Prenatal glucocorticoid exposure programs adrenal PNMT expression and adult hypertension

P Nguyen[1,2], S Khurana[1], H Peltsch[1,2], J Grandbois[1,2], J Eibl[1,4], J Crispo[1,3], D Ansell[1,2] and T C Tai[1,2,3,4]

[1]Medical Sciences Division, Northern Ontario School of Medicine, Sudbury, Ontario, Canada
Departments of [2]Biology, [3]Chemistry and Biochemistry and [4]Biomolecular Sciences Program, Laurentian University, Sudbury, Ontario, Canada

Correspondence should be addressed to T C Tai
Email
tc.tai@nosm.ca

Abstract

Prenatal exposure to glucocorticoids (GCs) programs for hypertension later in life. The aim of the current study was to examine the impact of prenatal GC exposure on the postnatal regulation of the gene encoding for phenylethanolamine *N*-methyltransferase (PNMT), the enzyme involved in the biosynthesis of the catecholamine, epinephrine. PNMT has been linked to hypertension and is elevated in animal models of hypertension. Male offspring of Wistar-Kyoto dams treated with dexamethasone (DEX) developed elevated systolic, diastolic and mean arterial blood pressure compared to saline-treated controls. Plasma epinephrine levels were also elevated in adult rats exposed to DEX *in utero*. RT-PCR analysis revealed adrenal PNMT mRNA was higher in DEX exposed adult rats. This was associated with increased mRNA levels of transcriptional regulators of the PNMT gene: Egr-1, AP-2, and GR. Western blot analyses showed increased expression of PNMT protein, along with increased Egr-1 and GR in adult rats exposed to DEX *in utero*. Furthermore, gel mobility shift assays showed increased binding of Egr-1 and GR to DNA. These results suggest that increased PNMT gene expression via altered transcriptional activity is a possible mechanism by which prenatal exposure to elevated levels of GCs may program for hypertension later in life.

Key Words
- fetal programming
- hypertension
- adrenal
- phenylethanolamine *N*-methyltransferase
- Wistar-Kyoto rat
- glucocorticoids

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Introduction

The hypothesis of the Fetal Origins of Adult Disease, proposed by Barker (Barker 1995, Barker *et al*. 2006) associates low birth weight with the onset of cardiovascular disease later in life, a phenomenon otherwise known as fetal programming. This is mediated in part by adaptation of the fetus to adverse conditions that are encountered during development *in utero*. Examples of such stressors include maternal nutrient restriction, hypoxia, physical trauma, as well as exposure to alcohol and nicotine. There is a large and growing body of evidence linking an adverse prenatal environment to the fetal programming of cardiovascular disease in adult life (Vehaskari & Woods 2005, Cottrell & Seckl 2009, Alexander *et al*. 2015). Low birth weight and developmental programming of hypertension has been correlated with impaired kidney development and dysregulated renin-angiotensin-aldosterone system (Baum 2010, Goyal *et al*. 2010), impaired vascular structure and function (Pladys & Sennlaub 2005, Ligi *et al*. 2010), as well as altered activity of the hypothalamic–pituitary–adrenal (HPA) (Bouret 2009, Boyne *et al*. 2009), and the sympatho-adrenal (SA) axes (Young 2002, Johansson *et al*. 2007). Further, studies

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have observed that exposure to high doses of glucocorticoids (GCs) during fetal development, generated either as a consequence of maternal stressors or administered antenatally also reduces birth weight and leads to development of essential hypertension later in life (Edwards *et al*. 1993, Drake *et al*. 2007). The role of GCs in the fetal programming of hypertension has been investigated in sheep (Dodic *et al*. 2002), rats (Benediktsson *et al*. 1993, Levitt *et al*. 1996, Ortiz *et al*. 2002, O'Regan *et al*. 2008, Habib & Gattineni 2011), as well as in humans (Karemaker *et al*. 2006). However, the exact mechanisms by which GC-mediated fetal programming of hypertension occurs requires further investigation.

GCs are hormones which play a major role in the development of various organ systems *in utero* (Drake *et al*. 2007). They are also an important component of the HPA axis as part of the body's response to stress, acting upon the adrenal medulla to regulate the biosynthesis and secretion of the catecholamine epinephrine, a neurotransmitter/neurohormone that is physiologically significant in the sympathetic control of blood pressure and cardiovascular activity (Borkowski & Quinn 1984, Wong 2006). Its synthesis and release is regulated by the HPA and SA axes during the neuroendocrine response to stress (Axelrod 1976, Wong 2006). Elevated plasma levels of epinephrine are observed in humans with essential hypertension (Buühler 1982) as well as various animal models of hypertension, including the spontaneously hypertensive rat (SHR) (Jablonskis & Howe 1994).

