Adrenal steroidogenesis following prenatal dexamethasone exposure in the spiny mouse

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Abstract

Antenatal stress disturbs the development of the fetal hypothalamic–pituitary–adrenal axis and adrenal steroidogenesis. We investigated the effect of brief maternal exposure to high glucocorticoids (dexamethasone (DEX)) at mid- and late-pregnancy on adrenal structure and production of steroids in spiny mouse. Pregnant spiny mice were treated for 60 h with 125 µg/kg DEX or saline s.c. by osmotic minipump at day 20 (0.5) or 30 (0.75) of gestation. Immunohistochemical expression of steroidogenic acute regulatory-protein (StAR), 3β-hydroxysteroid dehydrogenase (3βHSD), 17-hydroxylase, 17-20lyase (P450C17), and cytochrome b5 (CYTB5) was determined in adrenals on postnatal (P) day 170 ± 20. DHEA, testosterone, and cortisol were measured by RIA. Maternal DEX at 20 days significantly reduced the expression of STAR, P450C17 (CYP17A1), and CYTB5 in the adrenal zona reticularis (ZR) of adult offspring, with greater change in male vs female offspring ($P < 0.05$). Plasma DHEA was decreased in male offspring from DEX-treated (6.84 ± 1.24 ng/ml) vs saline-treated (13 ± 0.06 ng/ml; $P = 0.01$) dams, and the DHEA:cortisol ratio was lower in males ($P < 0.05$). Testosterone levels increased in male offspring from DEX (266.03 ± 50.75 pg/ml) vs saline (83.47 ± 32.3 pg/ml, $P < 0.05$)-treated dams. DEX treatment at 0.75 gestation had no significant effect on any parameters measured. This study shows that brief exposure to excess glucocorticoid has long-term impacts on the ZR and adrenal steroidogenesis, affecting the secretion of DHEA and testosterone in male offspring, an effect produced at 0.5 but not at 0.75 gestation. DHEA is important for brain development, and its suppression in adult life might contribute to the neurobehavioral pathologies that can arise after illness and stress during pregnancy.

Key Words

- DHEA
- glucocorticoids
- prenatal stress
- adrenal cortex
- P450C17

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Introduction

Epidemiological data suggests that fetal development is affected by a number of stresses during gestation, which can lead to alterations in the regulation of stress-sensitive

physiology into adult life (Adams *et al.* 1993, Brosnan 2001, Goodyer *et al.* 2001, Cotter & Pariante 2002, Anisman & Merali 2003, Barker 2004). This developmental

'programming' of physiological, endocrine, and behavioral functions during pregnancy is thought to be mediated by maternal glucocorticoids. At high concentrations, the free fraction of circulating maternal glucocorticoids can cross the placental and fetal blood-brain barriers (Zarrow *et al.* 1970, Herbert 1998), where they affect fetal hypothalamic-pituitary-adrenal (HPA) activity and modify the development of several organ systems, particularly the brain. One outcome of maternal physical or psychological illness during pregnancy is an increased risk of neurodevelopmental alterations in the fetus, which can lead to several psychiatric illnesses postnatally, including mood disorders and schizophrenia (Hammen *et al.* 1987, Mednick *et al.* 1988, Larsson *et al.* 2005).

A large body of evidence suggests that chronic maternal stress may increase the incidence of preterm birth, developmental delays, and psychiatric and behavioral abnormalities in children. Because increased secretion of cortisol is a common feature of illness and stress, this steroid has become a candidate as a 'programming factor' in prenatal stress. However, other adrenal steroids may be involved. The androgen DHEA is particularly interesting because of its anti-glucocorticoid actions in the brain (Kimonides *et al.* 1998, Cardounel *et al.* 1999, Kimonides *et al.* 1999, Kurata *et al.* 2004), and because adrenal secretion has been shown to be altered in several disease states and psychopathologies (Goodyer *et al.* 2001). For example, decreased plasma DHEA and/or its sulfated derivative DHEAS occurs in patients with rheumatoid arthritis, coronary artery disease, anorexia nervosa, anxiety, depression, and obsessive compulsive disorder (Barrett-Connor *et al.* 1986, Wilder 1996, Goodyer *et al.* 1998, Gordon *et al.* 1999, Wolkowitz & Reus 2000, Harris *et al.* 2001, van Niekerk *et al.* 2001, Bigos *et al.* 2009). HPA axis dysregulation has been described for psychosis in general, and for schizophrenia in particular (Cotter & Pariante 2002), and includes increased basal cortisol secretion (Ryan *et al.* 2004), increased adrenocorticotrophic hormone (ACTH)/cortisol responsiveness to the dexamethasone (DEX)/corticotrophic-releasing hormone challenge test (Lammers *et al.* 1995), and increased plasma DHEA concentrations in severely psychotic male subjects and medicated patients with chronic schizophrenia (Oades & Schepker 1994, di Michele *et al.* 2005).

ACTH is the endogenous adrenal secretagogue for both cortisol and DHEA (Kalimi *et al.* 1994), for both of which 17 α -hydroxypregnenolone is the direct precursor. DHEA synthesis in the adrenal gland and brain is mediated by the enzyme P450c17, in association with the hemo-protein cytochrome b5 (CYTB5; Compagnone & Mellon

1998, Mapes *et al.* 1999, Zwain & Yen 1999, Tagawa *et al.* 2006). In the brain, this important androgen is a potent anti-glucocorticoid (Kalimi *et al.* 1994, Kimonides *et al.* 1999), which may be highly significant in the context of prenatal stress and ensuing brain pathology in humans. DHEA has been shown to protect hippocampal neurons against the neurotoxic effects of both glutamate analogs and glucocorticoids, where it is thought to modulate neuronal survival through stress-activated protein kinase-related intracellular pathways (Kimonides *et al.* 1998).

