Testosterone is involved in mediating the effects of prenatal stress in male guinea pig offspring

Amita Kapoor[1] and Stephen G. Matthews[1,2,3]

[1]*Departments of Physiology,* [2]*Obstetrics and* [3]*Gynaecology and Medicine, Faculty of Medicine, University of Toronto, Medical Sciences Building, 1 King's College Circle, Toronto, Ontario, M5S 1A8, Canada*

Non-technical summary Studies in humans have demonstrated a link between stress during pregnancy and altered behaviour and stress reactivity in children. In guinea pigs, we have previously shown that a short period of maternal stress during gestation leads to increased anxiety, elevated basal cortisol levels and decreased testosterone levels in adult males. We hypothesized that restoring testosterone to normal levels in the adult males born to prenatally stressed mothers would reverse the changes in behaviours and endocrine function. We found differences in attention and anxiety-related behaviours and basal stress endocrine activity between the prenatally stressed and control males. Administration of testosterone reversed the behavioural differences in the prenatally stressed offspring. There was, however, little effect of postnatal testosterone administration on stress-related endocrine activity. This study provides new information to begin to address the mechanism underlying the interplay between prenatal stress, gonadal steroids and postnatal behaviours.

Abstract A link exists between stress during pregnancy and altered hypothalamic–pituitary–adrenal (HPA) activity and behaviour in children. In the guinea pig, male offspring born to mothers that were exposed to stress during pregnancy demonstrated increased anxiety, basal cortisol levels and decreased testosterone concentrations. Testosterone is known to inhibit HPA function and anxiety behaviours. Therefore, we hypothesized that restoring plasma testosterone would ameliorate the differences observed in HPA function and behaviour. Pregnant guinea pigs were exposed to a stressor during the period of rapid fetal brain growth (prenatal stress, PS) or left undisturbed (control, C). Behaviour in an open-field and prepulse inhibition (PPI) of the acoustic startle reflex (ASR) was assessed in juvenile offspring. In adulthood, male offspring were divided into four groups: Control + sham gonadectomy (GDX), control + GDX + testosterone replacement, PS + sham GDX and PS + GDX + testosterone. Male offspring were retested in the open-field and PPI. Basal HPA activity was also assessed. As juveniles, PS males exhibited significantly lower ASR ($P < 0.05$) and elevated PPI. In adulthood, PS male offspring exhibited significantly decreased PPI ($P < 0.02$) and this was reversed by administration of testosterone. We also found that adult PS offspring exhibited significantly less activity in the open-field ($P < 0.05$) and administration of testosterone increased ambulatory activity in PS animals. Basal plasma adrenocorticotrophin hormone (ACTH) levels were significantly greater in PS animals and there was a trend towards reversal by administration of testosterone in PS males. In conclusion, prenatal stress results in male guinea pig offspring that exhibit age-dependent differences in ambulatory activity, sensorimotor gating and HPA activity. In adulthood, the behavioural changes are reversed by replacement of plasma testosterone.

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Corresponding author S. G. Matthews: Department of Physiology, Faculty of Medicine, University of Toronto, Medical Sciences Building, 1 King's College Circle, Toronto, Ontario, M5S 1A8. Canada. Email: stephen.matthews@utoronto.ca

Abbreviations ACTH, adrenocorticotrophin hormone; ADHD, attention deficit hyperactivity disorder; ASR, acoustic startle response; AUC, area under the curve; AVP, arginine vasopressin; C, control, CBG; corticosteroid binding globulin; GDX, gonadectomy; HPA; hypothalamic–pituitary–adrenal; PND, postnatal day; PPI, prepulse inhibition; PS, prenatal stress; PVN, paraventricular nucleus; S, sham; SHBG, sex hormone binding globulin; T, testosterone-replaced.

Introduction

Studies in humans are demonstrating a strong link between maternal anxiety and/or elevated cortisol levels during pregnancy and altered behaviour and hypothalamic–pituitary–adrenal (HPA) axis activity in children (O'Connor *et al.* 2003, 2005; Davis *et al.* 2007). Specifically, children born to mothers that experienced elevated stress during pregnancy are more likely to demonstrate increased stress responsiveness (Davis *et al.* 2010; Tollenaar *et al.* 2011), emotional and behavioural problems (O'Connor *et al.* 2003; Ramchandani *et al.* 2005), a greater likelihood of development of attention deficit hyperactivity disorder (ADHD) (Rodriguez & Bohlin, 2005) and subsequently, greater severity of ADHD symptoms (Grizenko *et al.* 2008). Studies in animal models are revealing similar results in endocrine function and behaviours. Adult rats born to mothers exposed to stress during pregnancy exhibit altered basal and stress-induced HPA axis activity (Weinstock *et al.* 1998; Brunton & Russell, 2010). Behaviourally, they exhibit hyperactivity during adulthood (Weller *et al.* 1988), altered prepulse inhibition (PPI) and acoustic startle response (ASR) (Burton *et al.* 2006).

A large component of neuroendocrine development occurs postnatally in the rat (Dobbing & Sands, 1979). Therefore, the fetal rat brain is relatively immature compared to the human during gestation. However, similar outcomes are seen in species in which the brain maturation occurs earlier. Rhesus macaque infants born to mothers exposed to stress during pregnancy exhibit abnormal social behaviour (Clarke & Schneider, 1993) and alterations in HPA axis activity (Clarke & Schneider, 1997; Schneider *et al.* 2004). In the guinea pig, we have shown that male offspring born to mothers that were exposed to a moderate stressor during the period of the fetal brain growth spurt, demonstrate increased anxiety behaviour and elevated basal plasma cortisol levels. These males also exhibited significantly lower plasma testosterone levels (Kapoor & Matthews, 2005).