The biosynthesis of epinephrine is regulated by phenylethanolamine *N*-methyltransferase (PNMT), the terminal enzyme in the catecholamine biosynthetic pathway. In hypertensive rats, adrenal PNMT mRNA, protein and enzyme activity are elevated compared to their non-hypertensive counterparts resulting in the increased adrenergic function associated with hypertension (Axelrod 1976, Reja *et al*. 2002). Numerous genetic linkage studies have investigated PNMT as a candidate gene for hypertension in rats and humans (Kaneda *et al*. 1988, Hoehe *et al*. 1992, Koike *et al*. 1995). The differences in PNMT expression in hypertension are not attributed to polymorphisms within the PNMT gene suggesting that altered regulation of the PNMT gene can account for changes in its expression (Nguyen *et al*. 2009, Khurana *et al*. 2015). Studies to date have identified key regulatory transcription factors involved in trans-activation of the PNMT promoter in adrenal chromaffin and PC12 cells, such as Egr-1, Sp1, AP-2, HIF-1 α, MAZ and the glucocorticoid receptor (GR) (Ebert *et al*. 1994, Tai *et al*. 2002, 2009, 2010*a*, Her *et al*. 2003). GCs are key regulators of PNMT, and we have previously demonstrated that the induction of PNMT gene expression *in vitro* upon GC administration is mediated by GC response elements (GRE) within the PNMT promoter (Tai *et al*. 2002). Further, altered activity of the transcription factors regulate PNMT not only *in vitro* (Tai *et al*. 2002, 2010*a*), but also in immobilization stress (Tai *et al*. 2007, Wong *et al*. 2008) as well as in a genetic model of hypertension, the SHR (Nguyen *et al*. 2009).

The aim of this study was to investigate the effects of prenatal GC administration on the molecular mechanisms involved in the dysregulation of adrenal PNMT in a fetal programming model of hypertension, by examining expression of transcriptional regulators of the PNMT gene in male offspring of Wistar-Kyoto (WKY) dams injected with dexamethasone (DEX). Results from this study show changes in key transcription factors regulating PNMT expression in the adrenal glands of DEX-treated male WKY offspring, coincident with elevated adrenal PNMT expression. This suggests that altered regulation of adrenal PNMT expression, along with coincident elevated epinephrine biosynthesis, is an important component of GC-induced fetal programming of adult hypertension.

Materials and methods

Animals

Male ($n=3$) and female ($n=12$) WKY rats were obtained from Charles River Laboratories (Montreal, QC, Canada) at 10 weeks of age. All animals were supplied with food (Harlan Teklad standard rat chow; Indianapolis, IN, USA) and water *ad libitum*. All animal procedures were approved by the Laurentian University Animal Care Committee, in accordance with guidelines from the Canadian Council on Animal Care.

Breeding and dosing regimen

After acclimating to housing conditions for 2 weeks, males were introduced to virgin females overnight during estrous cycles until vaginal plugs were observed (denoted day 1 of pregnancy). Pregnant females were caged individually for the remainder of pregnancy. Upon entering the third trimester (gestational days 15–21), pregnant female WKY rats were administered daily s.c. injections of DEX (0.1 mg/kg per day), or saline vehicle (control group; 4% ethanol/0.9% saline solution) (Levitt *et al*. 1996, Oliveira *et al*. 2006).

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Physiological measurements

Male offspring of WKY dams were weaned at 3 weeks of age, and consequently subject to weekly blood pressure measurements via a non-invasive tail-cuff plethysmography method (CODA 6; Kent Scientific, Torrington, CT, USA) as previously described (Feng *et al.* 2008, Nguyen *et al.* 2009). Animals were adapted to the measurement process during the first week of measurement. For a given session, animals were introduced into plexiglass holders kept on thermal heating pads set to 30 °C with blood pressure measurement cuffs placed on the base of the tail. Animals were acclimated to measurement conditions for 10 min prior to 30 consecutive blood pressure measurements over 30 min. Body weights were also monitored during the measurement period. All measurements were conducted during the light cycle, between 0800 h and 1700 h.

Tissue collection

Following blood pressure measurements at 17 weeks of age, animals were anesthetized with i.p. administration of ketamine (Ketalean; Bimeda, Cambridge, ON, USA) and xylazine (Rompun; Bayer, Etobicoke, ON) (75 mg ketamine, 5 mg xylazine per kg body weight), then sacrificed by immediate decapitation, as described previously (Nguyen *et al.* 2009). Adrenal glands were frozen on dry ice, and subsequently stored at −80 °C until use.

RNA extraction

Total RNA was extracted from left adrenal glands of WKY rats via mechanical disruption of tissue (TissueLyser; Qiagen) in Trizol Reagent (Sigma–Aldrich) as described previously (Nguyen *et al.* 2009). Isolated total RNA pellets were resuspended in diethylpyrocarbonate (DEPC)-treated nuclease-free water and concentrations determined using spectrophotometric measurement of absorbance at 260 nm (NanoDrop; Nanodrop Technologies, Wilmington, DE, USA).