The relationship between DHEA and cortisol in the developing fetus has been difficult to examine in conventional laboratory rodents, as rat and mouse adrenal glands do not express P450c17, and adrenal DHEA production is insignificant at any stage of life (Van Weerden *et al.* 1992). Moreover, the major adrenal glucocorticoid in these species is corticosterone (Van Weerden *et al.* 1992, Ishimura & Fujita 1997). The spiny mouse (*Acomys cahirinus*) has a relatively long gestation (38–39 days) (Brunjes *et al.* 1989), and in contrast to other rodents their adrenal glands express both P450C17 (CYP17A1) and accessory hemoprotein CYTB5, and secrete both cortisol and DHEA from at least mid-gestation (Quinn *et al.* 2013). The aim of this study was to determine the long-term consequences of a brief exposure to maternally administered glucocorticoid, as a model of transient stress in pregnancy, on the synthesis of DHEA and cortisol in adult offspring. A low dose of the synthetic glucocorticoid DEX was used in this study to model a transient rise in maternal glucocorticoid. Importantly, DEX is a synthetic glucocorticoid that binds preferentially to the glucocorticoid receptor, and not the mineralocorticoid receptor (Welberg & Seckl 2001), and therefore mimics the effects of endogenous glucocorticoids acting through the glucocorticoid receptor. This model has previously been shown to lead to reduced nephron numbers, altered renal gene expression in the fetal spiny mouse (Dickinson & Walker 2007a), and altered male sexual behavior in laboratory rat, both outcomes of which are also observed after maternal stress caused by immobilization and crowding stress (Holson *et al.* 1995, Hayashi *et al.* 1998). The timing of the excess glucocorticoid exposure was chosen to occur either just before or just after differentiation of the major steroidogenic zones of the adrenal cortex, the evidence of which was shown previously (Quinn *et al.* 2013), and is extended in the present study by examining the expression and localization of STAR, P450C17, CYTB5, and 3 β -hydroxysteroid dehydrogenase (3 β HSD) in the spiny mouse adrenal gland at both 20 and 30 days of gestation (0.5 and 0.75 term

respectively). Finally, as previous studies have found that the outcomes of adverse *in utero* conditions with respect to increased glucocorticoids can be more prominent in male than in female offspring (Aiken & Ozanne 2013), and the incidence of neurological illness, such as schizophrenia, is also greater in males (Johnston & File 1991, Iacono & Beiser 1992), we also determined the sex difference in response to this maternal glucocorticoid treatment.

Materials and methods

Animals

All experimental procedures were approved by School of Biomedical Sciences, Animal Ethics Committee, Monash University and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Spiny mice were bred and housed as described previously (Dickinson & Walker 2007b).

DEX administration

Osmotic micro pumps (Azlet, model 1003D) were implanted s.c. in pregnant spiny mice at either 20 days of gestation (12 dams) or 30 days of gestation (12 dams), as described previously (Dickinson *et al.* 2007), and they received either DEX (Sigma–Aldrich; 125 µg/kg body weight; $n=6$ dams at 20 days and $n=6$ dams at 30 days), or saline ($n=6$ dams at 20 days and $n=6$ dams at 30 days) administered over 60 h. Untreated (control group) dams ($n=6$ dams at 20 days and $n=6$ dams at 30 days) received no surgical treatment. Each pregnant spiny mouse was then allowed to deliver naturally.

Sample collection

Pups were weaned at 40 days of postnatal age and housed with the same sex groups of up to four animals until they reached 170 ± 20 days of age, when they were killed and blood and tissue collected. Male ($n=6$) and female ($n=6$) spiny mice at 170 ± 20 days of postnatal age of each treatment (control, saline, and DEX) and fetal age (20 vs 30 days of gestation) group were killed by decapitation and blood was collected from the trunk vessels. Plasma was obtained by centrifugation (5 min at 3000 g, 4 °C) and stored at -20 °C before DHEA, cortisol, and testosterone RIA. Owing to restrictions in plasma volume, control (untreated) group plasma was not available for testosterone RIA. All adrenal glands were collected through a midline abdominal incision. Both adrenal glands were

excised, and the left gland was weighed and immersed in 4% paraformaldehyde for 24 h before being processed and paraffin embedded for histology and immunohistochemistry (IHC). Right adrenal glands were weighed and frozen at -80 °C for immunoblot analysis ($n=2$ per sex). Adrenal glands were also collected from untreated fetal male and female spiny mice at 20 days of gestation ($n=6$) and 30 days of gestation ($n=11$), and processed as mentioned earlier. Blood and tissue collection always occurred between 1030 and 1130 h.

Hormone assays

Cortisol was extracted from plasma using dichloromethane, and total plasma cortisol was measured by RIA as described previously (Bocking *et al.* 1986) using cortisol as standard (H-4001, Sigma Chemical Company). The values obtained by RIA were corrected for the extraction recovery (mean recovery, 74.6%). The intra-assay coefficient of variation (CV) was 2.1% at 1.5 ± 0.26 ng/ml and 7.7% at 64.5 ± 2.09 ng/ml. The assay sensitivity was 0.37 ng/ml. DHEA was measured in spiny mouse plasma using a commercially available RIA kit according to manufacturer's instructions (DSL8900, Beckman Coulter, Brea, CA, USA). The cross-reactivity of the antisera used in the assay was determined by the manufacturer and was $<0.75\%$ with other structurally similar steroids. For plasma, the intra-assay CV was 2.9% at 2.5 ± 0.75 ng/ml and 5.3% at 7.5 ± 2.25 ng/ml. The assay sensitivity was 0.2 ng/ml. Plasma testosterone concentration was measured using a commercially available RIA kit according to manufacturer's instructions (IM1119, Immunotech, Marseilles, France). The cross-reactivity of the antisera used in the assay was determined by the manufacturer and was $<0.75\%$ with other structurally similar steroids. The assay sensitivity for serum testosterone was 15.6 pg/ml. The intra-assay CV was 8.0%.

