Differential effects of prenatal stress and glucocorticoid administration on postnatal growth and glucose metabolism in rats

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Abstract

Glucocorticoid administration during pregnancy programmes cardiovascular and metabolic functions in the adult offspring. Often, the control procedures are stressful *per se* and raise maternal glucocorticoid concentrations. This study compared the effects of maternal injection with dexamethasone (dex, 200 μg/kg) or saline with no treatment from 15 to 20 days of rat pregnancy on offspring growth and glucose metabolism. Near term, maternal corticosterone concentrations were higher in the saline-treated dams and lower in the dex-treated dams relative to untreated animals. In both male and female offspring, growth rate was measured for 14 weeks, and glucose tolerance was assessed between 12 and 13 weeks together with body fat content and plasma concentrations of insulin, leptin, and corticosterone between 14 and 15 weeks. Offspring liver was collected at different ages and was analyzed for glycogen content and gluconeogenic enzyme activity. Compared with untreated animals, both dex and saline treatments altered postnatal growth although adult body weight was unaffected. The two treatments had different effects on adult insulin concentrations and on hepatic glycogen content and gluconeogenic enzyme activities both pre- and postnatally. Relative to untreated animals, adult glucose tolerance was improved by maternal saline injection in males but not in females, while it was impaired in female offspring but not in male offspring of the dex-treated dams. Adult glucose tolerance was related to male body fat content but not to female body fat content. Dex and saline treatments of pregnant rats have differential sex-linked effects on the growth and glucose metabolism of their offspring, which indicates that the programming actions of natural and synthetic glucocorticoids may differ.

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Introduction

Human epidemiological studies have shown that impaired intrauterine growth is associated with an increased risk of cardiovascular, metabolic, and other diseases in later life (Barker 1994). These associations have led to the concept that adult disease can originate *in utero* as a result of developmental programming of key tissues and organ systems during suboptimal intrauterine conditions associated with poor fetal growth (Gluckman *et al*. 2008). Experimentally, prenatal programming of postnatal metabolism has been demonstrated in a number of species using a range of different techniques to induce intrauterine growth restriction (IUGR) including maternal stress and glucocorticoid administration (see McMillen & Robinson 2005, Fowden *et al*. 2006, Seckl 2008). Similarly, in naturally occurring IUGR in polytocous species, low birth weight is associated with adult glucose intolerance and altered fat deposition (Poore & Fowden 2002, 2004). In both naturally occurring and experimentally induced IUGR, the programmed alterations in postnatal glucose handling are associated with functional changes in a range of tissues involved in growth and glucoregulation, including several endocrine systems (see McMillen & Robinson 2005, Fowden *et al*. 2006).

In pregnant rats, maternal treatment with the synthetic glucocorticoid, dexamethasone (dex), via a number of different routes leads to IUGR and glucose intolerance of the adult offspring, accompanied by changes in the liver, fat, and skeletal muscle, and in circulating concentrations of insulin, leptin, and corticosterone in the adults (Muneoka *et al*. 1997, Nyirenda *et al*. 1998, 2006, Smith & Waddell 2000, Sugden *et al*. 2001, Cleasby *et al*. 2003, O'Regan *et al*. 2004, Wyrwoll *et al*. 2006, 2008). Similarly, stresses during rat pregnancy that raise maternal glucocorticoid concentrations, such as restraint, noise, and isolation, induce IUGR and alter glucose handling and hypothalamic–pituitary–adrenal (HPA) axis function in the adult offspring (Barbazanges *et al*. 1996, Vallée *et al*. 1996, Maccari *et al*. 2003, Lesage *et al*. 2004, D'mello & Lin 2006). Indeed, in pregnant rats, procedures such as saline injection, often used as a control in studying prenatal origins of adult disease, lead to elevated maternal and fetal corticosterone concentrations, which may programme tissue development *per se* (Ward & Weisz 1984, Barbazanges *et al*. 1996). Consequently, the stress of injection may mimic,

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in part, the programming effects of synthetic glucocorticoids and may mean that the comparison of offspring from dex- and saline-treated, or vehicle-treated, dams may be examining different types or degrees of intrauterine programming. However, little is known about the effects of commonly used control procedures, such as maternal saline or vehicle injection, on glucose metabolism of their offspring. The current study, therefore, compared the effects of maternal injection with dex or saline with no treatment during rat pregnancy on the growth and glucose metabolism of their male and female offspring.

Materials and Methods

Animals

A total of 32 virgin female Wistar rats aged between 12 and 15 weeks were used (Fig. 1). They were maintained at 22 °C in a 12 h light:12 h darkness cycle and were fed a standard laboratory chow throughout the study (Standard Breeding Diet No. 3, Special Diet Services, Essex, UK). Females were housed overnight with a male Wistar rat (minimum age 15 weeks) and were checked daily for the presence of a copulatory plug. The day after mating was taken as day 0 of pregnancy (term is 21·5 days). Before mating, all females were housed in groups, whereas after mating, they were housed individually to allow the measurement of daily food intake. Dams were weighed on days 0, 15, and 20 of pregnancy.

Experimental procedures

All experimental procedures were carried out under the Animals (Scientific Procedures) Act 1986. On days 15–19 of pregnancy inclusive, 22 dams were injected subcutaneously with either saline (400 μl, 0·9% w/v, $n=11$) or dex (200 μg/kg, dex 21-phosphate sodium salt, Sigma, in 400 μl saline, $n=11$, Fig. 1). Administration of dex to pregnant rats at this dose and stage of gestation is known to programme cardiovascular and metabolic functions in the adult offspring (Nyirenda *et al.* 1998, Cleasby *et al.* 2003, Drake *et al.* 2004, O'Regan *et al.* 2004). Ten dams acted as controls and received no treatment (Fig. 1). Between 0800 and 1000 h on day 20, a subset of each group ($n=6$ control, $n=7$ saline, and $n=7$ dex dams) was anesthetized with a mixture of sodium pentobarbitone and chloral hydrate (equithesin, 0·6 ml/100 g body weight, i.p., and 9·6% pentobarbital sodium, BDH Chemical Ltd, Poole, England; 42·6% chloral hydrate, Sigma–Aldrich Co). Once anesthetized, a ventral incision was made in the abdomen and a 2-ml blood sample was collected from the uterine vein for the measurement of blood glucose and plasma corticosterone concentrations (Fig. 1). Fetuses were removed individually from each horn, weighed, and killed by decapitation immediately after delivery. A small blood sample (<20 μl) was obtained from the severed vessels in the fetal neck for the measurement of blood glucose concentrations. The fetal liver was removed, weighed, and then flash-frozen in liquid nitrogen for subsequent analyses of glycogen content and gluconeogenic enzyme activities (Franko *et al.* 2009). When all fetuses were delivered, the dam was killed with a lethal dose of anesthetic (200 mg/kg sodium pentobarbitone, i.v., Dolethal, Vetoquinol UK Ltd, Buckingham, UK). Maternal blood samples were centrifuged at 4 °C, and the plasma was stored at −20 °C until the measurement of corticosterone concentrations.