Semi-quantitative RT-PCR

Two micrograms of total RNA was treated with DNAse I (Sigma–Aldrich), and cDNA subsequently synthesized using Moloney Murine Leukemia Virus Reverse Transcriptase enzyme (Promega) as previously described (Nguyen *et al.* 2009). PCR was performed in 25 μl reaction volumes containing 125 ng of cDNA, using GoTaq Flexi DNA polymerase (Promega) along with 200 μM of dNTPs, and 25 ng of forward and reverse primer sequences specific for PNMT, tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), chromogranin A, Egr-1, Sp1, GR, AP-2 and cyclophilin (housekeeping control) (Sigma Genosys, Oakville, ON, USA). The primer sequences used for the amplification were the same as previously described (Nguyen *et al.* 2009). PCR products were resolved on 1.5% agarose gels and stained with ethidium bromide.

Protein extraction

Protein was extracted from right adrenal glands of WKY rats through mechanical disruption of tissue (TissueLyser; Qiagen) in RIPA lysis buffer solution (25 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 2 mM EDTA, 1% sodium deoxycholate, 1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma–Aldrich) and protease inhibitors (Complete mini-EDTA free protease inhibitor cocktail tablet; Roche Diagnostics), as previously described (Nguyen *et al.* 2009). Lysates were incubated on ice for 10 min prior to centrifugation (12 000 ***g*** for 20 min at 4 °C) to pellet cell debris, after which supernatant was collected and stored at −80 °C. Protein concentrations were determined by the Bradford method (BioRad).

Western immunoblotting

Western blot analysis of protein targets was performed as previously described (Tai *et al.* 2002, 2007, 2009). Briefly, 50 μg of total protein per sample was resolved on 12% SDS-polyacrylamide gels and subsequently transferred to PVDF membranes (BioRad) in transfer buffer (48 mM Tris, 39 mM glycine, 0.037% SDS, 20% methanol) at 100V for 1 h. Transfer and equal loading of protein were verified by Ponceau S stain (Sigma). Membranes were incubated for 1h at 4°C in blocking solution containing 5% skim milk in Tris-buffered saline with Tween-20 (TBST; 10 mM Tris-HCl, 150mM NaCl, 0.05% Tween-20), then rinsed in TBST (3×10 min) before overnight incubation with primary antibodies specific for rat PNMT (Immunostar, Hudson, WI, USA; 1:3000 dilution), Egr-1 (C-19, Santa Cruz Biotechnologies; 1:1000), Sp1 (PEP-2, Santa Cruz; 1:2500), GR (M-20, Santa Cruz; 1:2000), AP-2 (Millipore, Billerica, MA, USA, 1:2000) in 5% skim milk-TBST. Membranes were then rinsed in TBST (3×10 min) and incubated with HRP-conjugated secondary IgG (Sigma; 1:5000) in 5% skim milk-TBST for 1 h, followed by a final series of rinses in TBST (3×10 min). Proteins were then detected by enhanced chemiluminescence (Haan & Behrmann 2007) and subsequent exposure to film (Pierce, Rockford, IL, USA).

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Gel shift mobility assay

GMSAs were performed using 20 μg of total protein, combined with double-stranded oligonucleotides for Egr-1, Sp1, GR and AP-2 which were 5′ end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Promega) as described previously (Tai *et al*. 2002, Nguyen *et al*. 2009). Proteins and probes were incubated in binding buffer (20 mM Tris-HCl (pH 7.5), 40 mM KCl, 12% glycerol, 0.5 mg/ml bovine serum albumin, 5 mM $MgCl_2$, 1 mM DTT and 4 μg poly (dA-dT)) in 25 μl reaction volumes. Supershift assays were conducted to confirm protein/DNA complexes, wherein antibodies specific for Egr-1, Sp1, GR and AP-2 were added to binding reactions. After incubation on ice for 1 h, protein/DNA binding complexes were resolved on 7% polyacrylamide gels with subsequent exposure to film (Pierce).

Plasma corticosterone levels

Trunk blood was collected in EDTA-coated blood collection vials (Becton Dickinson, Mississauga, CA, USA) and centrifuged at 1500 ***g*** for 20 min. Plasma was subsequently collected and stored at −80 °C until use. Corticosterone levels were determined in duplicate for each sample ($n=4$ per group) using Milliplex immunodetection assays according to the manufacturer's protocol (Millipore).

Plasma epinephrine levels

Catecholamine levels were quantified using a CAT ELISA kit as per manufacturer's protocol (Immunobiological Laboratories International, Toronto, CA, USA). Plasma samples ($n=4$ per group) were first extracted on extraction plates coated with boronate affinity gel, with subsequent acylation and release. Plasma samples, along with catechol-*O*-methyltransferase (COMT), and standards for epinephrine were added to 96-well microtiter plates coated with anti-rabbit IgG. COMT metabolizes epinephrine into metanephrine. Following metabolism, anti-serum for epinephrine was added to the plate, which was allowed to incubate for 2 h at room temperature. Plates were washed six times using a 1X wash buffer containing TBS-T. Enzyme conjugate was then added for a 1 h incubation period at room temperature, followed by another series of washes prior to the addition of the substrate solution, which was allowed to incubate for 40 min at room temperature. A stop solution (1M NaOH, 0.25M EDTA) was added to stop the substrate reaction before measuring optical density using a spectrophotometer at 405 nm. Sample concentrations were determined from standard curves.