Hemotoxylin and eosin staining

Hemotoxylin and eosin staining was carried out to assess the cell morphology of adrenal glands. Paraffin-embedded sections (5 µm) were stained in alum hemotoxylin for 5 min and then rinsed in running distilled water. Sections were then differentiated with 0.3% acid alcohol for 1–2 s. The sections were then rinsed in running distilled water and then in Scott's tap water, followed by distilled water again. The sections were then stained with eosin for ~ 5 s.

Immunohistochemistry

IHC was carried out on 5 µm paraffin-embedded sections. The sections were deparaffinized in xylene and antigen retrieval was carried out in 0.01 M citric acid buffer (pH 6) using a microwave oven (three bursts of 5 min duration). The sections were then rinsed in PBS (0.1 M, pH 7.4) incubated with 3% hydrogen peroxide for 20 min at room temperature to block endogenous peroxidase activity, and then incubated with 5% normal goat serum in 0.1 M PBS containing 3% BSA for 45 min at room temperature to block non-specific binding. The sections were then incubated for 12 h at 4 °C with primary antibody in 0.1 M PBS (Table 1). The sections used for STAR negative controls were incubated overnight with antibody pre-incubated with antigen (STAR blocking peptide (sc-23524P)) in place of the primary antibody. The sections were then washed three times in PBS and incubated for 1 h at room temperature with the appropriate IgG secondary antibody (biotinylated anti-rabbit (P450c17, Cytb5), anti-goat (3βHSD, StAR), or anti-mouse (tyrosine hydroxylase (TOH)) at 1:250 dilution (all Vector Laboratories, Burlingame, CA, USA). Antibody binding was visualized using streptavidin HRP (1:200; Amersham Biosciences), with metal-enhanced diaminobenzidine (Pierce Biotechnology, Inc., Rockford, IL, USA) as the chromogen.

Immunoblotting

Immunoblotting was used to verify the specificity of the STAR protein in the adult spiny mouse. Total protein from male and female adult spiny mice (170 days old) was extracted from snap-frozen adrenals and concentration determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Scoresby, VIC, Australia). Protein (35 µg) was subjected to a reducing SDS-PAGE and transferred onto a PVDF membrane (Immobilon-P, Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk in PBS/0.5% Tween-20 (PBS/T) before incubation

with primary antibody overnight at 4 °C (polyclonal goat anti-STAR; 1:200, Santa Cruz Biotechnology). The membrane was then incubated with secondary antibody (1:1000 rabbit anti-goat HRP, Serotec, Oxford, UK) at room temperature for 1 h and the immune-labeled protein visualized using an Immobilon Western Chemiluminescent HRP substrate (Millipore).

Quantification of histology and IHC

Sections were viewed using an Olympus microscope and images were captured using a Nikon digital camera and DP2 BSW computer program. For each adrenal gland, the results of three fields of view per section, per adrenal zone (zona reticularis (ZR), zona fasciculata (ZF), and zona glomerulosa (ZG)), for six sections per adrenal gland, were averaged for each animal and then the results averaged for all animals in each group. Densitometric analysis using ImageJ (National Institutes of Health, Bethesda, MA, USA) was used to calculate the percentage area of the field of view occupied by positive staining. The color images were converted to gray scale, and the positive signal was expressed as the relative number (%) of pixels above background (threshold). Data from DEX/saline-treated animals are expressed as a percent change from untreated control animals.

Data analysis and statistical analysis

All data are expressed as mean ± S.E.M. with only one male and one female offspring from any 1 l sampled at 170 ± 20 days of age used for the analysis. Statistical significance was determined using statistical analysis software (Prism, GraphPad Software, Inc., La Jolla, CA, USA). Adrenal weight-to-body weight ratio differences between groups were assessed using one-way independent measures ANOVA, with the Tukey's test applied *post hoc*. Treatment-dependent changes in the plasma concentrations of DHEA, testosterone, and cortisol were analyzed using the two-way

Table 1 Primary antibodies used for immunohistochemical analysis

Primary antibody	Antigen	Source	Dilution
STAR protein	Polyclonal goat anti-human StAR (K-20) (sc-23524)	Santa Cruz Biotechnology	1:100
P450C17	Polyclonal rabbit anti-bovine P450c17	Alan Conley (Univ. Davis, CA, USA)	1:1000
Cytochrome b5	Polyclonal rabbit anti-human cytochrome b5	Alan Conley	1:1000
3β-Hydroxysteroid dehydrogenase	Polyclonal goat anti-human 3β-hydroxysteroid dehydrogenase	Santa Cruz Biotechnology	1:100
Tyrosine hydroxylase	Monoclonal mouse anti-rat tyrosine hydroxylase	Boehringer (Mannheim, Germany)	1:1000

independent measures ANOVA with the Bonferroni's *post hoc* test. Treatment-dependent differences between the DEX and saline groups in percentage of positive enzyme expression relative to controls (untreated), as determined by IHC, were analyzed using the two-way independent measures ANOVA with the Bonferroni's *post hoc* test. Statistical significance was accorded when $P < 0.05$. Where there were no significant differences between males and females (steroidogenic enzyme expression at each of 20 and 30 days of gestation; enzyme expression between the control (untreated) and saline-treated animals for both males and females at 170 ± 20 days of age; and DEX vs saline adrenal structure as shown by H&E and TOH IHC), only representative images of male adrenals have been shown.