The remaining four dams in each of the saline and dex groups received another injection on day 20 and were then allowed to deliver naturally without further treatment,

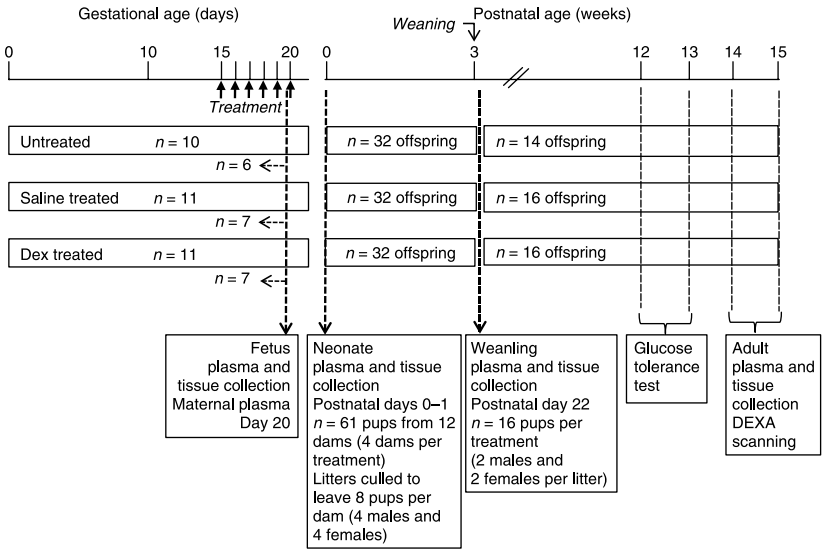

Figure 1 Schematic diagram of the experimental protocol indicating the sequence of experimental procedures and the numbers of dams and offspring involved at each stage of the experiment.

together with the four remaining control dams (Fig. 1). On day 1 of postnatal life, all litters were culled to eight pups so that four females and four males nearest to the median weight for the litter were reared by their mother (Fig. 1). The rest of each litter was killed by decapitation (Fig. 1). A small blood sample (<20 μl) was taken from the severed neck vessels to measure blood glucose, and the liver from culled neonates was collected into liquid nitrogen. Reared pups were weighed weekly until 21 days of postnatal age when they were weaned, and then on day 22, half of each litter (two males and two females) were selected randomly for culling by cervical dislocation. A blood sample (<20 μl) and the liver were collected from each of the culled weanlings ($n=16$ controls, $n=16$ saline, and $n=16$ dex offspring from 4 dams per treatment, Fig. 1). The remaining two males and two females from each litter were individually marked and fed standard laboratory chow (Standard Breeding Diet No. 3, Special Diet Services) until the end of experimental procedures between 14 and 15 weeks of postnatal age (Fig. 1). They were housed in groups by sex with a control, saline-treated individual, and dex-treated individual in each cage. Offspring were weighed weekly from weaning to 14 weeks for the calculation of fractional growth rate (FGR).

Between postnatal weeks 12 and 13, a glucose tolerance test was carried out after an overnight fast ($n=14$ control, $n=16$ saline, and $n=16$ dex from 4 dams per treatment, Fig. 1). Glucose (0·2 g/kg as a 20% solution) was injected intraperitoneally, and blood samples (<20 μl) for the measurement of blood glucose concentrations were collected from a single nick in the tail vein at −15 and 0 min before and at 15, 30, 45, 60, 90, and 120 min after glucose administration. At the end of the glucose tolerance test, the rats were returned to their cages until between 14 and 15 weeks when they were weighed and euthanized by cervical dislocation in the fed state for the collection of blood (2 ml) and liver samples (Fig. 1). After removal of the liver, the fat content of the abdominal region of the carcass was measured using dual emission X-ray absorption (DEXA) scanning (LUNAR PIXImus Densitometer, GE Lunar Corporation, Madison, WI, USA). The scan included peritoneal, omental, mesenteric, perirenal, and subcutaneous fat deposits in the abdominal cavity. Thereafter, the discrete peritoneal and perirenal fat deposits were excised and weighed.

Biochemical analyses

Blood glucose concentrations were measured using a handheld glucometer (Lifespan, Ortho-Clinical Diagnostics, High Wycombe, UK). Concentrations of insulin, leptin, and corticosterone were measured in adult plasma by RIA using commercially available kits (insulin and leptin, Linco Research, Saint Charles, MO, USA; corticosterone, Immu-Chem, Orangeburg, NY, USA). For each hormone, all samples were analyzed in a single assay. The lower limits of sensitivity of the assay for insulin, leptin, and corticosterone were 0·1, 0·06, and 10 ng/ml respectively. The intra-assay coefficient of variation was ≤10% for all three assays. The hepatic glycogen content and activities of glucose-6-phosphatase (G6Pase, EC 3.1.3.9) and phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.49) were measured in the liver from the postnatal offspring and from, at least, four fetuses from each litter (two of each sex) using established methods described previously (Franko *et al*. 2007, 2009). Briefly, G6Pase activity was measured as phosphate production from glucose-6-phosphate, while total hepatic PEPCK activity was estimated by measuring ^{14}C incorporation into malate (Fowden *et al*. 1993). For both gluconeogenic enzymes, tissue was homogenized in ice-cold sucrose (0·25 M) and activity was measured over a 10-min period at 37 °C. Hepatic glycogen content was measured as glucose produced by amyloglucosidase activity in 10 min at 55 °C (Franko *et al*. 2007). The inter-assay coefficients of variation of a fetal liver homogenate in the glycogen, G6Pase, and PEPCK assays were 9, 11·8, and 5% respectively. Hepatic protein content was determined using the Lowry assay. All tissue and plasma analyses were done in duplicate. Measurements of the fat content of the region of interest by DEXA scanning were validated biochemically in ten additional Wistar rats using the chloroform/methanol method of fat extraction (Folch *et al*. 1957). Body fat content measured by DEXA scanning was linearly related to the fat content determined biochemically as follows: $y=0{\cdot}79x+1{\cdot}11$, $n=10$, $r=0{\cdot}955$, and $P<0{\cdot}01$, where y is the percentage fat content measured by DEXA scanning and x is the percentage fat content determined biochemically.