Testosterone is known to have an inhibitory effect on HPA axis activity and anxiety behaviour. Early studies demonstrated that castration increased the glucocorticoid response to physical stress, an effect that was ameliorated by a single testosterone injection (Gaskin & Kitay, 1971). More recently, it has been demonstrated that testosterone replacement decreased the adrenocorticotrophin (ACTH) response to restraint stress in GDX male rats. GDX without testosterone replacement was shown to increase both the ACTH and corticosterone response to psychological and physical stressors (Handa *et al.* 1994; Seale *et al.* 2004). Behaviourally, administration of androgens to GDX male rats resulted in an increased amount of exploratory behaviour in an open-field and in an elevated plus maze (Edinger & Frye, 2005). Testosterone has also been shown to have a negative relationship with PPI. Post-pubertal castrated rhesus macaques exhibited significantly higher PPI than their intact counterparts (Morris *et al.* 2010) and in castrated male rats, administration of the PPI disruptor 5-OH-DPAT, a $5HT_{1A}$ (serotonin receptor 1A) agonist, was significantly less effective than in intact rats (Gogos & van den Buuse, 2003).

Numerous studies have demonstrated that prenatal stress affects sex steroid levels in male offspring. Indeed, male rats born to mothers exposed to stress during pregnancy exhibited decreased rates of male copulatory behaviour, which was associated with a decrease in plasma testosterone levels (Ward, 1972). This may be due to decreased androgen exposure during gestation, as the prenatal surge in testosterone was attenuated in the fetuses of dams exposed to stress during pregnancy (Ward *et al.* 2003). Male guinea pigs born to mothers exposed to an unstable social environment throughout pregnancy exhibited lower plasma testosterone levels during the period of female sexual receptivity compared to male offspring born to mothers that remained in a stable social environment during pregnancy (Kemme *et al.* 2007). Male rat offspring born to mothers exposed to restraint stress during the last week of pregnancy exhibited significantly decreased plasma testosterone levels, elevated basal ACTH levels and a blunted response to stress (Richardson *et al.* 2006). Thus, evidence suggesting a role for testosterone in mediating the effects of prenatal stress on HPA axis activity and behaviour in male offspring is accumulating.

In the current study, we hypothesized that the decreased testosterone levels in male guinea pig offspring born to mothers exposed to stress during the fetal brain growth spurt, at least in part, mediates the differences in behaviour and endocrine activity exhibited by these animals. Hence, increasing plasma testosterone levels to those of control male offspring would ameliorate the differences in anxiety and attention-related behaviours and HPA axis activity.

Methods

Animals

Female guinea pigs (400–500 g) (Hartley strain, Charles River Canada, St Constant, PQ, Canada) were mated in our animal facility as described previously (Dean & Matthews, 1999). This method produces accurately time-dated pregnant guinea pigs. Food (Guinea Pig Chow 5025, Ralston Purina International, Leis Pet Distributing Inc., Wellesley, ON, Canada) and water were available *ad libitum*. The animals were kept in a 12:12 h light–dark cycle, with lights off at 19.00 h. Room temperature was 23°C. All studies were performed according to protocols approved by the Animal Care Committee at the University of Toronto, in accordance with the Canadian Council for Animal Care.

Pregnant guinea pigs were exposed to a strobe light for 2 h, from 09.00 h to 11.00 h, on gestational day (GD) 50, 51 and 52 (PS, $n = 15$). We have previously demonstrated that stress during this time period results in robust activation of the HPA axis in pregnant guinea pigs (Kapoor & Matthews, 2005). A control group of pregnant guinea pigs ($n = 12$) was left undisturbed throughout gestation except for routine maintenance. All animals were allowed to deliver normally. Normal litter size is two to three fetuses. Animals were weaned on postnatal day (PND) 25, tested in an open-field (30 min) and placed into individual clear polycarbonate cages. Animals were within visual, auditory and olfactory contact of at least two other animals at all times. Individual housing was a requirement for catheterization in adulthood. Offspring remained undisturbed except for behavioural testing and biweekly cage maintenance. On PND 75, male offspring were divided into four groups: control offspring with sham GDX (C–S, $n = 8$), control offspring with GDX plus testosterone replacement (C–T, $n = 8$), PS offspring with sham GDX (PS–S, $n = 10$) and PS offspring with GDX plus testosterone replacement (PS–T, $n = 8$). No more than one male from each litter was in any group. After GDX or sham surgeries, they were allowed to recover then subjected to a series of behavioural tests and salivary cortisol sampling. Animals were then catheterized to allow for blood sampling and measurement of HPA axis activity.

Gonadectomy and testosterone replacement

On PND 75, male guinea pig offspring were either surgically gonadectomised (GDX) and replaced with a testosterone pellet (7.5 mg; Innovative Research of America, Sarasota, FL, USA) or sham gonadectomised and a placebo pellet inserted to control for the stress of surgery. A pilot study was carried out to determine the pellet concentration that would yield plasma testosterone values similar to those previously determined in control adult male guinea pigs (Kapoor & Matthews, 2005). GDX was carried out as previously described (Anderson & Froimovitch, 1974; McGlinn *et al.* 1976). Briefly, male guinea pigs were anaesthetized using isoflurane (2–3%, IsoFlo, Isoflurane USP, Abbott Laboratories, Limited, Saint-Laurent, Quebec). Once the animal was anaesthetized, an incision parallel to the long axis of the body was made through the scrotal skin and tunica albuginea. The testicle was pushed through the incision and the seminiferous tubules were carefully separated from the tunica albuginea. The vas deferens and testicular artery were ligated, cut and testis was removed as a single mass. Testosterone and placebo pellets were subcutaneously inserted laterally to the neck. Animals were allowed to recover for 3 days following the surgery.