Results

Steroidogenic enzyme expression in the spiny mouse adrenal gland at 20 and 30 days of gestation, and at 170 ± 20 days of age in control (untreated) animals

Fetal adrenal development There was a large increase in the size of the fetal adrenal gland between 20 days of gestation (Fig. 1A, B, C and D) and 30 days of gestation (Fig. 1E, F, G and H), accompanied by increased expression of STAR, 3 β HSD, P450C17, and CYTB5 over this time (0.5–0.75 gestation). At 20 days of gestation, there was cytoplasmic staining of both STAR and CYTB5 (Fig. 1A and C) throughout the whole adrenal gland, but not P450C17 or 3 β HSD protein. By 30 days of gestation,

Figure 1

Immunohistochemical localization of STAR (A, E and I), P450C17 (B, F and J), CYTB5 (C, G and K), and 3 β HSD (D, H and L) in the developing adrenal gland of male spiny mouse at 20 days of gestation (A, B, C and D) and 30 days of gestation (E, F, G and H), and at 170 days of postnatal (P) age (I, J, K and L).

Insert in (A) shows negative control (antibody pre-incubated with antigen) for the STAR protein at 20 days of gestation. Scale bar = 50 μ m in (A, B, C and D) and 100 μ m in (E, F, G, H, I, J, K and L).

STAR protein was predominantly localized in the middle of the presumptive cortex, while CYTB5 immunoreactivity was still present in both the medulla and cortex, although with clearly decreased expression in the outer region of the cortex, the presumptive ZG (Fig. 1E and G). At 20 days of gestation, the expression of P450C17 in the fetal adrenal gland was very low and no expression of β HSD could be detected (Fig. 1B and D). However, by 30 days of gestation P450C17 was widely distributed, with strong expression in cells surrounding the presumptive medulla and occupying most of the cortex (Fig. 1E). β HSD immunoreactivity was present in cells surrounding the medulla and also in the ZG (Fig. 1H).

Adult adrenal gland At 170 ± 20 days of age, STAR protein was expressed throughout the adrenal cortex, with no greater or lesser expression evident for the ZG, ZF, and ZR (Fig. 1I). P450C17 immunoreactivity was observed in the ZR and ZF only, with the strongest expression in the ZF (Fig. 1J). CYTB5 expression was present throughout the adrenal cortex and the medulla in the adult gland (Fig. 1K). β HSD expression was found predominantly in the ZF and ZG in the adrenal gland of adult offspring (Fig. 1L).

Effect of maternal DEX treatment on body and adrenal weights and adrenal morphology at 170 ± 20 days of age

DEX treatment at 20 days of gestation did not significantly affect the body weight of 170 ± 20 -day-old male or female offspring, whereas treatment at 30 days of gestation resulted in a significant increase in body weight of male spiny mice (Table 2). The adrenal glands of male and

female offspring were not significantly affected by the DEX treatment at either 20 or 30 days of gestation (Table 2). Hematoxylin and eosin staining of the adrenal cortex and TOH immunohistochemical analysis of the adrenal medulla showed no obvious gross structural differences between the male and female adult offspring of saline and DEX-treated mothers, irrespective of whether the DEX had been administered at 20 days of gestation (Fig. 2A, B, C and D) or 30 days of gestation (Fig. 2E, F, G and H).

Effect of DEX treatment during pregnancy on steroidogenic enzyme expression at 170 ± 20 days of age

STAR After maternal DEX at 20 days of gestation, STAR expression was significantly decreased in the ZR of male (percentage of positive staining relative to control: DEX $-13.73 \pm 4.67\%$ vs saline $+5.59 \pm 4.18\%$; $P < 0.05$), but not in female adult offspring (DEX $-2.02 \pm 4.94\%$ vs saline $+3.77 \pm 3.78\%$), whereas it was increased in the ZG of males (DEX, $+33.09 \pm 9.26\%$ vs saline $+3.82 \pm 0.15\%$; $P < 0.05$) and decreased in ZG of females (DEX $-37.84 \pm 9.14\%$ vs saline $+4.25 \pm 0.23\%$; $P < 0.001$; Fig. 3D and E). In contrast, DEX treatment at 30 days of gestation had no significant effect on STAR expression in any cortical zone of the adrenal gland at 170 ± 20 days of age (Fig. 3J).

P450C17 DEX treatment at 20 days of gestation had the effect of significantly decreasing P450C17 immunoreactivity in the ZR of male offspring only (percentage of positive staining relative to control: DEX $-61.12 \pm 6.56\%$ vs saline $+1.69 \pm 2.96\%$; $P < 0.001$; Fig. 4E), with no effects on expression in the ZF of either sex. DEX treatment at

Table 2 Body weight (BW), combined adrenal weight (absolute (mg)) and adrenal weight expressed relative to BW in 170 ± 20 -day-old-offspring of saline and dexamethasone (DEX)-treated mothers at 20 and 30 days of gestation. Values are expressed as mean $\pm$ S.E.M.

	20 Days of treatment				30 Days of treatment			
	Control (n=6)	Saline (n=6)	DEX (n=6)	P value (saline vs DEX)	Control (n=7)	Saline (n=6)	DEX (n=6)	P value (saline vs DEX)
Male offspring								
Body weight (g)	39.9 ± 0.3	32.5 ± 1.0	33.8 ± 0.7	NS	36.5 ± 1.3	38.9 ± 0.5	42.7 ± 0.7	<0.01
Adrenal weight (mg)	NA	NA ^a	NA	NA	20.1 ± 0.02	23 ± 1.0	47 ± 17.0	NS
Adrenal weight (mg/g BW)	NA	NA	NA	NA	0.5 ± 0.2	0.6 ± 0.03	1.2 ± 0.5	NS
Female offspring								
Body weight (g)	29.9 ± 1.66	30.4 ± 1.1	29.3 ± 0.7	NS	32.9 ± 0.7	35.3 ± 0.939	36.9 ± 1.226	NS
Adrenal weight (mg)	NA	NA	NA	NA	22.5 ± 0.01	23 ± 2.0	33 ± 18.0	NS
Adrenal weight (mg/g BW)	NA	NA	NA	NA	0.68 ± 0.01	0.65 ± 0.071	0.873 ± 0.453	NS

NS, not significant.