Statistical analyses

Means (±S.E.M.) are presented throughout. Tissue and blood samples were collected at four ages: fetuses, neonates, weanlings, and adults between 14 and 15 weeks of postnatal age. The effect of treatment in the pregnant dams was assessed by one-way ANOVA with the Holm–Sidak *post hoc* test (Sigmastat 3.5; Systat Software Inc., Point Richmond, CA, USA). For the offspring, the effects of treatment and sex were assessed by linear mixed model using the Sidak *post hoc* test using the dam as a subject with each offspring and time, or age, as repeated measures as appropriate (SPSS 16.0, SPSS Inc., Chicago, IL, USA). When sex of the offspring or an interaction between maternal treatment and sex of the offspring was identified as a significant influence, male and female data were analyzed separately by linear mixed model using the Sidak *post hoc* test. Associations between the adult variables were assessed by linear mixed model using generalized estimating equations clustered on the dam to allow for nonindependence of littermates (Stata v10.1, Statacorp., College Station, TX, USA). FGR from birth to weaning and from weaning to 14 weeks was calculated for each animal as weight increment over the entire period expressed as grams gained per week per gram starting weight at birth or weaning respectively.

Results

Pregnancy outcome

Dam weight was not significantly different between the groups on days 0 and 15 of pregnancy, but by day 20, it was significantly lower in the dex-treated animals than in the control animals (Table 1). Maternal weight gain was, therefore, similar in the three groups from 0 to 15 days, but relative to the controls, it was significantly less in the saline- and dex-treated groups during the treatment period (Table 1). Weight gain over the treatment period was also significantly less in the dex-treated dams than in the saline-treated dams (Table 1). Maternal food intake did not differ between groups before or during the treatment period (Table 1). Litter size was also similar in the three groups (Table 1). On day 20, maternal concentrations of plasma corticosterone were highest in dams treated with saline and lowest in those receiving dex (Table 1). There were no significant differences in maternal blood glucose concentrations with treatment on day 20 (Table 1).

Offspring growth and morphometry

Compared with the controls, dex treatment reduced body weight of the offspring *in utero*, at birth, during suckling, and for 4 weeks after weaning ($P<0{\cdot}01$, all cases, Fig. 2). Body weight of offspring from the saline-treated dams was similar to that of controls *in utero*, intermediate between the values of the control and dex-treated groups at birth (Fig. 2A), and significantly less than control values during suckling and from weaning to 7 weeks ($P<0{\cdot}01$, all cases, Fig. 2B and C).

Table 1 Mean (±S.E.M.) food intakes and absolute and incremental (Δ) body weights at different periods of pregnancy, litter size, and concentrations of blood glucose and plasma corticosterone on day 20 of pregnancy in untreated, control rat dams ($n=10$ dams or for corticosterone and glucose $n=6$ dams) and those treated with saline or dexamethasone from days 15 to 19 of pregnancy ($n=11$ dams or for corticosterone and glucose $n=7$ dams in each treated group)

	Control	Saline	Dexamethasone
Food intake (g/day per rat)			
1–10 days	21·1±0·6	20·4±0·9	21·6±1·0
11–15 days	25·0±1·1	22·5±1·1	25·3±1·0
16–20 days	24·9±0·7	24·2±1·1	24·7±1·2
Body weight (g)			
Day 0	260±11	258±10	268±10
Day 15	312±11	305±12	315±13
Day 20	388±10^{a}	364±14^{a,b}	347±15^{b}
Δ 0–15 days	52±6	47±3	46±4
Δ 15–20 days	76±8^{a}	59±4^{b}	31±4^{c}
Litter size	13·4±0·7	13·9±0·5	14·3±0·7
Corticosterone (ng/ml)	652±59^{a}	818±52^{b}	465±25^{c}
Glucose (mmol/l)	5·00±0·61	4·90±0·9	4·33±0·42

Values within rows with different letters as superscripts are significantly different from each other ($P<0{\cdot}01$, one-way ANOVA).