Quantitation and statistical analysis

Images of resolved PCR gels and autoradiographs were documented using Chemidoc XRS imaging system (BioRad), with subsequent densitometric analysis performed with QuantityOne software (BioRad). All data is represented as mean ± S.E.M. For the blood pressure measurements, the data set was analyzed by fitting each data set to a second order polynomial equation (Least Squares method for fitting), followed by Extra Sum-of-Squares *F* Test to compare the parameters of each equation to determine if the curves were significantly different or if one curve would adequately fit both data sets. For all the other experimental measurements, a *t*-test was performed between the saline and DEX groups. All analyses were done using GraphPad Prism software (GraphPad, La Jolla, CA, USA). Values of $P \leq 0.05$ were considered statistically significant.

Results

Physiological measurements

Blood pressure was measured in male offspring from 4 to 17 weeks of age (Fig. 1A, B and C). Average systolic blood pressure was higher in the DEX offspring as early as 4 weeks of age, and this persisted throughout the remainder of the measurement period, up to 17 weeks of age. Elevations of similar magnitude were also observed in the DEX-treated offspring with respect to diastolic blood pressure, and mean arterial blood pressure over the same time period. Body weight was also monitored during the measurement period (Fig. 1D). DEX-treated male offspring had lower average body weight than their saline counterparts from 7 weeks of age up to 17 weeks of age.

PNMT expression and plasma epinephrine concentration

Semi-quantitative RT-PCR was performed to compare levels of PNMT gene expression. At 17 weeks of age, adrenal PNMT mRNA levels were significantly higher within DEX-treated offspring compared to that of their saline-treated counterparts (Fig. 2A; 1.5-fold). The transcripts of TH and DBH from the catecholamine biosynthetic pathway were also elevated in the adrenals of prenatally DEX-exposed animals (data not shown).

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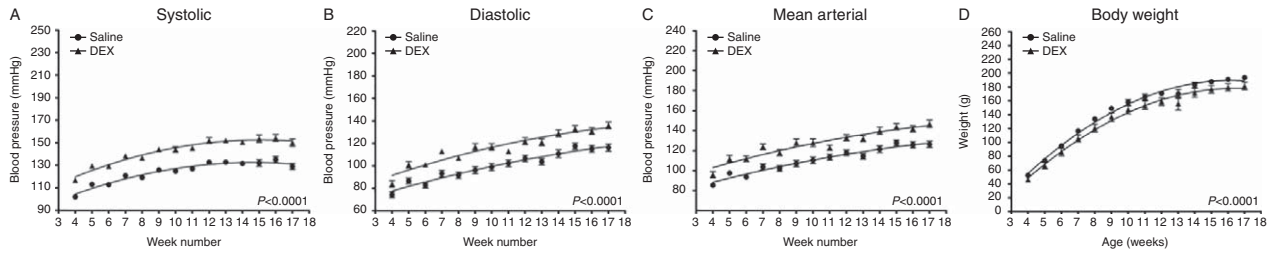

Figure 1
Blood pressure and body weight of male offspring born to saline or DEX-treated Wistar-Kyoto dams. The systolic (A), diastolic (B) and mean arterial blood pressure (C) were elevated, while body weight was lower (D) in prenatally DEX-exposed offspring. The data set was analyzed by fitting each data set to a second order polynomial equation (Least Squares method for fitting), followed by Extra Sum-of-Squares *F* Test to compare the parameters of each equation to determine if the curves were significantly different or if one curve would adequately fit both data sets. We found significant differences between the curves (saline vs DEX, $P<0.0001$). Please note that the *Y*-axes do not begin at 0 for Fig. 1A, B and C.

Western Blot analysis showed increased levels of PNMT protein present in DEX-treated offspring (Fig. 2B 1.44-fold). Plasma corticosterone and epinephrine levels were also assessed; there were no significant changes in plasma corticosterone levels in DEX-treated offspring compared to saline controls (Fig. 2C), while plasma epinephrine levels were increased in DEX-treated rats (Fig. 2D; twofold).

Alteration of transcriptional regulators of PNMT

To determine if altered PNMT expression in the DEX-treated offspring could be due to elevated transcriptional activity of PNMT, semi-quantitative RT-PCR was performed to examine expression of transcription factors Egr-1, Sp1, GR and AP-2 (Fig. 3). Egr-1 mRNA levels were highly elevated in DEX-treated offspring compared