^aAdrenal weights were not available (NA) at time of post mortem.

Figure 2

Hematoxylin and eosin staining (A, C, E and G) and tyrosine hydroxylase (TOH) immunohistochemistry (B, D, F and H) in the adrenal gland of adult (170 days of postnatal age) offspring of saline (A, B, E and F) and DEX-treated dams (C, D, G and H). (A, B, C and D) Male adult offspring from dams treated with DEX or saline at 20 days of gestation. (E, F, G and H) Male adult offspring from dams treated at 30 days of gestation. Scale bars: 100 μ m in A, C, E and G; and 500 μ m in B, D, F and H.

30 days of gestation had no effect on P450C17 expression in the ZR or ZF of either male or female spiny mice at 170 $\pm$ 20 days of age (Fig. 4J). As mentioned earlier, P450C17 was not present in the ZG of the adult adrenal gland.

Cytochrome b5 DEX treatment at either 20 or 30 days of gestation had the effect of reducing CYTB5 expression in the ZR and ZF of the adrenal cortex of male offspring, with no significant effect on expression in those zones of the female adrenal cortex, and no effect on the ZG in either sex (Fig. 5A, B, C, D, E, F, G, H and I).

3 β -Hydroxysteroid dehydrogenase DEX treatment had no significant effect on 3 β HSD expression throughout

the adrenal gland of male and female spiny mice at 170 $\pm$ 20 days of age (Fig. 6A, B, C, D, E, F, G, H, I and J), although there was a trend ($0.05 < P < 0.1$) for decreased expression of 3 β HSD in the ZG of the female offspring after treatment at 20 days of gestation. 3 β HSD expression was limited to the ZF and the ZG in all females regardless of treatment (Fig. 6G and I). Unexpectedly however, the male offspring of mothers exposed to prenatal DEX at 30 days of gestation showed increased immunoreactivity of 3 β HSD in the ZR (Fig. 6H), a result not found in the saline-treated control males (Fig. 6F) or untreated age-matched control males (see Fig. 1L).

Plasma steroid levels in adult offspring exposed to maternal DEX or saline treatment during pregnancy

The DEX treatment at 20 days of gestation resulted in significantly decreased plasma concentrations of DHEA in 170 $\pm$ 20-day-old male offspring (DEX 6.84 $\pm$ 1.24 ng/ml vs saline 13.06 $\pm$ 0.27 ng/ml; $P < 0.05$), but not in female spiny mice at this age (Fig. 7A). In the males, there was a trend ($P = 0.27$) for plasma cortisol to be increased after the DEX treatment (Fig. 7B), and the plasma DHEA:cortisol ratio was significantly decreased, a change not observed in female offspring (Fig. 7C). After DEX treatment at 20 days of gestation, plasma testosterone concentrations were significantly higher in adult male offspring (DEX 266.03 $\pm$ 50.75 pg/ml vs saline 83.47 $\pm$ 32.3 pg/ml; $P < 0.05$), an effect not observed in females at this age (Fig. 7D).

DEX treatment at 30 days of gestation had no effect on the absolute and relative concentrations of DHEA and cortisol in either sex (Fig. 7E, F and G), although significant differences in the plasma concentration of cortisol between male (351.03 $\pm$ 59.69 ng/ml) and female (724.99 $\pm$ 94.24 ng/ml; $P < 0.01$) spiny mice were noted (Fig. 7F). After treatment at 30 days of gestation there were no significant differences in testosterone concentrations between the DEX – vs saline – treated adult offspring, but as expected, adult males showed significantly higher circulating testosterone concentrations compared to females (Fig. 7H).

Discussion

The effects of the DEX treatment at 20 or 30 days of gestation are summarized in Table 3. The principal finding of this study is that a pulse of glucocorticoid delivered at 0.5, but not at 0.75, of pregnancy in the spiny mouse significantly alters the developmental trajectory of the

Figure 3

Immunohistochemical localization (A, B, C, D, F, G, H and I) and percentage positive image relative to control (E and J) of STAR protein in the zona reticularis (ZR), zona fasciculata (ZF), and zona glomerulosa (ZG) in male and female adult offspring from dams treated with saline or dexamethasone (DEX) at 20 days of gestation (A, B, C, D and E) or 30 days of gestation (F, G, H, I and J). Inserts in (A, B, C and D) show high magnification of STAR expression in the ZR (A and C) and ZG (A, B, C and D) in the adult offspring

of 20 days of gestation DEX- or saline-treated mothers. Inserts in (F and H) show negative control (no primary antibody) adrenal glands of male offspring of mothers treated with DEX or saline at 30 days of gestation. Insert in (G) shows immunoblot verification of enzyme expression and antibody specificity for STAR protein in male (lane 1) and female (lane 2) spiny mouse adult adrenal glands at 170 days of age. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ DEX vs saline; scale bar = 100 μ m.

fetal male spiny mouse adrenal glands, resulting in decreased secretion of DHEA at 170 ± 20 days after birth. At 20 days (0.5) gestation, the protein STAR and the accessory hemoprotein CYTB5 were expressed in the adrenal gland, but the enzymes P450c17 and 3 β HSD were not expressed at this stage. However, by 30 days (0.75) of gestation, all four enzymes were expressed, and the adrenal cortex had clearly differentiated into zones that prefigured the definitive development of the ZR, ZF, and ZG.