Behavioural analysis

Open-field. Ambulatory activity and thigmotaxis (time spent in the outer 14 cm of the arena) in a novel open-field (42 cm × 42 cm; 30 min) were determined twice using an Opto-Max animal activity meter (Columbus Instruments, Columbus, OH, USA), on PND 25 (at the time of weaning) and PND 78 (after GDX–T or sham surgery), as we have described previously (Kapoor & Matthews, 2005; Emack *et al.* 2008). Open-field analysis of the individual offspring was performed between 08.00 h and 10.00 h in a room with an ambient temperature of 23°C and standard fluorescent lighting, in which noise was minimized.

Prepulse inhibition and acoustic startle response. Assessment of sensorimotor gating and the acoustic startle response in male guinea pig offspring was undertaken at PND 30 (prepubertal) and again at PND 79 (after GDX and testosterone replacement or sham surgery). A single test unit (SR-Lab, San Diego Instruments, San Diego, CA, USA) was used. The acoustic startle cubicle was sound attenuated and equipped with a ventilation fan and house light. The animal holder was mounted on top of the startle platform that detected and transduced motion of the animal. The session was started with a 5 min acclimatization period with 70 dB background noise level that was continued throughout the test session. Animals received four pulses of 120 dB, 30 ms long, 10 kHz in order to establish baseline startle responses. These responses were not used to calculate PPI or ASR. Next, guinea pigs were tested on 60 trials. The trials consisted of 8 startle trials (120 dB, 10 kHz, 30 ms), 32 prepulse and startle trials that assessed PPI (8 for each prepulse intensity) and 4 no-stimulus trials. The PPI trials consisted of prepulses of 3, 6, 9 and 12 dB (10 kHz, 20 ms) above background (70 dB) followed by startle pulses. The pulses were initiated 100 ms after the onset of prepulses. The inter-trial interval ranged from 10 to 20 s. The trials were

presented in a pseudo-random fashion. Average responses during the 100 ms period following the termination of the startle stimulus were recorded. PPI was calculated according to the following formula: [1 – (startle amplitude on prepulse and startle trials/startle amplitude on pulse alone trials)] × 100 = % inhibition. ASR was calculated by taking an average of the startle amplitude on the pulse-alone trials. PPI has previously been tested in guinea pigs (Rehn *et al.* 2004) and the protocol described above was validated in pilot studies in our laboratory (A. Kapoor & S. G. Matthews, unpublished data).

Endocrine analysis

Saliva was collected for cortisol on PND 78, prior to the open-field (0 min), immediately after the open-field (30 min) and during the recovery phase (60 and 120 min), as we have previously described (Dunn *et al.* 2010). Briefly, cotton buds (Unilever, HPC-NA, Greenwich, CT, USA) were placed in the guinea pig's mouth and they were allowed to chew the bud for 15–20 s. Saliva was collected from the cotton buds by centrifugation (3000 rpm, 2 min) and stored at −20°C until use. Minimal animal handling is required for this collection technique.

On PND 80, catheters were surgically implanted in the carotid artery and attached to a swivel system (Lomir Biomedical Inc., Notre-Dame-de-I'lle-Perot, PQ, Canada) above the cage, as described previously (Liu & Matthews, 1999). This allowed full rotation of the catheter and unrestricted movement of the guinea pig. Repeated sampling of animals catheterized in this way does not result in activation of the HPA axis. On PND 83, blood samples were taken every 2 h from 07.00 h to 19.00 h for measurement of basal plasma ACTH and cortisol. Plasma was also collected on PND 82 and PND 83 for determination of plasma testosterone levels. Blood was collected into tubes containing EDTA–Trasylol, and plasma was separated by centrifugation and stored at –20°C. Upon completion of endocrine tests, animals were left undisturbed for at least 48 h prior to being killed by decapitation. Brains, pituitaries and adrenals were collected and weighed.

Double-antibody and coated tube radioimmunoassay kits (ICN Biomedical Inc., Costa Mesa, CA, USA) were used to determine plasma ACTH, cortisol and testosterone concentrations. These assays have been previously used in the guinea pig (Banjanin *et al.* 2004; Kapoor & Matthews, 2005). A high-sensitivity ELISA was used to measure levels of cortisol in guinea pig saliva (Salimetrics LLC, PA, USA) (Emack *et al.* 2008; Kapoor & Matthews, 2008). The intra-assay coefficients of variation were < 5% for all assays. All samples were processed in the same assay to negate inter-assay variability.

Statistical analysis

All data were expressed as mean ± standard error of the mean (S.E.M.). For all tests, significance was set at $P < 0.05$. Data were statistically analysed using *t* tests and analysis of variance (ANOVA). *t* tests were used for juvenile inner zone time, ASR and the salivary cortisol response to the open-field net area under the curve (AUC). One-way ANOVAs were used for juvenile ambulatory activity (time) and adult ASR. Two-way repeated measures ANOVA were used for juvenile ambulatory activity in the open-field (activity × time), juvenile PPI (% PPI × intensity) and juvenile salivary cortisol response to the open-field (salivary cortisol × time). Two-way ANOVAs were used for adult time spent in the centre of the open-field, adult salivary cortisol response to the open-field net AUC, adult plasma ACTH and cortisol total AUC, and adult basal plasma testosterone levels (prenatal stress × testosterone replacement). Three-way repeated measures ANOVA was used to analyse adult ambulatory activity, PPI, the salivary cortisol response to the open-field, plasma ACTH and cortisol (prenatal stress × testosterone replacement × time).