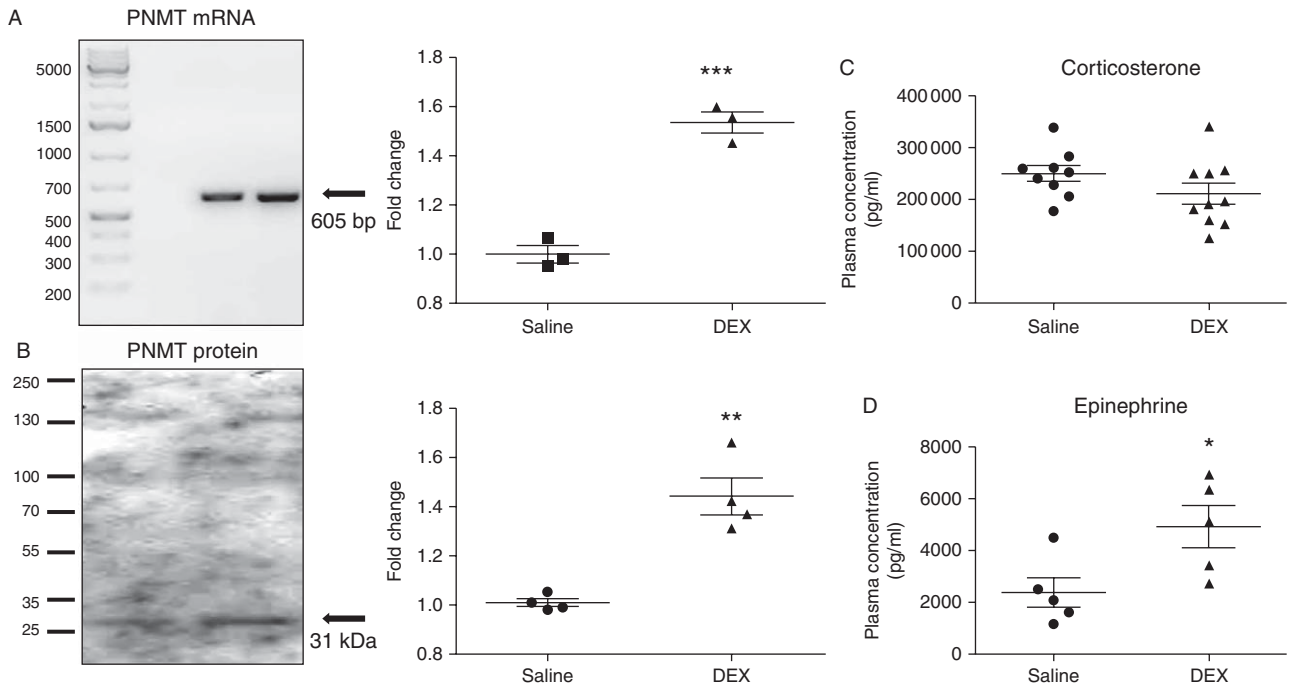

Figure 2
Effects of prenatal DEX exposure on PNMT expression and plasma corticosterone and epinephrine levels. Representative images of (A) semi-quantitative RT-PCR performed on rat adrenal mRNA, (B) Western immunoblotting performed on rat adrenal whole-cell lysate for PNMT, as well as plasma corticosterone (C) and (D) epinephrine levels determined by immunoassay. Fold changes between saline controls vs DEX-treated males are presented as mean ± S.E.M. (normalized to cyclophilin A control in PCR, and Ponceau S for the Western blot). Significant difference between groups designated as $*P\leq0.05$, $**P\leq0.005$, $***P\leq0.0001$. Please note that the *Y*-axes do not begin at 0 for the graphical representation in Fig. 2A and B.

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to saline-treated controls (2.5 fold). Similar increases were found with respect to AP-2 (1.9-fold), and GR mRNA in DEX-treated offspring (1.9-fold). Analysis of Sp1 mRNA showed not as much of an increase, but was still significant (1.18-fold). In addition, Western Blot analysis showed increased levels of Egr-1 (1.5-fold) and GR (1.7-fold) compared to SAL males (Fig. 4). No differences in Sp1 or AP-2 protein were observed between groups. The differences in expression of Egr-1 and GR protein are comparable to the changes in their respective mRNA levels. The ability of these proteins to bind and activate the PNMT promoter was also assessed using gel shift mobility assays (Fig. 5). In DEX-treated male offspring, there was increased binding of Egr-1 (1.7-fold) as well as GR to DNA (2.0-fold) compared to saline-treated rats. This is reflective of the increased levels of Egr-1 and GR mRNA and protein present in DEX treated males. No significant changes in binding were observed with AP-2, and only a slight increase with Sp1. DNA-protein complexes were confirmed by antibody supershift (data not shown).

Discussion

Antenatal GC administration has previously been used experimentally to program offspring for adult hypertension (Benediktsson *et al*. 1993, Levitt *et al*. 1996, Ortiz *et al*. 2002, O'Regan *et al*. 2008, Habib & Gattineni 2011). In this study, we demonstrate that administration of the synthetic GC, DEX during the third trimester of gestation results in elevated systolic, diastolic, and mean arterial blood pressure in WKY male rats. These changes are observed as early as 4 weeks of age, and are maintained up to 17 weeks of age. The administration of DEX has been well-established as a paradigm for programming of hypertension in rat offspring (Khurana *et al*. 2015). Our results are in agreement with previous studies that have shown programming of hypertension in offspring upon prenatal administration of DEX (Levitt *et al*. 1996, O'Regan & Kenyon 2004, Woods & Weeks 2005, Wyrwoll *et al*. 2007), corticosterone (Dodic *et al*. 2002, Singh *et al*. 2007) or betamethasone (Shaltout *et al*. 2009), during the third trimester of gestation.