Glucocorticoid exposure at 0.5 gestation resulted in changes in the expression of the key proteins of DHEA synthesis in the adult adrenal cortex, and many of these effects were greater in males. DEX given at 0.5 gestation resulted in a decrease in STAR, P450C17, and CYTB5

expression in the ZR of male adult offspring and, with exception of CYTB5, these changes were not observed in the ZF. As STAR is required in the rate-limiting step involved in all steroidogenic reactions (Clark *et al.* 1994, Miller & Auchus 2011), and CYTB5 allosterically modulates the 17-20lyase activity of P450C17 (Katagiri *et al.* 1995, Lee-Robichaud *et al.* 1995), it is likely that the decreased expression of these enzymes in the ZR may be responsible for the decreased levels of circulating DHEA that were present in the adult male offspring of these pregnancies.

In the ZF, expression of STAR, P450C17, and 3 β HSD was not significantly altered in offspring exposed to the mid-gestation maternal DEX treatment, and accordingly,

Figure 4

Immunohistochemical localization (A, B, C, D, F, G, H and I) and percentage positive image relative to control (E and J) of P450c17 in the ZR and ZF in male and female adult offspring of dams treated with saline or dexamethasone (DEX) at 20 days of gestation (A, B, C, D and E) or 30 days of gestation (F, G, H, I and J). (E) A significant decrease in P450c17 expression (expressed as percentage of positive staining/image area – see ‘Materials

and methods’ section) in the ZR of male adult offspring of mothers exposed to DEX at 20 days of gestation. *** $P \leq 0.001$ DEX vs saline; scale bar = 100 µm. DEX, dexamethasone; ZR, zona reticularis; ZF, zona fasciculata; ZG, zona glomerulosa; M, medulla; 20dGA-T, adult offspring of mothers treated at 20 days of gestation; 30dGA-T, adult offspring of mothers treated at 30 days of gestation.

cortisol levels did not differ significantly between the DEX and saline treatment groups. It is possible that the decrease in CYTB5 in the ZF may act to direct the synthesis of 17-hydroxy-pregnenolone to 17-hydroxy-progesterone rather than DHEA. Together, these findings indicate that maternal stress can program the development of the fetal adrenal gland so that there is diminished adrenal production of DHEA and preferential synthesis and secretion of cortisol. Such change of steroidogenesis also occurs in human patients with acute physiologic stress, such as burns (Lephart *et al.* 1987), serious illnesses (Carlström *et al.* 1990, Oberbeck & Kobbe 2010), and schizophrenia (Ritsner *et al.* 2004). Interestingly, testosterone and androstenedione concentrations have also been observed to decline in men

exposed to burn trauma, while cortisol concentrations were elevated (Dolecek 1989).

After DEX treatment at 20 days of gestation in the pregnant spiny mouse, there was increased expression of STAR protein in the adrenal gland of male offspring, but decreased expression in female offspring. There was also a decrease in 3βHSD expression in the ZG of female offspring, suggesting altered capacity of female offspring to produce aldosterone from this zone. Female offspring also showed a trend for this mid-gestation DEX treatment to decrease plasma DHEA and cortisol, suggesting there may be a general underactivity of the HPA axis in female offspring exposed to prenatal DEX treatment.

These effects of DEX treatment during pregnancy on the adult spiny mouse adrenal gland are consistent with

Figure 5

Immunohistochemical localization (A, B, C, D, F, G, H and I) and percentage positive image relative to control (E and J) of cytochrome b5 (CYTB5) in the ZR, ZF, and ZG in male and female adult offspring of dams treated with saline or DEX at 20 days of gestation (A, B, C, D and E) or 30 days of gestation (F, G, H, I and J). * $P \leq 0.05$ and ** $P \leq 0.01$ DEX vs saline.

Scale bar = 100 μ m. DEX, dexamethasone; ZR, zona reticularis; ZF, zona fasciculata; ZG, zona glomerulosa; M, medulla; 20dGA-T, adult offspring of mothers treated at 20 days of gestation; 30dGA-T, adult offspring of mothers treated at 30 days of gestation.

other studies, in which stress induced by undernutrition in rats, from day 10 of gestation until delivery, resulted in a decrease in several steroidogenic enzyme mRNAs in the adult adrenal gland (Khorram *et al.* 2008, 2011). In this model of nutritional maternal stress, adrenal profiles of steroidogenic enzymes in adult offspring were also markedly altered in a sex-specific manner, with significant decreases evident in *P450c17* (*Cyp17a1*) mRNA expression and increased *P450scc* (*Cyp11a1*) and aldosterone synthase expression in the male offspring (Khorram *et al.* 2011). These changes confirm that maternal stress in pregnancy provokes gender-specific effects in the development of the adrenal cortex, and the similarity to the DEX-induced changes observed in the current study further implicates maternal cortisol as the factor that 'programs' the development of the adrenal gland. Further research is

necessary to establish when major programming effects on adrenal enzyme expression occur (i.e. during fetal life, after birth, or in early postnatal or adolescent periods), and the possibility that these changes may, at least in part, be mediated at different levels of the HPA axis.

In contrast to the effects of DEX administration at 20 days of gestation, and despite significant increase in the relative and absolute weight of the adrenal gland in males, there were no significant changes in STAR or P450C17 expression in the ZR or ZF, or of 3 β HSD in the ZF following the DEX treatment at 30 days of gestation. Accordingly, the circulating levels of both DHEA and cortisol in these adult offspring did not significantly differ between the DEX and saline treatment groups. However, 3 β HSD expression in the ZF was significantly higher in males compared with females, a fundamental difference between

Figure 6

Immunohistochemical localization (A, B, C, D, F, G, H and I) and expression relative to control (E and J) of 3 β -hydroxysteroid dehydrogenase (3 β HSD) in the ZF and ZG in male and female adult offspring of saline and DEX treated mothers at 20 days of gestation (A, B, C, D and E) and 30 days of gestation (F, G, H, I and J). Insert in (H) shows high magnification of the ZR in male

adult offspring of mothers treated with DEX at 30 days of gestation.

