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

Amita Kapoor¹ and Stephen G. Matthews¹,²,³

¹Departments of Physiology, ²Obstetrics and ³Gynaecology and Medicine, Faculty of Medicine, University of Toronto, Medical Sciences Building, 1 King’s College Circle, Toronto, Ontario, MSS 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.
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 5HT1A (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 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

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.
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: \[1 - \text{pPPI} = \left(\frac{\text{startle amplitude on prepulse and startle trials/startle amplitude on pulse alone trials}}{\text{pulse-alone trials}}\right) \times 100\%\] 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-ÎIle-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 \(\times\) time), juvenile PPI (% PPI \(\times\) intensity) and juvenile salivary cortisol response to the open-field (salivary cortisol \(\times\) 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 \(\times\) 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 \(\times\) testosterone replacement \(\times\) 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 \((P < 0.05; \text{Fig. 1A})\). 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. 1B).

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. 2A). 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. 2B).

**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. 3A). This analysis also demonstrated that PS offspring exhibited significantly increased % PPI over the four prepulse intensities ($P < 0.02$; Fig. 3A).

In adulthood (PND 79), three-way repeated measures ANOVA analysis revealed a significant effect of intensity ($P < 0.0001$; Fig. 3B), again with % PPI positively associated with the level of prepulse ($P < 0.05$, Fig. 3B). 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 $\times$ 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. 4A). 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. 4B).

**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. 5A and inset). Similarly at PND 78, all groups exhibited a significant cortisol response to the open-field ($P < 0.0001$) 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 stress compared to controls (Fig. 5B 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. 6A). 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. 6A inset).

Three-way repeated measures ANOVA of basal plasma cortisol levels revealed a significant effect of time ($P < 0.0001$; Fig. 6B). There was also a trend towards an effect of prenatal stress ($P = 0.050$) and a trend towards...
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 \((P < 0.05, \text{Fig. 6B inset})\).

**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](image)

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 adrenocorticotropic (ACTH) and cortisol](image)

Basal plasma (A) adrenocorticotropic (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 serotonergic 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-HT1A 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 sensori-
motor 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
eeminence 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 cortisol 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.