Further, our study shows that DEX-treated offspring had a reduced level of growth compared to their saline-treated counterparts throughout the study period. Typically, DEX administration leads to offspring with reduced birth weight (Levitt *et al*. 1996), with accelerated postnatal growth (O'Regan & Kenyon 2004), resulting in hypertension and obesity associated with metabolic syndrome. However, it has also been shown that offspring born with low birth weight can also experience slowed or stunted growth, with persistently reduced body weight throughout childhood without influence from

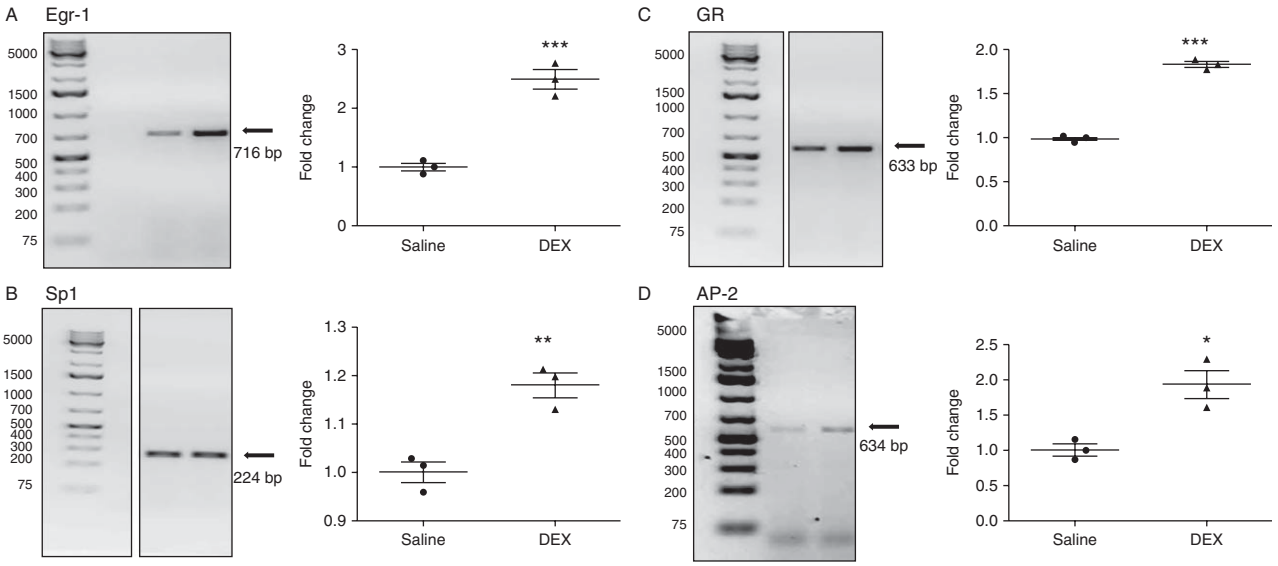

Figure 3
Effects of prenatal DEX exposure on transcriptional regulators by RT-PCR. Representative images of semi-quantitative RT-PCR performed on rat adrenal mRNA for Egr1 (A), Sp1 (B), GR (C) and AP-2 (D). Fold changes in expression levels between saline controls vs DEX-treated males are presented as mean ± S.E.M. (normalized to cyclophilin A). Significant difference between groups designated as $*P \leq 0.05$, $**P \leq 0.005$, $***P \leq 0.0001$. Please note that the *Y*-axis does not begin at 0 for the graphical representation in Fig. 3B.

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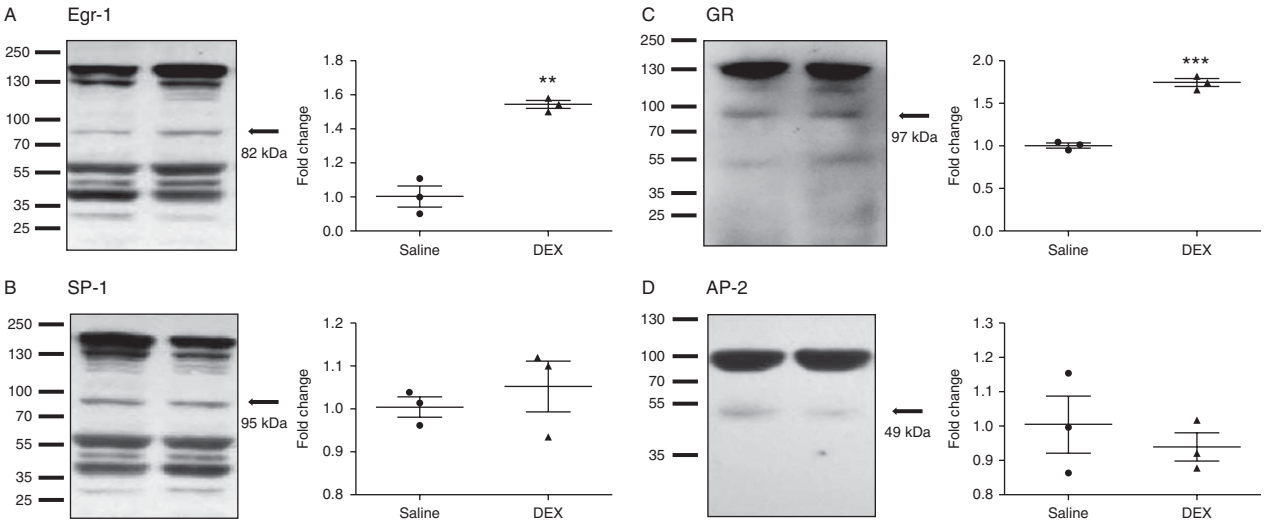