* $P \leq 0.05$ DEX vs saline. Scale bar = 100 μ m. DEX, dexamethasone; ZR, zona reticularis; ZF, zona fasciculata; ZG, zona glomerulosa; M, medulla; 20dGA-T, adult offspring of mothers treated at 20 days of gestation; 30dGA-T, adult offspring of mothers treated at 30 days of gestation.

males and females (regardless of treatment). In addition, in the male offspring of mothers exposed to DEX at 30 days of gestation show an evidence of 3 β HSD immunoreactivity in ZR, a finding which was unexpected, given it did not occur in females, or in any offspring treated at 20 days of gestation with DEX, nor was it observed during normal development of the ZR in this species (Quinn *et al.* 2013), perhaps resulting in a decrease in DHEA biosynthesis. The observations show that increased glucocorticoids of maternal origin can profoundly affect the developmental trajectory of the adrenal gland. The effects depend on the time of gestation and the developmental status of the adrenal gland.

Humans infants younger than 5 years old exhibit a poorly developed adrenal ZR that also expresses 3 β HSD (Rainey *et al.* 2002), but when the ZR expands at

adrenarche the 3 β HSD content falls restricting steroidogenesis to the Δ 5-pathway throughout adolescence and adulthood, thus maintaining DHEA-S production (Auchus & Rainey 2003). While 3 β HSD activity is essential for the synthesis of aldosterone and cortisol in the ZG and ZF, 3 β HSD expression in the ZR might have a negative impact on the biosynthesis of DHEA as it would lead to the synthesis of progesterone from pregnenolone, or of 17-OH progesterone from 17-OH pregnenolone, rather than the formation of DHEA. Thus, 3 β HSD expression in the ZR of the DEX-treated offspring in the spiny mouse, together with the decrease in CYTB5 in both the ZR and the ZF, may represent an enzymatic environment in which steroid flux is directed away from the Δ 5-pathway toward cortisol synthesis, as well as converting DHEA itself to androstenedione. Indeed, there was a trend of increased

Figure 7

(A and E) Plasma DHEA concentrations; (B and F) plasma cortisol concentrations; (C and G) the DHEA:cortisol ratio; and (D and H) plasma testosterone concentrations in adult offspring of pregnant dams treated with either saline or DEX at 20 (A, B, C and D) or 30 (E, F, G, H) days of

gestation. * $P \leq 0.05$ and ** $P \leq 0.01$. DEX, dexamethasone; 20dGA-T, adult offspring of mothers treated at 20 days of gestation; 30dGA-T, adult offspring of mothers treated at 30 days of gestation.

cortisol production in the male offspring exposed to DEX after treatment at 30 days of gestation, although the level of circulating cortisol was still below that of the females in both the DEX and saline treatment groups.

Other studies looking at the effects of glucocorticoid stress during late pregnancy have shown varying results. Waddell *et al.* (2010) found that DEX administered from day 13 to term in pregnant rats did not significantly alter the expression of *Star* mRNA in the adrenal gland of adult offspring, although plasma and urinary corticosterone and urinary aldosterone were elevated, indicative of enhanced adrenal responsiveness. Low-dose DEX given to rats during late pregnancy has been shown to reduce birth weight and result in hypertension, hyperglycemia, and hyperinsulinemia in adult offspring (Lindsay *et al.* 1996, Holness & Sugden 2001, O'Regan *et al.* 2004). Studies in sheep, pigs, and guinea pigs have shown increased HPA axis activity and anxiety-like behaviors in adult offspring (Cadet *et al.* 1986, Lingas & Matthews 2001, Sloboda *et al.* 2002, Jarvis *et al.* 2006), as well as increased adrenal the expression of *Mc2r* mRNA (Waddell *et al.* 2010), the gene encoding the ACTH receptor (Hirsch *et al.* 2011). These studies strongly suggest that maternal glucocorticoids mediate the effects of maternal stress on the development of the HPA axis in the fetus, and the results of this study in the spiny mouse show that the fetal adrenal gland is particularly vulnerable to high levels glucocorticoids even if they occur only transiently at mid-gestation. Previous studies have further shown that there is a reduction in glucocorticoid feedback sensitivity in late gestation in the guinea pig (Kapoor *et al.* 2006). Plasma cortisol concentrations in the fetal spiny mouse decrease from 30 days of

gestation until the day of birth (Lamers *et al.* 1986, Quinn *et al.* 2013), suggesting that there is a developmental change in sensitivity to glucocorticoid feedback within the HPA axis after 30 days (0.75) of gestation.

This study has also shown that plasma testosterone concentrations were increased after the DEX treatment

Table 3 Summary of the effects of maternal administration of dexamethasone (DEX) at 20 or 30 days of gestation in male and female offspring at 170

DEX treatment at	20dGA		30dGA	
	Male	Female	Male	Female
Body weight	—	—	↑	—
Adrenal weight	—	—	↑	—
Plasma cortisol	—	—	—	—
DHEA	↓	—	—	—
DHEA:cortisol	↓	—	—	—
Testosterone	↑	—	—	—
STAR				
ZR	↓	—	—	—
ZF	—	—	—	—
ZG	↑	↓	—	—
P450c17				
ZR	↓	—	—	—
ZF	—	—	—	—
ZG	—	—	—	—
Cytb5				
ZR	—	—	—	—
ZF	—	—	—	—
ZG	—	—	—	—
3βHSD				
ZR	—	—	↑	—
ZF	—	—	—	—
ZG	—	—	—	—