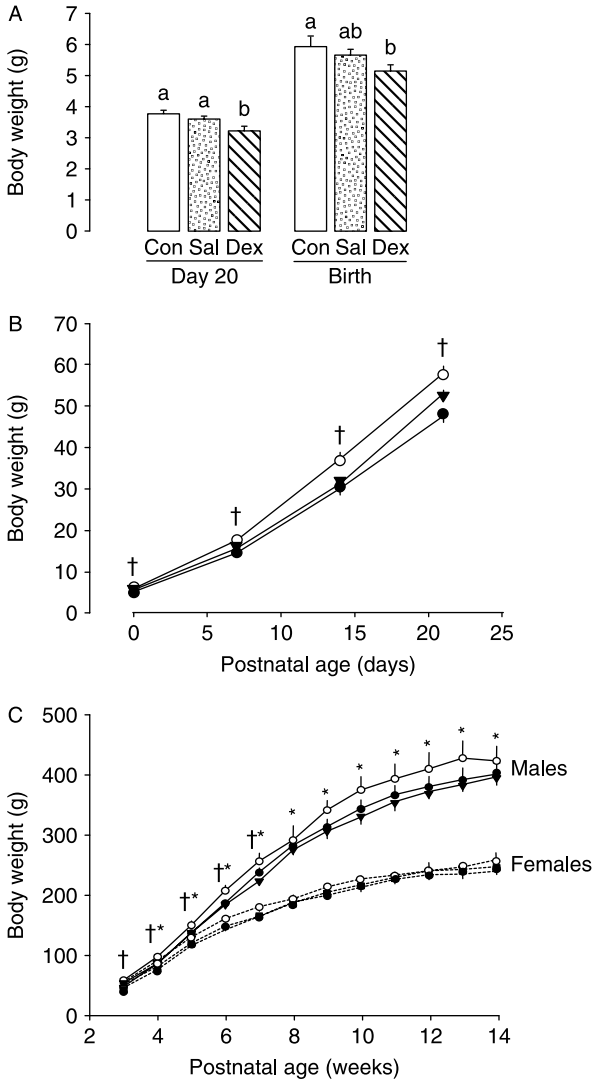

Figure 2 Mean (±S.E.M.) body weights of the offspring of untreated, control rat dams, and dams treated with saline or dexamethasone from day 15 to day 19 (fetuses) or day 20 of pregnancy (all other offspring) (A) on day 20 of pregnancy and at birth (Con, open columns; Sal, stippled columns; Dex, striped columns $n=4$–7 l per treatment at each age), (B) during suckling from birth to weaning (Con, open circles; Sal, triangles; Dex, filled circles, $n=4$ l per treatment group with four male and four female pups in each litter), and (C) in males (solid lines) and females (dashed lines) from weaning to 14 weeks of postnatal age (Con, open circles, $n=7$ males and $n=7$ females from four dams; Sal, triangles, $n=8$ males and $n=8$ females from four dams; Dex, filled circles, $n=8$ males and $n=8$ females from four dams). At each age in (A), columns with different letters are significantly different from each other ($P<0{\cdot}01$), while columns sharing common letters are not significantly different from each other ($P>0{\cdot}05$, linear mixed model). In (B) and (C), significant effects of maternal treatment ($^{\dagger}P<0{\cdot}01$) and/or sex of the offspring ($^{*}P<0{\cdot}01$) are shown by linear mixed model. There were no significant effects of sex of the offspring on body weight in (A) or (B).

By postnatal week 8, maternal treatment no longer had a significant influence on offspring body weight (Fig. 2C). Body weights of male and female offspring from the saline- and dex-treated dams, although still numerically smaller, were not significantly different from those of the controls at the time of the glucose tolerance test or at tissue collection (Table 2). Sex of the offspring had no significant effect on the body weight of fetal, newborn, or weanling pups ($P>0{\cdot}05$, all cases). However, by postnatal week 4, males weighed more than females and remained heavier thereafter (Fig. 2C). There was no interaction between maternal treatment and sex of the offspring in determining body weight at any of the ages studied ($P>0{\cdot}05$, all cases, Fig. 2C, Table 2).

FGR, calculated as weight increment from birth to weaning, was unaffected by maternal treatment or sex of the offspring ($P>0{\cdot}05$, both cases, Table 2). In contrast, FGR from weaning to 14 weeks was influenced by both maternal treatment and sex of the offspring ($P<0{\cdot}05$, both cases, Table 2). Over this period, FGR was significantly less in females than in males (Table 2). In males, postweaning FGR was higher in offspring of the dex-treated dams than in the other groups with no significant difference between the control and saline-treated groups (Table 2). In females, maternal treatment had no effect on the postweaning FGR (Table 2).

In adults between 14 and 15 weeks, the fat content of the abdominal region measured by DEXA scanning was influenced by both maternal treatment ($P<0{\cdot}02$) and sex of the offspring ($P<0{\cdot}001$). Females had less fat than males irrespective of treatment (Table 2). In the males, fat content was significantly lower in offspring of the saline-treated animals than in the other groups ($P<0{\cdot}05$, Table 2). A similar trend was observed in the females, but this did not reach statistical significance ($P>0{\cdot}05$, Table 2). When perirenal and peritoneal fat contents were calculated gravimetrically, the values were influenced by maternal treatment ($P<0{\cdot}02$) and sex of the offspring ($P<0{\cdot}05$), with significantly lower values in the dex-treated group than in the control group ($P<0{\cdot}02$). This effect of dex appeared to be more pronounced in males than in females (Table 2). When data from all adult animals were combined, there were significant positive relationships between the DEXA percentage fat content and postweaning FGR ($P<0{\cdot}001$, $n=46$ offspring from 12 dams) when differences in current weight were taken into account. Percentage fat content calculated gravimetrically was not related to postweaning FGR ($P>0{\cdot}05$, $n=46$ offspring from 12 dams).

Plasma hormone concentrations

Between 14 and 15 weeks, plasma leptin concentrations were significantly lower, while plasma corticosterone concentrations were significantly higher in females than in males irrespective of treatment (Table 2). Neither of these hormone concentrations was affected by maternal treatment (Table 2). In contrast, plasma insulin concentrations were influenced by both maternal treatment and sex of the offspring with lower values in females than in males (Table 2). Insulin levels were significantly higher in the saline-treated group than in the dex-treated group with intermediate values in the controls ($P<0{\cdot}02$, Table 2). When data from all adult offspring were combined, plasma leptin concentrations were positively related to DEXA fat content ($P<0{\cdot}016$, $n=32$ offspring from 11 dams). Conversely, plasma corticosterone concentrations were inversely related to DEXA fat content ($P<0{\cdot}012$, $n=40$ offspring from 12 dams). In contrast, insulin concentrations were unrelated to DEXA fat content ($P>0{\cdot}05$), but were positively related to postweaning FGR ($P<0{\cdot}025$, $n=35$ offspring from 12 dams). No significant relationships were observed between any of these hormone concentrations and percentage fat content calculated gravimetrically ($P>0{\cdot}05$, all cases). Nor were any of these hormone concentrations related to fat content (DEXA or excised) when males and females were considered separately ($P>0{\cdot}05$, all cases, $n=16$–20 offspring from 12 dams).

Glucose metabolism

Glucose concentrations At all ages studied, blood glucose concentrations in the fed state were unaffected by maternal treatment or sex of the offspring ($P>0{\cdot}05$). The mean blood glucose concentrations in the fetuses, neonates, and weanlings were $4{\cdot}3\pm0{\cdot}2$ mmol/l ($n=19$ litters), $3{\cdot}9\pm0{\cdot}2$ mmol/l ($n=12$ litters), and $8{\cdot}9\pm0{\cdot}2$ mmol/l ($n=43$ pups from 12 dams) respectively. Adult glucose concentrations in the fasted state were significantly less than those in the fed state and were also unaffected by maternal treatment or sex of the offspring (Table 2).