Figure 4
Analysis of the transcriptional regulators of PNMT by Western immunoblot. Representative images of semi-quantitative western blots performed on rat adrenal mRNA for Egr1 (A), Sp1 (B), GR (C) and AP-2 (D). Fold changes in expression levels between saline controls vs DEX-treated males are presented as mean ± S.E.M. (equal loading confirmed by Ponceau S staining). Significant difference between groups designated as $^{**}P \leq 0.005$, $^{***}P \leq 0.0001$. Please note that the *Y*-axes do not begin at 0 for the graphical representation in Fig. 4A, B and D.

environmental insults such as poor living conditions, and still develop adult hypertension (Barker *et al.* 2006). It is likely that the population of offspring in this study had developed hypertension according to this paradigm. Therefore, prenatal stresses on the offspring indeed program for hypertension later in life. However, the severity of hypertension and other associated risk factors for cardiovascular disease can be influenced greatly by environmental factors (Alexander 2007).

This study provides the first evidence that prenatal GC administration leads to increased PNMT gene expression in adult adrenal glands via altered transcriptional activity,

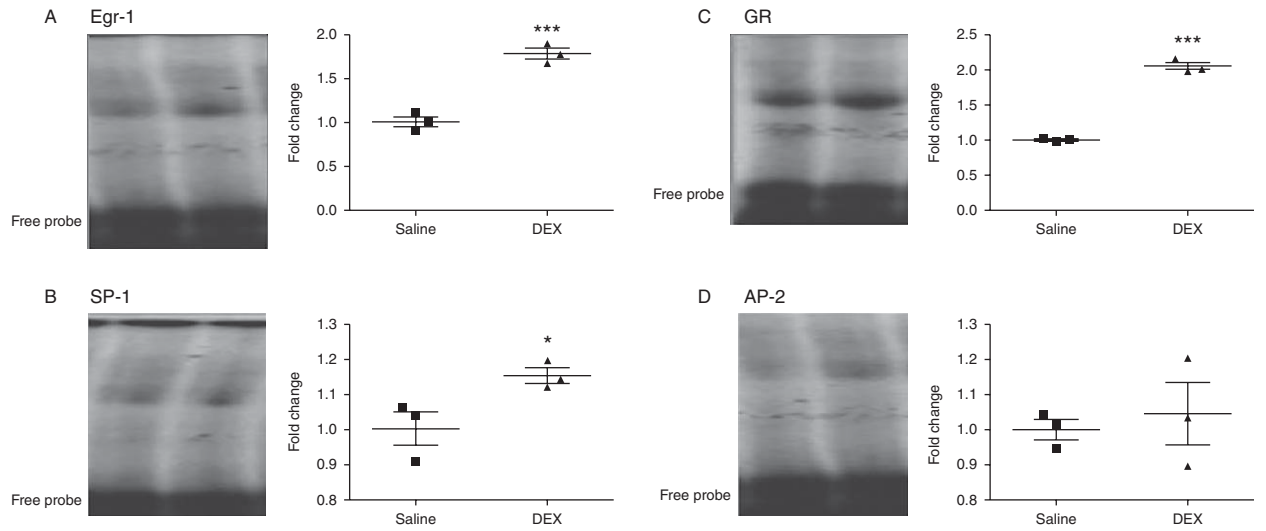

Figure 5
Comparison of affinity for binding the PNMT promoter sequence of adrenal gland protein. Representative panel of Gel Mobility Shift Assays (GMSA) performed on rat adrenal whole-cell lysates with radiolabeled oligonucleotides specific for Egr1 (A), Sp1 (B), GR (C) and AP-2 (D). Fold changes in binding levels between saline control vs DEX-treated males are presented as mean ± S.E.M. Significant difference between groups is designated by $^{*}P \leq 0.05$, $^{***}P \leq 0.0001$. Please note that the *Y*-axes do not begin at 0 for the graphical representation in Fig. 5B and D.