Results

Testosterone replacement

Testosterone levels after GDX and testosterone replacement or sham were: control–sham (C–S) 5.22 ± 0.26 ng ml^{-1}; control–testosterone (C–T) 5.34 ± 0.69 ng ml^{-1}; prenatal stress–sham (PS–S) 3.68 ± 0.45 and prenatal stress–testosterone replacement (PS–T) 5.33 ± 0.38 ng ml^{-1}. Two-way ANOVA of plasma testosterone levels averaged for each animal on PND 82 and 83 revealed significant main effects of prenatal stress ($P < 0.01$) and testosterone replacement ($P < 0.05$). PS males exhibited significantly lower plasma testosterone levels compared to the control males and testosterone-replaced males exhibited significantly higher plasma testosterone levels compared to the sham-operated males.

Open-field activity

Ambulatory activity in an open-field was summed at 5 min intervals for the 30 min exposure. In juvenile males (PND 25), two-way ANOVA revealed no overall effect of prenatal stress on ambulatory activity in the open-field, though there was an overall significant effect of time ($P < 0.05$; Fig. 1*A*). Repeated measures ANOVA of ambulatory activity over 5 min intervals for each treatment group revealed there was a significant effect of time in the control animals ($P < 0.01$) with activity decreasing over time, however there was no effect of time

in the male PS offspring. There was no effect of prenatal stress on time spent in the inner zone of the open-field at PND 25 (Fig. 1*B*).

In adult offspring, analysis of ambulatory activity in an open-field by three-way repeated measures ANOVA revealed a significant effect of prenatal stress ($P < 0.05$). Compared to control offspring, PS males exhibited significantly lower ambulatory activity over the 30 min period (Fig. 2*A*). There was also a significant effect of testosterone replacement, with testosterone-replaced animals exhibiting increased ambulatory activity compared to sham operated ($P < 0.05$). Adult male PS offspring that were not testosterone-replaced exhibited activity that began at a low baseline and maintained this decreased level throughout the 30 min open-field exposure. GDX and testosterone replacement in the control offspring did not result in any significant change in activity compared to the control sham GDX group. Two-way ANOVA analysis of the time spent in the inner zone of the open-field revealed no effect of prenatal stress or testosterone replacement (Fig. 2*B*).

Prepulse inhibition (PPI) and acoustic startle response (ASR)

Two-way ANOVA analysis of PPI in juvenile male offspring (PND 30) revealed a significant positive association with prepulse intensity on percentage PPI (% PPI; $P < 0.0001$; Fig. 3*A*). This analysis also demonstrated that PS offspring exhibited significantly increased % PPI over the four prepulse intensities ($P < 0.02$; Fig. 3*A*).

In adulthood (PND 79), three-way repeated measures ANOVA analysis revealed a significant effect of intensity ($P < 0.0001$; Fig. 3*B*), again with % PPI positively associated with the level of prepulse ($P < 0.05$, Fig. 3*B*). This analysis also revealed an effect of prenatal stress with PS male offspring exhibiting decreased % PPI compared to control offspring ($P < 0.02$). There was also a significant prenatal stress × testosterone interaction ($P < 0.03$), such that testosterone replacement in the PS males restored % PPI to values obtained in control offspring.

t test analysis for the ASR in PND 30 males revealed that those offspring born to mothers exposed to stress during

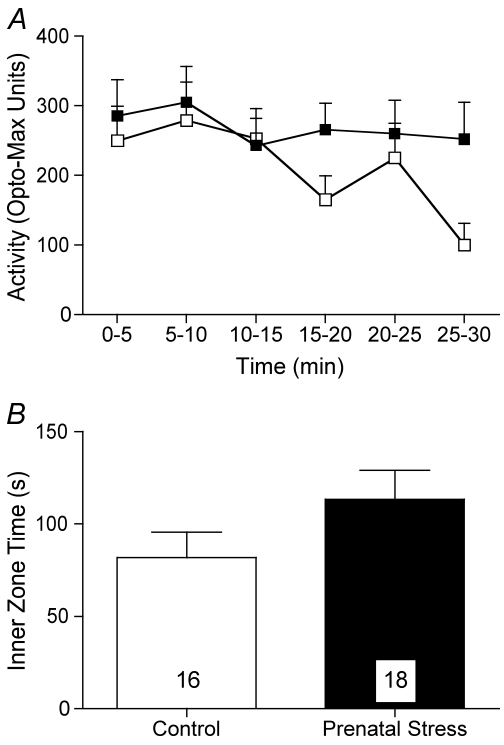

Figure 1. Behaviour (mean ± S.E.M.) in an open-field (30 min) of postnatal day (PND) 25 male guinea pig offspring born to mothers exposed to a high-frequency strobe light for 2 h on gestational days 50, 51 and 52 (prenatal stress, PS) or undisturbed throughout pregnancy (C)
A, ambulatory activity for C (□) and PS (■) in the open-field summed over 5-min intervals. *B*, time spent in the inner zone of the open-field over the 30 min open-field exposure. Animal numbers are indicated within bars.

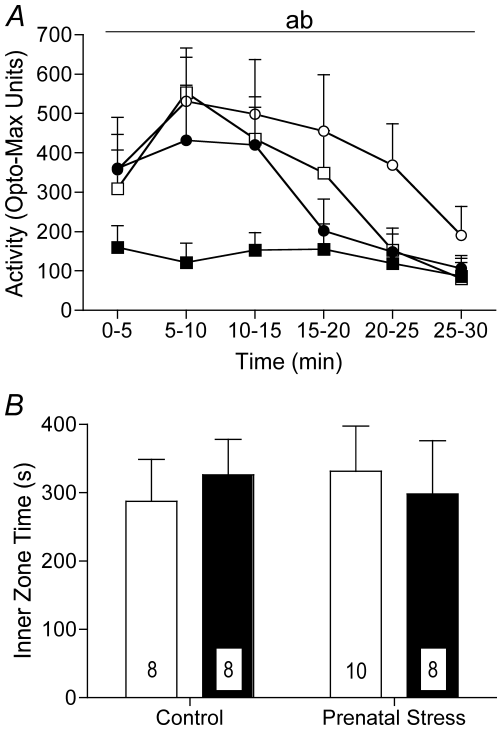

Figure 2. Behaviour (mean ± S.E.M.) in an open-field (30 min) of postnatal day (PND) 78 male guinea pig PS and C offspring
Adult male offspring were either gonadectomized (GDX) and replaced with testosterone (C–T, PS–T) or sham GDX operated (C–S, PS–S). *A*, ambulatory activity in the open-field summed over 5 min intervals (C-S (□); C–T (○); PS–S (■); PS–T (●)). *B*, time spent in the inner zone of the open-field over the 30 min open-field exposure. Open bars indicate sham, filled bars indicate GDX + T. Animal numbers are indicated within bars. 'a' indicates $P < 0.05$ PS *vs.* control, 'b' indicates $P < 0.05$ sham *vs.* GDX + T. Animal numbers are indicated within bars.