References

Anderson M & Froimovitch M (1974). Simplified method of guinea pig castration. Can Vet J 15, 126–127.
Banjani S, Kapoor A & Matthews SG (2004). Prenatal glucocorticoid exposure alters hypothalamic–pituitary–adrenal function and blood pressure in mature male guinea pigs. J Physiol 558, 305–318.
Bergman K, Glover V, Sarkar P, Abbott DH & O’Connor TG (2010). In utero cortisol and testosterone exposure and fear reactivity in infancy. Horm Behav 57, 306–312.
Bingham B, Gray M, Sun T & Viala V (2010). Postnatal blockade of androgen receptors or aromatase impair the expression of stress hypothalamic-pituitary-adrenal axis habituation in adult male rats. Psychoneuroendocrinology. 2001. Human studies of prepulse inhibition of startle: normal subjects, patient groups, and pharmacological studies. Psychopharmacology 156, 234–258.
Braff DL, Geyer MA & Swerdlow NR (2001). Human studies of prepulse inhibition of startle: normal subjects, patient groups, and pharmacological studies. Psychopharmacology 156, 234–258.
Brunton PJ & Russell JA (2010). Prenatal social stress in the rat programmes neuroendocrine and behavioural responses to stress in the adult offspring: sex-specific effects. J Neuroendocrinol 22, 258–271.
Burton C, Lovic V & Fleming AS (2006). Early adversity alters attention and locomotion in adult Sprague-Dawley rats. Behav Neurosci 120, 665–675.
Clarke AS & Schneider ML (1993). Prenatal stress has long-term effects on behavioral responses to stress in juvenile rhesus monkeys. Dev Psychobiol 26, 293–304.
Clarke AS & Schneider ML (1997). Effects of prenatal stress on behavior in adolescent rhesus monkeys. Ann N Y Acad Sci 807, 490–491.
Davis EP, Glynn LM, Schetter CD, Hobel C, Chicz-Demet A & Sandman CA (2007). Prenatal exposure to maternal depression and cortisol influences infant temperament. J Am Acad Child Adolesc Psychiatry 46, 737–746.
Davis EP, Glynn LM, Waffarn F & Sandman CA (2010). Prenatal maternal stress programs infant stress regulation. J Child Psychol Psychiatry.
Dean F & Matthews SG (1999). Maternal dexamethasone treatment in late gestation alters glucocorticoid and mineralocorticoid receptor mRNA in the fetal guinea pig brain. Brain Res 846, 253–259.
Dobbing J & Sands J (1979). Comparative aspects of the brain growth spurt. Early Hum Dev 3, 79–83.
Dunn E, Kapoor A, Leen J & Matthews SG (2010). Prenatal synthetic glucocorticoid exposure alters hypothalamic–pituitary–adrenal regulation and pregnancy outcomes in mature female guinea pigs. J Physiol 588, 887–899.
Edinger KL & Frye CA (2005). Testosterone’s anti-anxiety and analgesic effects may be due in part to actions of its 5α-reduced metabolites in the hippocampus. Psychoneuroendocrinology 30, 418–430.
Emack J, Kostaki A, Walker CD & Matthews SG (2008). Chronic maternal stress affects growth, behaviour and hypothalamo-pituitary-adrenal function in juvenile offspring. Horm Behav 54, 514–520.
Fenske M (1996). Measurement of salivary cortisol in guinea pigs. J Exp Anim Sci 38, 13–19.
Frye CA, Babson A & Walf AA (2010). Self-administration of 3α-androstanediol increases locomotion and analgesia and decreases aggressive behavior of male hamsters. Pharmacol Biochem Behav 86, 415–421.
Frye CA, Edinger KL, Lephart ED & Walf AA (2010). 3α-androstanediol, but not testosterone, attenuates age-related decrements in cognitive, anxiety, and depressive behavior of male rats. Front Aging Neurosci 2, 15.
Garcia AM, Cardenas FP & Morato S (2005). Effect of different illumination levels on rat behavior in the elevated plus-maze. Physiol Behav 85, 265–270.
Gaskin JH & Kitay JI (1971). Hypothalamic and pituitary regulation of adrenocorticoid function in the hamster: effects of gonadectomy and gonadal hormone replacement. Endocrinology 89, 1047–1053.
Gogos A & Van Den Bause M (2003). Castration reduces the effect of serotonin-1A receptor stimulation on prepulse inhibition in rats. Behav Neurosci 117, 1407–1415.
Grizenko N, Shayan YR, Polotskaia A, Ter-Stepanian M & Van Den Buuse M (2003). Castration reduces the effect of serotonin-1A receptor stimulation on prepulse inhibition in rats. Behav Neurosci 117, 1407–1415.
Grizenko N, Shayan YR, Polotskaia A, Ter-Stepanian M & Van Den Buuse M (2003). Castration reduces the effect of serotonin-1A receptor stimulation on prepulse inhibition in rats. Behav Neurosci 117, 1407–1415.
Hanza RJ, Nunley KM, Lorenza SA, Louise JP, McGivern RF & Bollnow MR (1994). Androgen regulation of adrenocorticotropic and corticosterone secretion in the male rat following novelty and foot shock stressors. Physiol Behav 55, 117–124.
Huizink AC, Bartels M, Rose RJ, Pulkkinen L, Eriksson CJ & Kaprio J (2008). Cholesterol exposure as stressor during pregnancy and hormone levels in adolescent offspring. J Epidemiol Commun Health 62, e5.
Kapoor A & Matthews SG (2005). Short periods of prenatal stress affect growth, behaviour and hypothalamic–pituitary–adrenal axis activity in male guinea pig offspring. J Physiol 566, 967–977.
Kapoor A & Matthews SG (2008). Prenatal stress modifies behaviour and hypothalamic–pituitary–adrenal function in female guinea pig offspring: effects of timing of prenatal stress and stage of reproductive cycle. Endocrinology 149, 6406–6415.
O'Connor TG, Kaiser S & Sachser N (2007). Prenatal maternal programming determines testosterone response during social challenge. *Horm Behav* 51, 387–394.

Kleinhaus K, Steinfeld S, Balaban J, Goodman L, Craft TS, Malaspina D, Myers MM & Moore H (2010). Effects of excessive glucocorticoid receptor stimulation during early gestation on psychomotor and social behavior in the rat. *Dev Psychobiol* 52, 121–132.

Koenig JJ, Elmer GI, Shepard PD, Lee PR, Mayo C, Joy B, Hercher E & Brady DL (2005). Prenatal exposure to a repeated variable stress paradigm elicits behavioral and neuroendocrinological changes in the adult offspring: potential relevance to schizophrenia. *Behav Brain Res* 156, 251–261.

Lehmann J, Stohr T & Feldon J (2000). Long-term effects of prenatal stress experiences and postnatal maternal separation on emotionality and attentional processes. *Behav Brain Res* 107, 133–144.

Liu L & Matthews SG (1999). Adrenocortical response profiles to corticotrophin-releasing hormone and adrenocorticotrophin challenge in the chronically catheterized adult guinea-pig. *Exp Physiol* 84, 971–977.

McCormick CM, Smythe JW, Sharma S & Meaney MJ (1995). Sex-specific effects of prenatal stress on hypothalamic-pituitary-renal responses to stress and brain glucocorticoid receptor density in adult rats. *Brain Res Dev Brain Res* 84, 55–61.

McGlenn SM, Shepherd BA & Martan J (1976). A new castration technic in the guinea pig. *Lab Anim Sci* 26, 203–205.

Mann C, Croft RJ, Scholes KE, Dunne A, O’Neill BV, Leung S, Copolov D, Phan KL & Nathan PJ (2008). Differential effects of acute serotonin and dopamine depletion on prepulse inhibition and p50 suppression measures of sensorimotor and sensory gating in humans. *Neuropsychopharmacology* 33, 1653–1666.