Hepatic glycogen content and gluconeogenic enzyme activities Hepatic glycogen content and activities of G6Pase and PEPCK were not affected by sex of the offspring at any of the ages studied ($P>0{\cdot}05$, all cases). Maternal dex treatment influenced hepatic glycogen content in the fetuses but not in the other groups of offspring (Fig. 3A). In fetuses, hepatic glycogen content was highest in the dex-treated group and lowest in the controls with intermediate values in the saline-treated group (Fig. 3A). A similar pattern of hepatic G6Pase activity with maternal treatment was observed in the fetuses (Fig. 3B). However, G6Pase activity was unaffected by maternal treatment at any of the postnatal ages studied (Fig. 3B). In contrast, hepatic PEPCK activity differed with maternal treatment at all ages studied, although the specific effects of treatment varied at each age (Fig. 3C). In fetuses, hepatic PEPCK activity was significantly higher in the treated group than in the untreated group, but it was not significantly different between saline and dex treatments (Fig. 3C). At birth, the saline-treated group had a lower PEPCK activity than the other two groups, while in weanlings, PEPCK activity was lowest in controls and highest in the dex-treated group with intermediate values in the saline-treated group (Fig. 3C). In adults, hepatic PEPCK

Table 2 Mean (±S.E.M.) values of body weight at the time of the glucose tolerance test and tissue collection, fractional growth rate (FGR) from birth to weaning and weaning to 14 weeks, abdominal fat content measured by dual emission X-ray absorption (DEXA) scanning and gravimetrically, and of plasma concentrations of leptin, insulin, and corticosterone at the time of tissue collection together with blood glucose concentrations in the fed state and after overnight fasting (basal) together with the maximum (max) and maximum increment (Δ max) in blood glucose levels during the glucose tolerance test in male and female adult offspring of untreated, control rat dams, and those treated with saline or dex from days 15 to 20 of pregnancy (n=5–8 males and females offspring from four dams in each treatment group)

	Treatment								
	Control		Saline		Dexamethasone		**Statistical analysis** (P value)[†]		
	Male	Female	Male	Female	Male	Female	Treatment	Sex	Interaction
Body weight (g)									
At glucose tolerance	394±19	230±6	368±7	225±5	376±19	227±7	NS	<0·001	NS
At tissue collection	430±18	258±7	403±8	249±5	413±21	248±8	NS	<0·01	NS
FGR (g/week per g)									
Birth – weaning	2·93±0·11	2·92±0·06	2·80±0·08	2·79±0·06	2·80±0·22	2·77±0·21	NS	NS	NS
Weaning – 14 weeks	0·576±0·030[a]	0·336±0·016[a]	0·584±0·014[a]	0·346±0·008[a]	0·690±0·025[b]	0·374±0·014[a]	0·040	<0·001	NS
Fat content %									
DEXA scanning	29·2±1·2[a]	25·2±2·0[a]	25·0±0·9[b]	22·2±1·1[a]	29·5±0·7[a]	24·7±0·5[a]	0·013	<0·001	NS
Gravimetric[‡]	2·9±0·2[a]	2·5±0·3[a]	2·4±0·2[a,b]	2·3±0·2[a]	2·1±0·1[b]	2·0±0·2[a]	0·015	0·045	NS
Leptin (ng/ml)	6·75±1·17	3·21±0·44	6·41±1·15	2·71±0·75	8·59±0·44	2·93±0·45	NS	<0·001	NS
Insulin (ng/ml)	1·86±0·45[a,b]	0·78±0·27[a]	2·49±0·40[a]	1·49±0·40[a]	1·03±0·13[b]	1·16±0·15[a]	0·041	0·006	NS
Corticosterone (ng/ml)	522±43	530±112	387±21	608±67	360±53	591±91	NS	0·049	NS
Glucose (mmol/l)									
Fed	6·4±0·1	6·6±0·2	6·4±0·1	6·5±0·2	6·7±0·3	6·5±0·1	NS	NS	NS
Fasted – Basal	4·8±0·2*	4·6±0·1*	5·0±0·1*	4·7±0·2*	4·6±0·1*	4·8±0·1*	NS	NS	NS
Max	12·9±0·4[a]	11·8±0·8[a]	12·9±0·8[a]	12·5±0·9[a,b]	13·0±0·6[a]	15·5±1·1[b]	0·49	NS	NS
Δ Max	8·0±0·9[a]	7·0±0·8[a]	7·9±0·7[a]	7·8±0·8[a,b]	8·4±0·6[a]	10·6±1·3[b]	0·043	NS	NS

For each sex within a row, values with different letters as superscripts are significantly different from each other (P<0·05, linear mixed model). *Significantly less than the corresponding value in the fed state (P<0·01, paired t-test).
[†]Linear mixed model (NS, not significant).
[‡]Peritoneal and perirenal fat deposits.