pregnancy exhibited a significantly lower ASR compared to control offspring ($P < 0.05$; Fig. 4*A*). This effect did not persist into adulthood. Two-way ANOVA revealed no effect of prenatal stress or testosterone replacement on the ASR in PND 79 male offspring (Fig. 4*B*).

Endocrine function

The salivary cortisol response to the stress of the open-field was measured at PND 25 and PND 78. At PND 25, both PS and C males exhibited a significant cortisol response ($P < 0.001$) to open-field exposure; however, there was no effect of prenatal stress on this response (Fig. 5*A* and inset). Similarly at PND 78, all groups exhibited a significant cortisol response to the open-field (Fig. 4*B*, $P < 0.0001$) and there was no effect of prenatal stress or testosterone replacement on salivary cortisol levels. Two-way ANOVA of the net AUC revealed a significant effect of PS on the salivary cortisol response to the open-field, with PS males exhibiting a decreased salivary cortisol response to stress compared to controls (Fig. 5*B* inset). There was no effect of testosterone replacement on the reduced salivary cortisol response to the open-field in the prenatally stressed adult offspring.

After catheterization surgery, plasma ACTH and cortisol levels were determined every 2 h from 07.00 h to 19.00 h. Three-way repeated measures ANOVA analysis of plasma ACTH levels revealed a significant effect of testosterone, with testosterone-replaced male offspring exhibiting significantly lower plasma ACTH levels over time compared to sham-operated animals ($P < 0.02$; Fig. 6*A*). There was a significant effect of time, with plasma ACTH levels increasing over the course of the day ($P < 0.001$). There was also a trend towards a prenatal stress, postnatal testosterone and time interaction ($P = 0.08$). There was a profound increase in plasma ACTH in the afternoon in the PS–sham animals compared to the control animals; however, testosterone replacement prevented the increase in plasma ACTH levels. Two-way ANOVA of the plasma ACTH AUC revealed that there was a trend towards an effect of testosterone replacement on total plasma ACTH levels, with testosterone-replaced PS and control males exhibiting decreased plasma ACTH levels ($P = 0.054$; Fig. 6*A* inset).

Three-way repeated measures ANOVA of basal plasma cortisol levels revealed a significant effect of time ($P < 0.0001$; Fig. 6*B*). There was also a trend towards an effect of prenatal stress ($P = 0.050$) and a trend towards

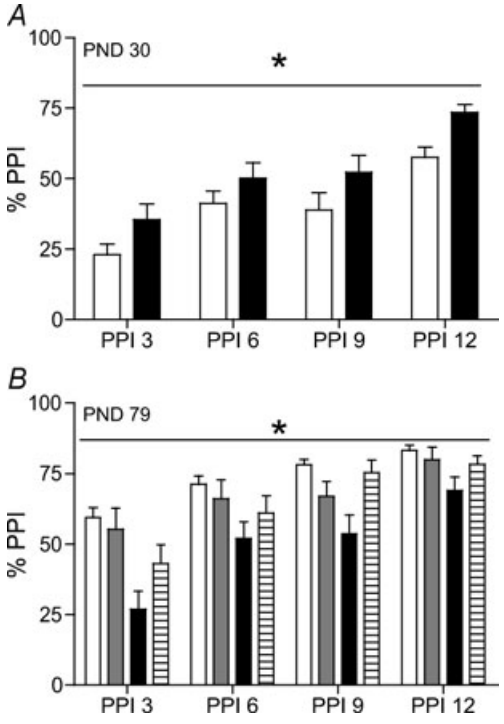

Figure 3. Per cent prepulse inhibition in response to an acoustic startle
Per cent prepulse inhibition (% PPI) in response to an acoustic startle with a prepulse of 3 dB (PPI3), 6 dB (PPI6), 9 dB (PPI9) and 12 dB (PPI12) above background in: *A*, postnatal day (PND) 30 male control males (open bars) or PS (black bars) or *B*, PND 79 male offspring that were either GDX and replaced with testosterone (C–T, grey bars; PS–T, black bars) or sham GDX (C–S, white bars; PS–S, striped bars). *indicates $P < 0.05$ between control and PS offspring.

