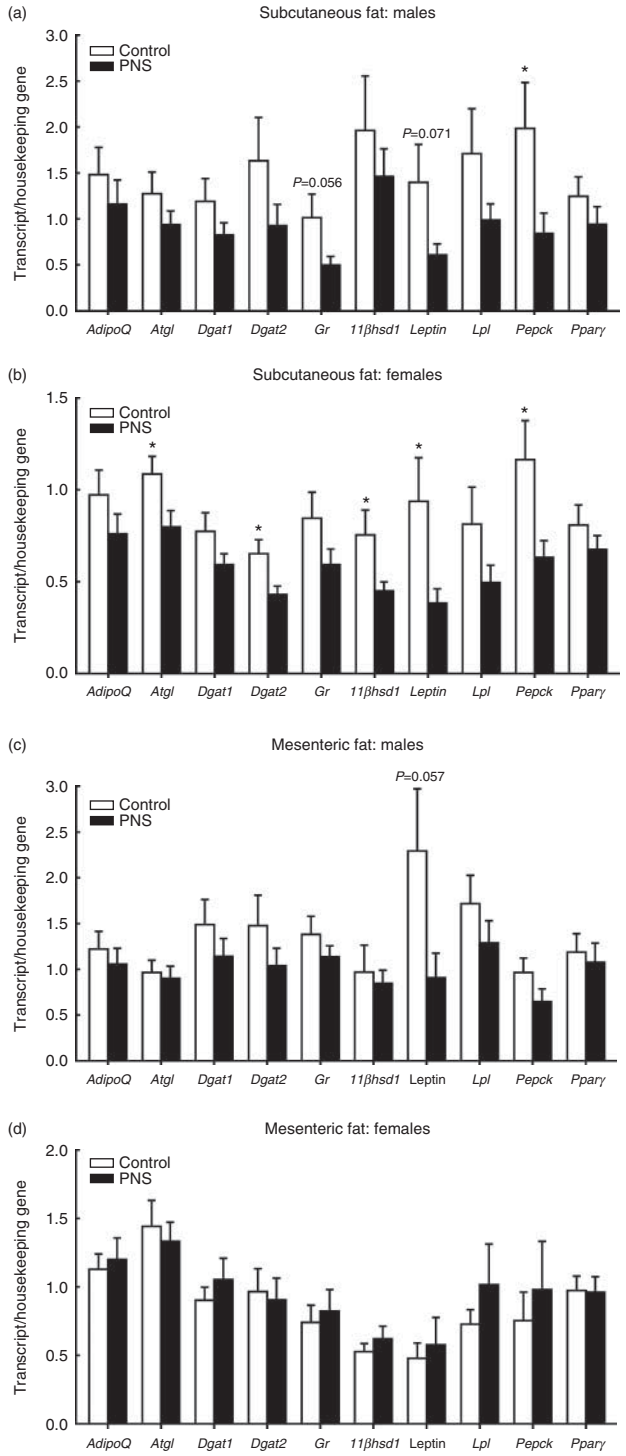


**Figure 6**
Gene expression in subcutaneous and mesenteric fat depots at 6 months. Prenatal stress (PNS) was associated with a significant reduction in subcutaneous fat in level of (a) *Pepck* mRNA in males and (b) 11β-hydroxysteroid dehydrogenase type 1 (*11βhsd1*), phosphoenolpyruvate carboxykinase (*Pepck*), adipose triglyceride lipase (*Atgl*) and diglyceride acyltransferase 2 (*Dgat2*) mRNAs in females. There were no changes in gene expression in mesenteric fat in either (c) males or (d) females. Statistics: $*P<0.05$ vs control group, Student's *t*-test. Data are normalised to the expression of the housekeeping gene cyclophilin A. Data are group means+S.E.M. Rat numbers=10/group. *AdipoQ*, adiponectin; *Atgl*, adipose triglyceride lipase; *Gr*, glucocorticoid receptor; *Lpl*, lipoprotein lipase; *Pparγ*, peroxisome proliferator-activated receptor γ.