Arrows, significant changes; —, no effect; GA, gestational age; P450c17, 17-hydroxylase and 17-20lyase; Cytb5, cytochrome b5; 3βHSD, 3β-hydroxysteroid dehydrogenase; ZR, zona reticularis; ZF, zona fasciculata; ZG, zona glomerulosa.

at 20 days of gestation. This was an unexpected finding, considering that testosterone is an important downstream metabolite of DHEA (Labrie *et al.* 2001), and that plasma DHEA was significantly lower in these DEX-treated offspring, a change likely to follow from the decreased expression of P450C17 in the adrenal gland. It is therefore likely that the high concentrations of testosterone in males are almost entirely testicular in origin, and so the possible effects of fetal DEX exposure on testicular function and the HPG axis are worthy of further investigation. The large difference in the plasma testosterone levels between the two groups treated with saline (tenfold difference 20 vs 30 days of gestation) suggests a greater effect of surgery at day 20 of gestation, possibly reflecting a shift in the maternal stress response or the expression and ratio of 11 β HSD1 and 2 in the placenta between these two stages. Plasma from the offspring of the 'control' group was not available for analysis (due to limited volumes), but the male offspring of mothers treated with both DEX and saline at 30 days of gestation showed testosterone within the same range as 140–180 days old males observed in the spiny mouse colony used in our study (H Dickinson, J Mamrot, S Douglas, M Weybury, L Wiadrowski, D W Walker & P Temple-Smith, 2013, unpublished observations). It therefore appears that surgery had a major re-programming effect on the male HPG axis, and further studies could determine if testicular development and the onset of puberty are also altered in these offspring. This is possible because a previous study has shown that increased fetal glucocorticoid exposure delays onset of puberty in postnatal rats (Smith & Waddell 2000).

It is certainly possible that stress caused by handling, surgery, and blood sampling may have influenced the circulating hormone concentrations reported in the current study. Previous studies in a variety of species have noted that even relatively short periods of handling stress can rapidly increase corticosterone concentrations in avian species, rodents, and reptiles (Harvey *et al.* 1980, Moore *et al.* 1991, Ryabinin *et al.* 1999), and although plasma levels of other hormones may change more slowly in response to stress, there is evidence in a variety of species that testosterone concentrations can decrease significantly within 15 min to an hour after handling stress (Wilson *et al.* 1979), and more extreme forms of stress such as social conflict (Huhman *et al.* 1991), electric foot shock, or immersion in cold water (Retana-Márquez *et al.* 2003). Importantly, surgery has also been shown to lower testosterone levels in males (Matsumoto *et al.* 1970), an effect that may, at least in part, be responsible for the

tenfold decrease in testosterone concentrations observed in the current study between offspring of mothers treated with saline at 20 and 30 days of gestation.

Other studies of acute maternal stress at mid-pregnancy have found similar outcomes in adult offspring. For example, in pregnant guinea pigs maternal stress induced by treatment with a strobe light for 2 h/day over 3 days resulted in increased basal cortisol levels and significantly decreased plasma testosterone concentrations in male offspring (Kapoor *et al.* 2006). In addition, acute restraint stress has also been shown to increase adult plasma corticosterone, and reduce plasma dihydrotestosterone and 3 α -androstenediol, but not the aromatized metabolite estradiol in rats (Walf & Frye 2012). Other studies in adult rats and mice (Pomerantz & Pitelka 1998, Kostic *et al.* 1999) have shown that acute and chronic immobilization stress can result in a decrease in serum androgen concentrations, mediated by increased nitric oxide (NO) signaling in the testes, which specifically inhibits P450c17 activity (Kostic *et al.* 1999). It has been suggested that NO inhibits the activity of cytochrome P450 complexes by binding to the heme iron (Quaroni *et al.* 1996) or to the sulfhydryl groups in these enzymes (Snyder *et al.* 1996). Although these experiments were carried out in the testes, it is possible that the inhibition of P450C17 by NO may occur in the adrenal gland or brain of the spiny mouse, warranting further investigation in this species. In the rat, immobilization stress has been shown to increase NO synthase enzyme activity in the anterior pituitary, adrenal cortex, and adrenal medulla in adult animals (Kishimoto *et al.* 1996), and prenatal stress has been shown to cause gender-dependent neuronal loss and oxidative stress in the brain (Zhu *et al.* 2004), perhaps related to the decrease in the production of endogenous and systemic DHEA by P450c17.

In humans there is evidence that low levels of circulating DHEA with normal levels of cortisol place the developing brain at risk for a range of neuropsychiatric disorders, including major depressive disorder, bi-polar disorder, and anxiety (Wolkowitz & Reus 2000, Goodyer *et al.* 2001, Harris *et al.* 2001, Ritsner *et al.* 2004). Low DHEA:cortisol ratios are observed in schizophrenia patients, and is positively associated with severity of depression, state and trait anxiety, trait anger, angry temperament, and hostility in such patients (Ritsner *et al.* 2004). As DHEA itself has been found to have potent anti-glucocorticoid and antioxidant effects in the brain (Bastianetto *et al.* 1999), it is possible that low circulating levels of DHEA may leave the brain increasingly vulnerable to oxidative damage. Interestingly, significantly

increased total nitrite levels and increased oxidant end-products by the reactions of NO with other free radicals have been observed in the plasma samples of human patients with schizophrenia (Black *et al.* 1999, Akyol *et al.* 2004).

In summary, this study demonstrates that the adrenal gland is an important target for glucocorticoid programming effects, and highlights the possibility that acute stress at a critical stage of adrenal development may determine steroidogenic enzyme expression and steroid synthesis and secretion in adult life. The outcome also depends on the sex of the fetus. The complex and developmentally sensitive interactions between cortisol, DHEA, and adrenal steroidogenic enzymes in the spiny mouse may provide an appropriate model for studying the effects of *in utero* exposure to glucocorticoids on the etiology of mental disorders with a presumed developmental origin.

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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