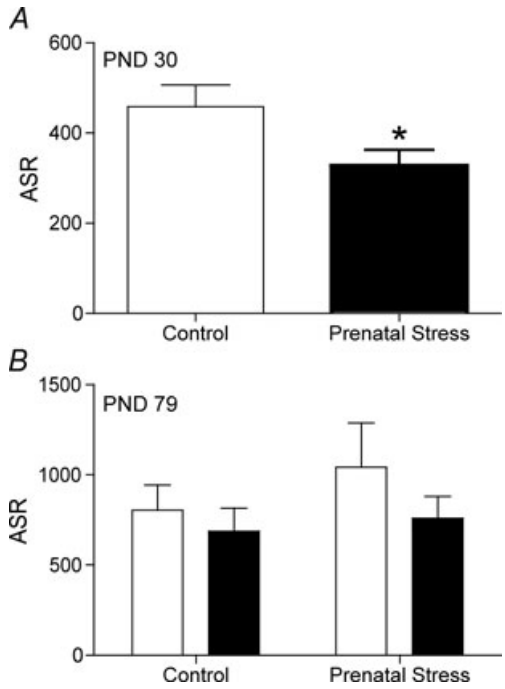

Figure 4. Acoustic startle response
Acoustic startle response (ASR) (mean ± S.E.M.) of: *A*, postnatal day (PND) 30 male control (open bars) or PS offspring (filled bars) or *B*, PND 79 male C and PS offspring that were either sham GDX (open bars) or GDX and replaced with testosterone (filled bars). *indicates $P < 0.05$ between control and PS offspring.

an interaction between prenatal stress and postnatal testosterone ($P = 0.084$). Two-way ANOVA of the total plasma cortisol AUC demonstrated a significant effect of prenatal stress, with PS animals exhibiting significantly higher plasma cortisol levels throughout the subjective day compared to controls ($P < 0.05$, Fig. 6*B* inset).

Discussion

In the present study, we have demonstrated that PS during the period of rapid fetal brain growth affects locomotor activity, sensorimotor gating (an index of attention) and the acoustic startle reflex (an index of fear) in male guinea pig offspring. There are also effects on stress-induced salivary cortisol levels during adulthood. Finally, we have demonstrated that the behavioural changes in prenatally stressed male offspring are, at least in part, dependent on PS-induced modification of plasma testosterone levels.

Measurement of plasma testosterone was undertaken after the catheterization surgery, approximately 6 days after behaviour in the open-field was assessed. Plasma testosterone levels were significantly lower in adult PS male offspring, and this is consistent with our previous studies (Kapoor & Matthews, 2005). GDX and testosterone replacement in the PS offspring increased plasma testosterone to levels seen in the controls.

Other studies have demonstrated effects of prenatal stress on HPA axis activity and sex steroid levels. Recently, in humans it was shown that the adolescent children of women who were in their third trimester of pregnancy during the Chernobyl disaster exhibited significantly higher levels of cortisol and testosterone (Huizink *et al.* 2008), demonstrating long-term programming of both glucocorticoids and sex steroids in humans. In rats, the

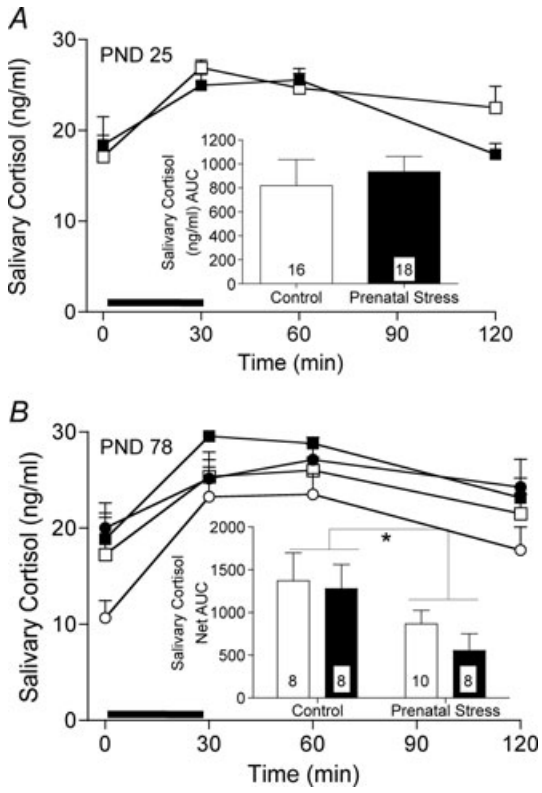

Figure 5. Salivary cortisol
Salivary cortisol (mean ± S.E.M.) in response to an open-field in (*A*) postnatal day (PND) 25 male guinea pig control (□) and PS (■) offspring. Net area under the curve (AUC) inset (*B*) PND 78 male C and PS guinea pig offspring. Adult male offspring were either GDX and replaced with testosterone (C–T (○); PS-T (●)) or sham GDX operated (PS-S (■); C-S (□)). Net AUC inset: open bars, sham; filled bars, GDX + T). Black line indicates time in open-field. *indicates $P < 0.05$ between PS and control offspring. Animal numbers are indicated within bars.