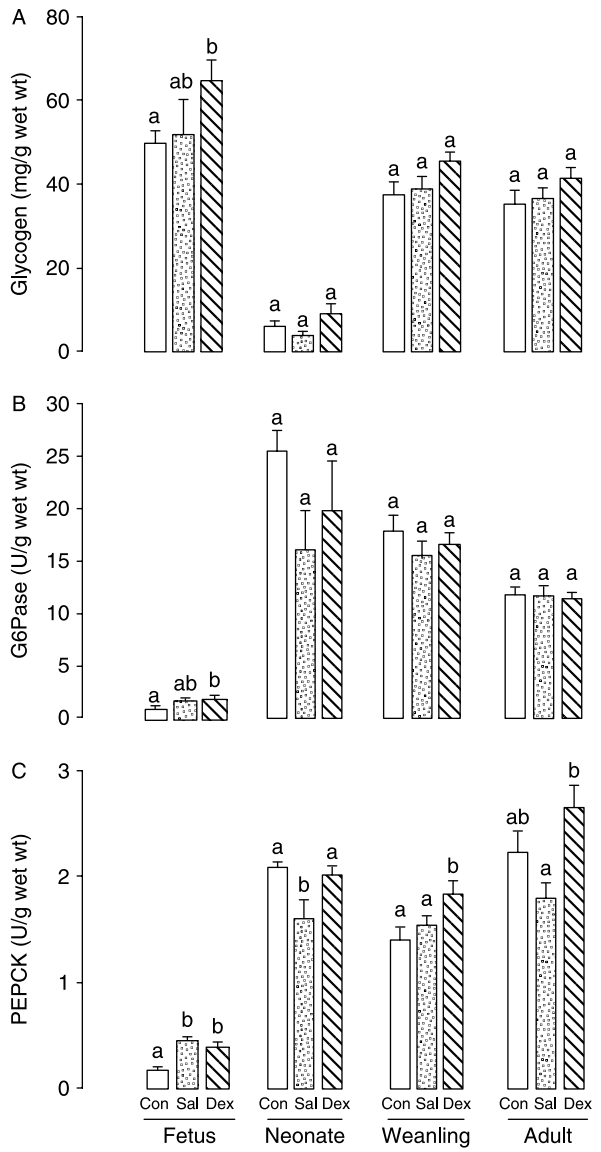

Figure 3 Mean (±S.E.M.) values of (A) glycogen content, (B) glucose-6-phosphatase (G6Pase) activity, and (C) phosphoenolpyruvate carboxykinase (PEPCK) activity of liver from the fetuses, neonates, weanlings, and adult offspring of untreated control dams (Con, open columns) or dams treated with saline (Sal, stippled columns) or dexamethasone (Dex, striped columns) from day 15 to day 19 (fetuses) or day 20 of pregnancy (all other offspring). For the fetuses and neonates, $n=4$–7 l for each treatment group. For weanlings and adults, $n=14$–16 individual offspring per treatment group with four dams per treatment. At each age, columns with different letters are significantly different from each other ($P<0{\cdot}02$), while columns sharing common letters are not significantly different from each other ($P>0{\cdot}05$, linear mixed model). In (A), (B), and (C), there were no significant effects of sex of the offspring at any age studied ($P>0{\cdot}05$, linear mixed model).

activity was higher in the dex-treated group than in the saline-treated group, but neither of these values was significantly different from that in the controls (Fig. 3C). At all ages studied, hepatic protein content was unaffected by either maternal treatment or sex of the offspring ($P>0{\cdot}05$, data not shown).

Adult glucose tolerance Adult glucose tolerance was affected by maternal treatment ($P<0{\cdot}003$) but not by sex of the offspring ($P>0{\cdot}05$, Fig. 4). In all animals irrespective of sex, maternal treatment influenced the glucose concentrations observed 15, 30, and 45 min after glucose administration (Fig. 4Ai). The maximum concentration and increment in blood glucose differed with maternal treatment but not with sex of the offspring (Table 2). For all animals, the area under the glucose curve (AUGC) was smallest for the saline-treated group and significantly less in the saline-treated animals than in the dex-treated animals with intermediate values in the controls (Fig. 4Aii). However, there was an interaction between maternal treatment and sex of the offspring in determining the time profile of the glucose concentration and the areas under the curve such that the specific effects of treatment depended on sex of the offspring ($P<0{\cdot}05$, all cases, Table 2). In males, glucose levels were significantly different with treatment 30 and 45 min after glucose administration, and AUGC was significantly less in the saline-treated group than in either of the other two groups (Fig. 4Bi and ii). No significant difference was observed in AUGC between male offspring of the control and dex-treated dams (Fig. 4Bii). There were also no significant differences in the maximum concentration or increment in blood glucose with maternal treatment in male offspring (Table 2). In contrast, in females, blood glucose concentrations were significantly different with treatment at 15 and 30 min, and AUGC was significantly greater in the dex-treated group than in the other two groups (Fig. 4Ci and ii). Unlike in males, there was no significant difference in AUGC between female offspring of the control and saline-treated dams (Fig. 4Cii). The maximum concentrations and increment in blood glucose in females were also significantly higher in the dex-treated group than in the control group in contrast to the males (Table 2).

There were no significant correlations between AUGC and current body weight, postweaning FGR, body fat content (DEXA or excised), hepatic glycogen content, or activities of G6Pase and PEPCK when data from all the adults were combined ($P>0{\cdot}05$, all cases). In males, AUGC was positively correlated to DEXA fat content ($P<0{\cdot}01$, $n=23$ offspring from 11 dams), but not to any of the other variables ($P>0{\cdot}05$, all other cases). In females, AUGC was positively correlated to postweaning FGR ($P<0{\cdot}041$, $n=22$ offspring from 12 dams) and was unrelated to any of the other variables ($P>0{\cdot}05$, all other cases).

Discussion

The results demonstrate that dex treatment and the stress associated with saline injection of rats during late pregnancy

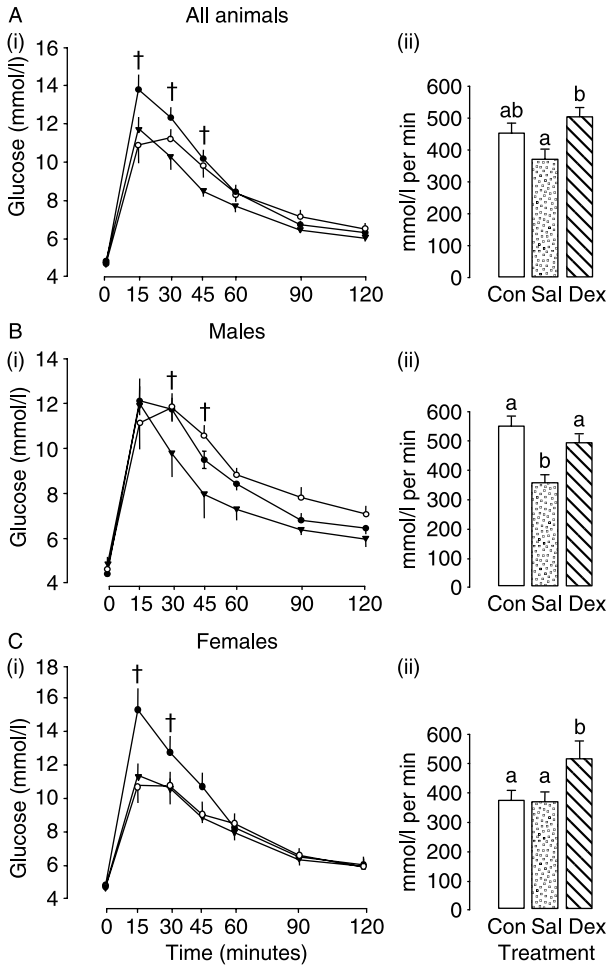

Figure 4 Mean (±S.E.M.) values of (i) blood glucose concentrations with respect to time and (ii) the area under the glucose curve for (A) all adult offspring irrespective of sex, (B) male offspring, and (C) female offspring of untreated control dams (Con, open circles and open columns, $n=7$ males and $n=7$ females from four dams) and of dams treated with saline (Sal, triangles and stippled columns, $n=8$ males and $n=8$ males females from four dams) or dexamethasone (Dex, closed circles and striped columns, $n=8$ males and $n=8$ females from four dams) from day 15 to day 20 of pregnancy. For (i), significant effect of maternal treatment $^{\dagger}P<0{\cdot}01$ (linear mixed model with maternal treatment, sex of the offspring, and time as factors, where appropriate). For (ii), columns with different letters are significantly different from each other ($P<0{\cdot}01$), while columns sharing common letters are not significantly different from each other ($P>0{\cdot}05$, linear mixed model).