Morris RW, Fung SJ, Rothmond DA, Richards B, Ward S, Noble PL, Woodward RA, Weickert CS & Winslow JT (2010). The effect of gonadectomy on prepulse inhibition and fear-potentiated startle in adolescent rhesus macaques. *Psychoneuroendocrinology* 35, 896–905.

O’Connor TG, Ben-Shlomo Y, Heron J, Goldberg J, Adams D & Glover V (2005). Prenatal anxiety predicts individual differences in cortisol in pre-adolescent children. *Biol Psychiatry* 58, 211–217.

O’Connor TG, Heron J, Goldberg J, Glover V & Team AS (2003). Maternal antenatal anxiety and behavioural/emotional problems in children: a test of a programming hypothesis. *J Child Psychol Psychiatry* 44, 1025–1036.

Otten W, Kanitz E, Courret D, Veissier I, Prunier A & Merlot E (2010). Maternal social stress during late pregnancy affects hypothalamic-pituitary-adrenal function and brain neurotransmitter systems in pig offspring. *Domest Anim Endocrinol* 38, 146–156.

Plappert CF, Rodenbucker AM & Pilz PK (2005). Effects of sex and estrous cycle on modulation of the acoustic startle response in mice. *Physiol Behav* 84, 585–594.

Ramchandani P, Stein A, Evans J, O’Connor TG & ALSPAC study team (2005). Paternal depression in the postnatal period and child development: a prospective population study. *Lancet* 365, 2201–2205.

Rehn AE, Van Den Buuse M, Copolov D, Briscoe T, Lambert G & Rees S (2004). An animal model of chronic placental insufficiency: relevance to neurodevelopmental disorders including schizophrenia. *Neuroscience* 129, 381–391.

Richardson HN, Zorrilla EP, Mandyam CD & Rivier CL (2006). Exposure to repetitive versus varied stress during prenatal development generates two distinct anxiogenic and neuroendocrine profiles in adulthood. *Endocrinology* 147, 2506–2517.

Rodriguez A & Bohlin G (2005). Are maternal smoking and stress during pregnancy related to ADHD symptoms in children? *J Child Psychol Psychiatry* 46, 246–254.

Schneider ML, Moore CF & Kraemer GW (2004). Moderate level alcohol during pregnancy, prenatal stress, or both and limbic-hypothalamic-pituitary-adrenocortical axis response to stress in rhesus monkeys. *Child Dev* 75, 96–109.

Schulz KM, Zehr JL, Salas-Ramirez KY & Sisk CL (2009). Testosterone programs adult social behavior before and during, but not after, adolescence. *Endocrinology* 150, 3690–3698.

Seale JV, Wood SA, Atkinson HC, Harbuz MS & Lightman SL (2004). Gonadal steroid replacement reverses gonadectomy-induced changes in the corticosterone pulse profile and stress-induced hypothalamic-pituitary-adrenal axis activity of male and female rats. *J Neuroendocrinol* 16, 989–998.

Tollenaar MS, Beijers R, Jansen J, Riksen-Walraven JM & de Weerth C (2011). Maternal prenatal stress and cortisol reactivity to stressors in human infants. *Stress* 14, 53–65.

Vlae V, Lee P, Sampson J & Wu J (2003). A testicular influence on restraint-induced activation of medial parvocellular neurons in the paraventricular nucleus in the male rat. *Endocrinology* 144, 3067–3075.

Vlae V & Meaney MJ (1996). The inhibitory effect of testosterone on hypothalamic-pituitary-renal responses to stress is mediated by the medial preoptic area. *J Neurosci* 16, 1866–1876.

Ward IL (1972). Prenatal stress feminizes and demasculinizes the behavior of males. *Science* 175, 82–84.

Ward IL, Ward OB, Affuso JD, Long WD 3rd, French JA & Hendricks SE (2003). Fetal testosterone surge: specific modulations induced in male rats by maternal stress and/or alcohol consumption. *Horm Behav* 43, 531–539.

Ward IL & Weisz J (1980). Maternal stress alters plasma testosterone in fetal males. *Science* 207, 328–329.

Ward IL & Weisz J (1984). Differential effects of maternal stress on circulating levels of corticosterone, progesterone, and testosterone in male and female rat fetuses and their mothers. *Endocrinology* 114, 1635–1644.
Weinstock M, Poltyrev T, Schorer-Apelbaum D, Men D & McCarty R (1998). Effect of prenatal stress on plasma corticosterone and catecholamines in response to footshock in rats. *Physiol Behav* **64**, 439–444.

Weller A, Glaubman H, Yehuda S, Caspy T & Ben-Uria Y (1988). Acute and repeated gestational stress affect offspring learning and activity in rats. *Physiol Behav* **43**, 139–143.

**Acknowledgements**

This work was supported by the Natural Sciences and Engineering Research Council of Canada (S.G.M.). A.K. was supported by scholarships from The Genesis Research Foundation–Obstetrics and Gynaecology Ontario Graduate Scholarships in Science and Technology and the Canadian Institutes of Health Research.