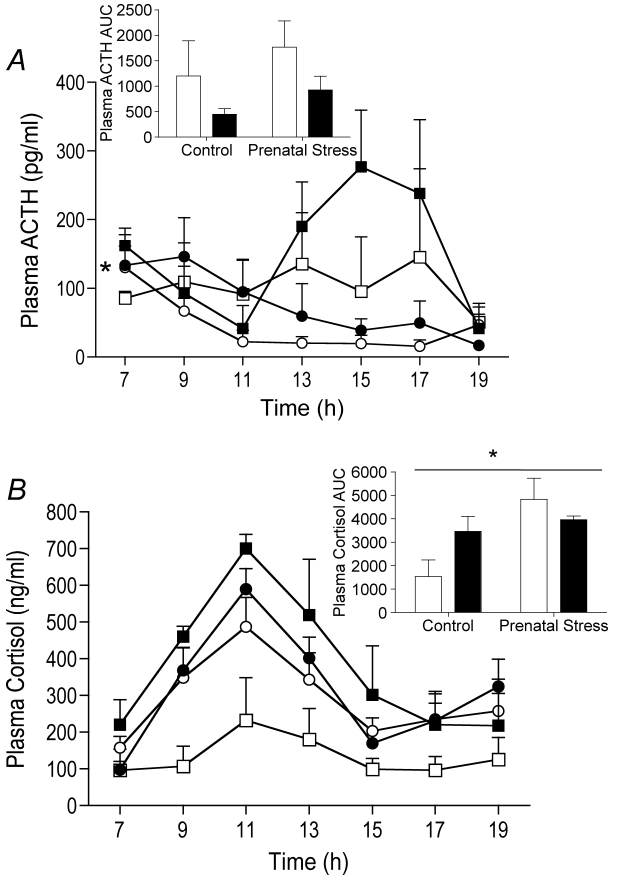

Figure 6. Plasma adrenocorticotrophin (ACTH) and cortisol
Basal plasma (*A*) adrenocorticotrophin (ACTH) levels from 07.00 h to 19.00 h in postnatal day (PND) 83 adult male control and PS guinea pig offspring. On PND 75, male offspring were GDX and replaced with testosterone (C–T, ○, $n = 8$; PS–T, ●, $n = 7$) or given a sham GDX surgery (C-S, □, $n = 4$; PS–S, ■, $n = 5$). Total area under the curve inset: open bars, sham; filled bars, GDX + T. *indicates $P < 0.05$ sham GDX *vs.* GDX + T. *B*, cortisol levels from 07.00 h to 19.00 h in adult male guinea pig offspring as described above. *indicates $P < 0.05$ PS *vs.* control animals.

seminal work by Ward *et al.* demonstrated that prenatal stress led to demasculinization and feminization of sexual behaviours (Ward, 1972). It was later shown that in a normal gestation, the male rat fetuses exhibited a surge in plasma testosterone levels on gestational days 18 and 19, but this rise in testosterone levels was absent in male fetuses whose mothers were stressed during pregnancy (Ward & Weisz, 1980). Furthermore, fetal corticosterone levels in response to maternal stress were found to be elevated only during the period of the stressor, while in the male fetuses, plasma testosterone levels also increased in response to the stressor and remained elevated (Ward & Weisz, 1984). This series of studies demonstrated that prenatal stress led to increases in both glucocorticoid and androgen secretion. In the current study, we did not measure testosterone or any other androgens in the pregnant guinea pigs exposed to stress. Further studies are required to determine if changes in maternal and/or fetal androgen levels are mediating programming of the hypothalamic–pituitary–gonadal axis in the prenatally stressed offspring.

In a hamster model, it has been demonstrated that adolescence is the most sensitive period for steroid-dependent organization of the brain (Schulz *et al.* 2009). In the current study, we replaced testosterone during adulthood, as we had shown in a previous study that plasma testosterone levels were significantly different between controls and prenatally stressed male offspring at PND 78 (Kapoor & Matthews, 2005). It is possible that the critical period for testosterone replacement is prior to or during puberty in our model and earlier replacement would have had a more substantial effect on reversing the outcomes associated with prenatal stress.

During adulthood, PS animals exhibited significantly lower ambulatory activity in the open-field and replacing testosterone in these animals increased activity to a level similar to that observed in controls. Previous studies have shown that androgens can facilitate locomotion. In rats, administration of androgens to GDX males resulted in increased exploratory behaviour in an open-field and in an elevated plus maze (Edinger & Frye, 2005). Recently, this was shown to be due to conversion of testosterone to its metabolite, 3α-androstanediol (Frye *et al.* 2010). Similar results were obtained in hamsters. Administration of 3α-androstanediol increased locomotor activity in an open-field (Frye *et al.* 2007). In the present study, it is possible that the reduced testosterone levels in the PS males led to lower levels of the 3α-androstanediol metabolite, and thus reduced activity in the open-field.

We have previously demonstrated that anxiety behaviour was increased in prenatally stressed juvenile and adult male offspring (Kapoor & Matthews, 2005). Interestingly, this was not observed in the current study. In the period since our previous study, all animal behavioural analyses have been relocated to purpose-built space with higher uniform light intensity. In rats, it has been shown that low levels of illumination led to increased exploration of an open-field (Garcia *et al.* 2005). Indeed, it appears that overall, time spent in the inner zone of the open-field were lower in the present study compared to our earlier study (Kapoor & Matthews, 2005). It is possible that this decreased movement in the inner zone was not sufficient to discriminate differences in thigmotaxis between control and prenatally stressed males. Another possible explanation for the discordant results is that in the present study animals were exposed to the GDX or sham surgery prior to open-field testing. The stress of a prior surgery may also have been a factor in the altered open-field behaviour.

PPI is a measure of sensorimotor gating. This is the process by which trivial or excess stimuli are 'gated-out' of awareness so that individual can focus attention on the most salient aspects of a stimulus-laden environment (Braff *et al.* 2001). In part, PPI has been shown to be dependent on serotoninergic and dopaminergic signalling (Mann *et al.* 2008). In the present study, sensorimotor gating was increased in juvenile PS offspring, as they displayed increased PPI compared to controls, but decreased in the adult PS offspring. A number of studies have demonstrated that PPI is disrupted in offspring exposed to stress during gestation; however, results have been mixed. Adult rat offspring born to mothers exposed to restraint stress three times during the last week of pregnancy, exhibited increased PPI compared to control offspring (Lehmann *et al.* 2000). In contrast, stress during the last week of pregnancy resulted in adult rat offspring that exhibited decreased PPI compared to controls across the range of prepulse intensities (Koenig *et al.* 2005). In the present study, the altered PPI may be suggestive of differences in serotonergic and/or dopaminergic signalling in male offspring whose mothers were exposed to stress during pregnancy. As the window of fetal neuroendocrine development targeted with the prenatal stress in the guinea pig was discrete, it will allow us to determine precisely how maternal stress affects developing signalling pathways in future studies.