have differential effects on the growth and glucose metabolism of their offspring. These effects depend, in part, on the age and sex of the offspring and are related to fat deposition and the plasma concentrations of insulin, leptin, and corticosterone in the adults. Compared with untreated controls, maternal dex treatment reduced offspring body weight *in utero* and at birth, whereas the stress associated with maternal saline injection did not. Both treatments reduced pup body weight during suckling and for 4 weeks after weaning, but only dex treatment led to a significantly higher postweaning FGR. The two treatments also had different effects on hepatic glucogenic capacity, particularly in fetal and early postnatal life. Furthermore, relative to controls, adult glucose tolerance was improved by prenatal stress in the males but not in the females, while it was impaired in the female offspring but not in the male offspring of the dex-treated dams. Since maternal corticosterone concentrations were elevated in the saline-injected dams relative to the other two groups, the current findings suggest that overexposure to both natural and synthetic glucocorticoids has programming effects *in utero*, but that their specific actions differ in a sex-linked manner in the rats. Thus, natural glucocorticoids may not entirely mimic the programming effects of synthetic glucocorticoids as shown previously in adult sheep over-exposed to glucocorticoids *in utero* during early gestation (Moritz *et al.* 2005).

Growth and body composition

Dex administration reduced maternal weight gain during the period of treatment and restricted prenatal and early postnatal growth of the pups in line with previous findings in rats treated with glucocorticoids during late pregnancy by injection, ingestion, and continuous infusion (Smith & Waddell 2000, Sugden *et al.* 2001, Swolin-Eide *et al.* 2002, Scheepens *et al.* 2003, O'Regan *et al.* 2004, Woods 2006). The reduced maternal weight gain during both dex and saline treatments in the current study was due, in part, to the reduced weight of the fetuses and placentas (not shown), and may also have reflected the differences in concentration of corticosterone and other stress hormones associated with injection, such as the catecholamines. It was not related to decreased food intake. However, reduced growth of maternal tissues, such as the mammary glands, may have compromised lactation relative to untreated dams, leading to lower pup weights during suckling. When the lactational constraint was lifted at weaning, there was a period of catch-up growth in both treated groups so that body weight was not significantly different between offspring of treated and untreated dams from 8 weeks of postnatal age. Similar catch-up growth after weaning has been observed in previous studies of maternal restraint stress and dex treatment during rat pregnancy (O'Regan *et al.* 2004, 2008, D'mello & Lin 2006). Indeed, the degree of catch-up growth appears to depend on litter size and is complete when litters are culled to eight pups, as in the present study, but not with larger pup numbers (O'Regan *et al.* 2004, 2008, Wyrwoll *et al.* 2006, 2008). Taken together, these observations suggest that nutritional constraint during suckling may contribute to the adult metabolic outcomes associated with prenatal stress and dex exposure.

The changes in prenatal and early postnatal growth induced by maternal dex and saline treatments were accompanied by altered fat distribution in the adult offspring. In the dex-treated group, the amount of excised peritoneal and perirenal fat relative to total body weight was reduced, particularly in

the males, consistent with the reduction in epididymal fat mass observed previously in adult males exposed to dex *in utero* (Sugden *et al.* 2001, Cleasby *et al.* 2003). However, relative to untreated controls, there was no apparent effect of dex treatment on fat content of the abdominal region measured by DEXA scanning. The disparity between the fat content measured gravimetrically and that measured by DEXA scanning in the present study suggests that fat distribution in the adult offspring is altered by maternal dex treatment with a decrease in peritoneal and perirenal fat and an increase in subcutaneous and other abdominal fat deposits relative to untreated controls. In contrast, prenatal stress induced by maternal saline injection reduced adult DEXA fat content compared with controls in parallel with combined peritoneal and perirenal fat weight, again to a greater extent in males than in females. Offspring of the saline-treated dams, therefore, appear to have a more proportionate reduction in adiposity than that observed in the dex-treated group. However, there was no influence of maternal treatment on plasma leptin concentrations in the adult offspring, despite the differences in fat deposition and the positive correlation observed between DEXA fat content and plasma leptin levels when all the data were combined regardless of maternal treatment or sex of the offspring. These findings are consistent with previous observations that postnatal hyperleptinemia does not develop in rats exposed prenatally to dex until at least 3 months after birth irrespective of the maternal route of steroid administration (Sugden *et al.* 2001, Cleasby *et al.* 2003, Wyrwoll *et al.* 2006). Differences in adult body fat content and distribution programmed *in utero* may, therefore, precede changes in circulating leptin concentrations in the rats.

The explanation for the greater sensitivity of males than of females to treatment-induced changes in fat deposition remains unclear, but it may be related to the higher fat content of the males overall. The percentage fat content of the adult male and female rats measured by DEXA scanning in the current study was similar to those reported previously for 6-month-old Wistar rats using the same methodology (Wyrwoll *et al.* 2006). When all the current data were combined, fat content measured by DEXA scanning was inversely related to the plasma corticosterone concentrations. Since corticosterone tends to mobilize fat stores during stressful conditions (Bouchard *et al.* 1993), males may have more fat than females between 14 and 15 weeks of age because they have lower corticosterone concentrations. Males also had higher levels of insulin than females in the present study, which would tend to favor fat deposition, although insulin concentrations were not directly correlated to the DEXA fat content of the adult offspring in either sex alone or when the data from both sexes were combined. The higher concentrations of insulin and leptin and lower concentrations of corticosterone observed in males than in females between 14 and 15 weeks in the present study are consistent with previous findings of adult rats at older ages (Sugden *et al.* 2001, Wyrwoll *et al.* 2006, 2008).