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resulting in elevated levels of circulating epinephrine. In particular, the observed elevation in PNMT expression in DEX-treated WKY offspring is associated with increased expression and activity of transcriptional regulators Egr-1 and GR. This is reflected for the most part in associated protein levels, similar to previous observations made in other animal models of hypertension such as the SHR (Reja *et al*. 2002, Nguyen *et al*. 2009), and experimental stress-induced models (Tai *et al*. 2007, Wong *et al*. 2008). In addition, Egr-1 and GR bind with greater affinity to DNA in the DEX-treated WKY offspring, suggesting these transcription factors are prominent in the dysregulation of PNMT in this model of hypertension. This finding suggests that prenatal GCs can alter expression of PNMT in adulthood. This has tremendous implications considering the role of epinephrine in blood pressure regulation and the link between PNMT and hypertension (Axelrod 1976, Hoehe *et al*. 1992, Koike *et al*. 1995, Wong 2006).

Plasma corticosterone levels were not significantly increased in the GC-programmed offspring compared to their saline-treated counterparts, an observation which contrasts some studies examining GC-induced hypertension (Levitt *et al*. 1996, Singh *et al*. 2007, O'Regan *et al*. 2008). Typically, such studies refer to the importance of the HPA and its potential as a programming target (Lesage *et al*. 2006). However, there are studies which have either found no changes, or even reduced plasma levels of corticosterone (Nyirenda *et al*. 2001, Lesage *et al*. 2004) in response to fetal programming by GC administration.

PNMT and epinephrine synthesis by adrenal chromaffin cells can be regulated by both the HPA and SA axis. In hormonal regulation of PNMT, chromaffin cells in the adrenal medulla are stimulated to produce epinephrine due to the secretion of GCs from the adrenal cortex, as a consequence of HPA activation. Additionally, neural regulation of PNMT is mediated by splanchnic nerve innervation as part of the SA system (Kvetňanský & Pacák 1995). It is suggested that the SA system can also be programmed for development of hypertension, with subsequent effects within the adrenal medulla, such as enhanced activity of chromaffin cells as early as 1 week of age (Molendi-Coste *et al*. 2006). Thus, increased activity of adrenal chromaffin cells may be dependent on dysregulation of either one or both the HPA and SA axes. In the SHR, a genetic model of hypertension, there is enhanced HPA activity mediated by increased levels of CRF mRNA, plasma ACTH and corticosterone (Djordjevic *et al*. 2007), as well as increased SA activity after periods of stress through increased levels of plasma epinephrine and norepinephrine (Kvetňanský & Pacák 1995). There is also increased activity of transcription factors Egr-1 and GR coincident with enhanced adrenal PNMT gene expression in SHR (Nguyen *et al*. 2009). The elevated activity of Egr-1 and GR observed in this study supports the notion of enhanced activity of both the HPA and SA axes in GC-programmed offspring, leading to increased PNMT expression and elevated epinephrine levels, which contribute to development of adult hypertension. As a key component of the neuroendocrine response to stress, epinephrine synthesis and secretion are both regulated by the HPA and SA axes (Axelrod 1976, Wong 2006). The HPA axis is required for adrenergic activation of PNMT gene expression (Kvetňanský & Pacák 1995), mediated via functional GC response elements (GREs) on the PNMT promoter which require activation by GR and also facilitate the recruitment of other transcription factors such as Egr-1 and AP-2 (Tai *et al*. 2002). PNMT expression in the adrenal medulla is also regulated by the SA system via splanchnic nerve innervation; this involves the neurotransmitters acetylcholine and pituitary adenylate cyclase-activating polypeptide (PACAP), whose signaling cascades regulate the transcription factors Egr-1, Sp1 and AP-2 (Morita *et al*. 1996, Wong & Tai 2002, Tai & Wong 2003, Tai *et al*. 2010*b*). Egr-1, expressed exclusively in adrenergic cells of the adrenal medulla (Criado *et al*. 1997), is well-known for its regulatory role in the expression of genes involved in catecholamine biosynthesis, particularly its induction associated with elevated PNMT expression in pheochromocytoma (Isobe *et al*. 2000). Also, it has been shown to be induced in response to stress *in vivo* together with GR (Tai *et al*. 2007). Furthermore, Egr-1, GR and AP-2 have been shown to synergistically activate the PNMT promoter (Wong *et al*. 1998, Tai *et al*. 2002, 2007), reinforcing the notion that elevated expression of these transcriptional regulators alter PNMT promoter activity, resulting in increased PNMT expression and epinephrine synthesis in GC-programmed offspring.

This study shows evidence of a molecular mechanism involved in the dysregulation of adrenal PNMT expression within a fetal programming model of hypertension, mediated by prenatal GC administration. Increased expression of the transcription factors Egr-1 and GR in the adrenal gland are associated with elevated PNMT expression levels, which subsequently lead to elevated epinephrine biosynthesis, likely contributing to the hypertensive phenotype of DEX-treated offspring. These results suggest that altered transcriptional regulation of PNMT expression through Egr-1 and GR is an important

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component of GC-induced fetal programming of adult hypertension, and this is mediated by enhanced activity of the sympathoadrenal system.

Declaration of interest

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

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