In the juvenile offspring of mothers stressed during pregnancy, ASR was significantly reduced. Acoustic startle is an aversive stimulus that can be used as a measure of innate fear response (Brocke *et al.* 2006). Therefore, this would suggest that prior to puberty, prenatally stressed male offspring exhibit decreased fear compared to their control counterparts. In juvenile and adult rats, the amplitude of the ASR was decreased in those whose mothers were treated with dexamethasone during pregnancy (Kleinhaus *et al.* 2010), suggesting that alterations in the ASR may be due to prenatal activation of the glucocorticoid receptor. Interestingly, it has recently been shown that amniotic fluid testosterone levels are positively associated with fear reactivity in male

infants (Bergman *et al.* 2010). Further studies are required to determine if fetal testosterone levels are altered by maternal stress exposure in our model. Another possible explanation to the reduced ASR being due to a decrease in fear reactivity is that the prenatally stressed juvenile males have reduced hearing ability.

During adulthood, PPI was significantly decreased in PS males that were not testosterone replaced. This was opposite to the results obtained in juvenile offspring, and highlights the importance of studying outcomes of prenatal stress longitudinally. In the present study, testosterone replacement restored PPI to control values. Human and animal studies have demonstrated that PPI is increased in males compared to females (Braff *et al.* 2005; Plappert *et al.* 2005). This would implicate testosterone as having a role in mediating this sex difference. Indeed, in rats, administration of a 5-$HT1_A$ receptor agonist disrupted PPI, and this was reversed by administration of testosterone (Gogos & van den Buuse, 2003). Therefore, we provide strong evidence that the decreased sensorimotor gating exhibited by male offspring born to mothers exposed to stress results from the reduction in testosterone in these animals, and this may be through altered serotonergic signalling. This complex interaction between sex steroids and sensorimotor gating requires further study.

Plasma ACTH levels were significantly lower in testosterone-replaced males. There is a large body of evidence in support of an inhibitory role of testosterone on HPA axis activation. In male GDX rats, the plasma ACTH and corticosterone response to restraint stress was negatively correlated with the level of testosterone replacement (Viau & Meaney, 1996). Furthermore, levels of arginine vasopressin (AVP) in the hypothalamic median eminence were lower in testosterone-replaced rats, and the ACTH response to stress was directly correlated with AVP levels in the median eminence (Viau & Meaney, 1996). In male rats, GDX resulted in increased c-fos expression in the parvocellular region of the paraventricular nucleus (PVN) in response to restraint stress and increased levels of AVP heteronuclear RNA (Viau *et al.* 2003). The effect of testosterone on HPA axis activity is probably androgen receptor mediated, as adult male rats administered flutamide (an androgen receptor antagonist) failed to demonstrate the normal decrease in corticosterone due to habituation after repeated stress (Bingham *et al.* 2011). We also found a trend towards an interaction between prenatal stress, postnatal testosterone and time on plasma ACTH levels. This strongly suggests that testosterone replacement in the PS males prevents the increase in plasma ACTH levels observed in the sham-operated PS offspring.

Plasma cortisol levels were significantly higher in both PS male groups compared to controls, and this is consistent with previous studies in our laboratory (Kapoor & Matthews, 2005). Interestingly, testosterone replacement did not correct the elevated plasma cortisol levels. In the present study, replacement of testosterone to control levels in the GDX offspring that had not been prenatally stressed resulted in an elevation in plasma cortisol levels. This is somewhat counterintuitive, and we would have predicted normal cortisol concentrations in these animals, as they were replaced precisely to control values. It is possible that the ratio of free testosterone *vs*. testosterone bound to sex hormone binding globulin (SHBG) was decreased in the testosterone-replaced males, such that lower levels of free, biologically active testosterone in these animals resulted in increased plasma cortisol levels. Further measurement of free plasma testosterone levels would be needed to assess this possibility; however, limited supply of plasma precludes this analysis in the current study.

We found that prenatally stressed adult males exhibited lower net area under the curve levels of salivary cortisol, representing a diminished cortisol response to the stress of the open-field. This is in contrast to basal plasma cortisol levels, which were higher in the prenatally stressed males. There also did not appear to be an effect of prenatal stress on the basal (time 0) salivary cortisol levels. Salivary cortisol is representative of free cortisol levels (Fenske, 1996). It is possible that since we were measuring total cortisol (free cortisol and that bound to corticosteroid binding globulin (CBG)) in plasma and free cortisol in saliva that the prenatally stressed males had higher levels of CBG-bound cortisol. Indeed, there are some studies that have demonstrated that CBG levels are affected by stress during gestation. Adult female rats whose mothers were stressed during the last week of pregnancy exhibited increased CBG levels, but this was not seen in the males (McCormick *et al.* 1995). Pigs whose mothers were exposed to social stress late in gestation exhibited lower CBG levels than their control counterparts (Otten *et al.* 2010). Thus, it is possible that CBG levels are mediating the elevated plasma cortisol levels in the prenatally stressed males. Unfortunately, due to limited plasma availability, we were unable to test this in the current study. It is also interesting to note that testosterone replacement failed to alter salivary or plasma cortisol levels, suggesting that in our model, glucocorticoid regulations is not affected by circulating androgen levels.

In conclusion, we have demonstrated that moderate prenatal stress during the fetal brain growth spurt, results in male guinea pig offspring that exhibit differences in ambulatory activity, sensorimotor gating, acoustic startle response and pituitary–adrenocortical activity. This was associated with a reduction in plasma testosterone levels in these animals. Replacement of plasma testosterone to control levels in adulthood resulted in reversal of the behavioural effects of prenatal stress, but not changes in HPA function. As is becoming clear in human and animal prenatal stress studies, programming of the

HPA axis and stress-related behaviours is intricately linked with other physiological pathways, including the hypothalamic–pituitary–gonadal axis. This study has begun to address the mechanism by which sex steroids influence behaviours and HPA axis function in prenatally stressed offspring. This new information will also be important for the development of follow-up studies in human cohorts, where mothers have experienced adversity during pregnancy.

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