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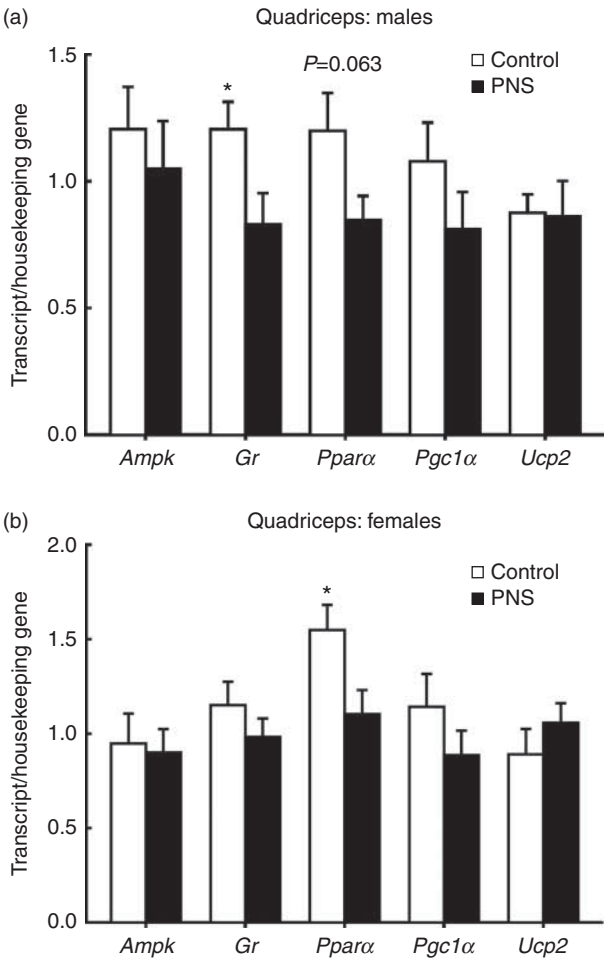


**Figure 7**
Gene expression in quadriceps muscle at 6 months. (a) Prenatal stress (PNS) was associated with reduced level of glucocorticoid receptor (*Gr*) mRNA in males. (b) PNS was associated with reduced level of *Pparα* mRNA in females. Statistics: $*P<0.04$ vs control group, Student's *t*-test. Data are normalised to the mRNA level of the housekeeping gene cyclophilin A. Data are group means+S.E.M. Rat numbers=10/group. *Ampk*, AMP-activated protein kinase; *Pgc1α*, peroxisome proliferator-activated receptor γ coactivator 1α; *Ucp2*, uncoupling protein 2.

was also reduced in subcutaneous fat in PNS females with a similar trend in mesenteric and subcutaneous fat of PNS males. Reduced leptin expression may lead to reduced leptin signalling to the central appetite and metabolism regulating neurones; indeed, developmental programming of leptin signalling is considered to be of importance in the development of the associated metabolic phenotype (Cottrell & Ozanne 2007).

In summary, PNS exposure resulted in effects on glucose–insulin and lipid homoeostasis and on peripheral gene expression in liver, adipose and skeletal muscle in the adult offspring, with particular effects on the expression of genes known to regulate lipid and glucocorticoid metabolism. Importantly, these changes are sex specific. The mechanisms that account for sex-specific programming effects are unclear but may include the effects of sex chromosomes or pre- and/or postnatal changes in sex steroid levels (Gabory *et al.* 2009), and our work further highlights the need for research aimed at understanding early life programming mechanisms in both sexes (Koletzko *et al.* 2011). Additionally, whether a more dramatic 'disease' phenotype would be observed in both male and female PNS offspring with ageing, poor postnatal diet or chronic stress exposure warrants further investigation.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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