Hepatic glucogenic capacity

Both maternal treatments altered the hepatic glucogenic capacity of male and female offspring relative to untreated controls. The most pronounced effects of treatment were observed in the fetuses, which suggest that hepatic glucogenic capacity *in utero* is glucocorticoid sensitive in rats as occurs in other species near term (Fowden *et al.* 1998). At birth, hepatic glycogen content and G6Pase activity were similar in the three groups of neonates, while hepatic PEPCK activity was lower in the newborn pups of the saline-treated dams than in the other two groups of neonates. In the saline-treated group, daily exposure to raised corticosterone concentrations via a maternal stress response to injection may have delayed the activation of the fetal HPA axis. In contrast, in the dex-treated group, withdrawal of the more potent synthetic steroid may have lifted negative feedback on the maternal and fetal HPA axes and allowed a rapid rebound in natural corticosterone concentrations (Fowden *et al.* 1998). At weaning, the effects of maternal treatment with dex, but not with saline, were still evident with raised PEPCK activity, but by between 14 and 15 weeks, neither treatment had a significant influence on the hepatic glucogenic capacity relative to untreated controls. These observations suggest that changes in PEPCK activity programmed *in utero* are more evident at times of major changes in nutrition at birth and weaning. However, hepatic PEPCK activity was higher in the adult offspring of the dex-treated dams than in those of the saline-treated dams as reported previously for offspring of dex- and vehicle-injected dams (Nyirenda *et al.* 1998, 2006). This glucocorticoid programmed upregulation of adult hepatic PEPCK activity is associated with increased expression of hepatocyte nuclear factor 4, a known transcription factor for the *Pck2* promoter (Nyirenda *et al.* 2006). In the current study, the difference in hepatic PEPCK activity between offspring of the saline- and dex-treated dams was observed in both adult males and females, whereas previously, this effect was observed only in males (Nyirenda *et al.* 1998, O'Regan *et al.* 2004). This discrepancy between studies may be due to differences in offspring age or dose of dex administered to their dams (Nyirenda *et al.* 1998, 2006, O'Regan *et al.* 2004). Overall, the current findings suggest that the hepatic glycogenolytic and gluconeogenic pathways have differential sensitivities to programming *in utero*, and that hepatic PEPCK activity is particularly prone to control by the developmental environment.

Glucose metabolism

Neither maternal treatment affected basal glucose concentrations at any age studied. In previous studies, maternal restraint stress has been shown to elevate fasting glucose concentrations in adult male offspring at 6 and 24 months but not at 3–4 months of postnatal age (Vallée *et al.* 1996, Lesage *et al.* 2004, D'mello & Lin 2006). Similarly, maternal dex treatment is associated with fasting hyperglycemia in

6-month-old male offspring in some but not in all previous studies depending on the route of maternal administration (Sugden *et al.* 2001, Drake *et al.* 2004, O'Regan *et al.* 2004). In the current study, both treatments influenced glucose tolerance of the adult offspring, although in different ways depending on sex of the offspring. In males, maternal saline injection improved glucose tolerance, measured as AUGC, while maternal dex treatment had little apparent effect on this measurement relative to untreated controls. The differing AUGC with treatment was largely due to differences in glucose clearance as the peak glucose concentration was similar in the three male groups. The cause of these differences in clearance remains unclear, but glucose tolerance was best in the saline-treated group, which had the highest plasma insulin concentration. There was also a positive correlation between male AUGC and DEXA fat content, which suggests that adipose tissue-derived factors reducing insulin sensitivity may also have contributed to the relative glucose intolerance of the fatter male groups (Stocker *et al.* 2005). In contrast, female glucose tolerance was unaffected by maternal saline injection, but it was impaired by dex treatment relative to untreated controls. The difference between female AUCG with treatment was primarily due to increased peak glucose concentrations in the dex-treated group. Since female fat content was unaffected by treatment, the glucose intolerance of females that were exposed to dex *in utero* may reflect poor first-phase insulin secretion or an increased rate of glucose absorption from the peritoneal cavity relative to the other groups. In addition, there may be sex-linked differences in the response of glucoregulatory hormones, such as insulin, corticosterone, and catecholamines, to i.p. injection of glucose, which contribute to the observed differences in adult glucose tolerance with maternal treatment.

In the present study, glucose tolerance was worse in the dex-treated group than in the saline-treated group in both sexes. The reason for this treatment difference appeared to depend on sex of the offspring with improved glucose clearance in the saline-treated group of males but impaired glucose tolerance in the dex-treated group of females relative to the untreated controls. In addition, hepatic PEPCK activity was highest in adult offspring of the dex-treated dams, irrespective of sex. Using an oral glucose tolerance test, a similar impairment of glucose tolerance coupled with high hepatic PEPCK activities has been observed in 6-month-old male offspring but not in female offspring of rats given dex by the same route for the same period of gestation as in the current study compared with vehicle-treated controls (O'Regan *et al.* 2004). The reasons for the differing responses of adult females prenatally exposed to dex between the current and earlier studies are unclear, but they may be related to differences in dex dose, vehicle composition, route of glucose administration, stage of estrous cycle, or offspring age at study (Nyirenda *et al.* 1998, O'Regan *et al.* 2004). However, taken together, these studies suggest that enhanced hepatic glucose production may also be a contributory factor to the glucose intolerance of adult rats prenatally exposed to dex (Seckl 2008). Abnormalities in hepatic glucose production associated with elevated PEPCK activity have also been observed in male offspring of rats that were deprived of protein during pregnancy (Burns *et al.* 1997, Desai *et al.* 1997).

Overall, the differences in intrauterine programming observed between maternal dex and saline treatments appear to be related, in part, to differences in body weight in fetal and early postnatal life and to altered fat content and distribution in the adult offspring. These differences in programming have implications for evaluating studies using control procedures that raise endogenous glucocorticoid concentrations. This may explain, in part, the differences in glucocorticocoid programming observed in previous studies using differing routes of steroid administration and, hence, control procedures (Sugden *et al.* 2001, Cleasby *et al.* 2003, Wyrwoll *et al.* 2006). However, since glucocorticoids alter prenatal development of many tissues involved in postnatal glucoregulation (Fowden *et al.* 1998), these hormones may be a common factor linking poor fetal growth during suboptimal conditions to developmental programming of postnatal glucose metabolism, particularly as many of the natural and experimental conditions leading to IUGR increase glucocorticoid exposure *in utero*.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of the research reported.

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