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Prenatal arsenic exposure alters the programming of the glucocorticoid signaling system during embryonic development

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Abstract

The glucocorticoid system, which plays a critical role in a host of cellular functions including mood disorders and learning and memory, has been reported to be disrupted by arsenic. In previous work we have developed and characterized a prenatal moderate arsenic exposure (50 ppb) model and identified several deficits in learning and memory and mood disorders, as well as alterations within the glucocorticoid receptor signaling system in the adolescent mouse. In these present studies we assessed the effects of arsenic on the glucocorticoid receptor (GR) pathway in both the placenta and the fetal brain in response at two critical periods, embryonic days 14 and 18. The focus of these studies was on the 11 β -hydroxysteroid dehydrogenase enzymes (11 β -HSD1 and 11 β -HSD2) which play a key role in glucocorticoid synthesis, as well as the expression and set point of the GR negative feedback regulation. Negative feedback regulation is established early in development. At E14 we found arsenic exposure significantly decreased expression of both protein and message in brain of GR and the 11 β -HSD1, while 11 β -HSD2 enzyme protein levels were increased but mRNA levels were decreased in the brain. These changes in brain protein continued into the E18 time point, but mRNA levels were no longer significantly altered. Placental HSD11B2 mRNA was not altered by arsenic treatment but protein levels were elevated at E14. GR placental protein levels were decreased at E18 in the arsenic exposed condition. This suggests that arsenic exposure may alter GR expression levels as a consequence of a prolonged developmental imbalance between 11 β -HSD1 and 11 β -HSD2 protein expression despite decreased 11HSDB2 mRNA. The suppression of GR and the failure to turn down 11 β -HSD2 protein expression during fetal development may lead to an altered set point for GR signaling throughout adulthood. To our knowledge, these studies are the first to demonstrate that gestational exposure to moderate levels of arsenic results in altered fetal programming of the glucocorticoid system.

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disclosure

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Keywords

Arsenic; Glucocorticoid; Placenta; Brain; Fetal; 11- β hydroxysteroid dehydrogenase isozymes

1. Introduction

Early developmental and adult exposure to arsenic is associated with a multitude of health problems, including cognitive (Rodriguez-Barranco et al., 2013; Rosado et al., 2007; von Ehrenstein et al., 2007; Wasserman et al., 2004), cardiovascular (McClintock et al., 2014; Moon et al., 2012; Wu et al., 2014), metabolic (Chen et al., 2009; Joshi and Shrestha, 2010; Lu et al., 2014) and metastatic (Ferrecio et al., 2013; Mostafa and Cherry, 2013; Wong et al., 1998; Xie et al., 2014) disorders that are manifested across the lifespan. The developmental origins of health and disease (DOHaD) theory (Gluckman et al., 2007; McMullen and Mostyn, 2009; Wadhwa et al., 2009) proposes that susceptibilities to chronic diseases are, in part, determined by physiologic changes initiated in utero in response to an altered prenatal environment. The theory posits that an adverse intra-uterine environment programs the development of the fetus to accommodate for this toxic environment by altering gene expressions. These adaptations are likely through epigenetic modifications. One system, the glucocorticoid signaling pathway has been demonstrated to be programmed in response to prenatal environments (Bolten et al., 2013; Conradt et al., 2013; Khulan and Drake, 2012; Lukaszewski et al., 2013; Reynolds, 2013) and these programming changes have been observed into adulthood (Goldstein et al., 2014; Khalife et al., 2013), including the transmission of the altered programming across generations (Luo et al., 2014). Further, there is support for the suggestion that altered glucocorticoid receptor (GR) programming could impact adult health (Brunton and Russell, 2011; Harris and Seckl, 2011; Merlot et al., 2008; Sarkar et al., 2008).

While there are many systems affected by arsenic, few have been implicated to play a role in all the various health problems associated with exposure. The GR signaling system, has been linked with cognitive deficits (Rodriguez et al., 2011), cardiovascular (Santos and Joles, 2012), metabolic (Sarr et al., 2012; Spencer, 2012), immune (Bellavance and Rivest, 2014) and metastatic diseases (Kitchin and Wallace, 2008; Schmitz et al., 2009). Several studies have demonstrated that arsenic produces specific disruptions in glucocorticoid transcriptional activity and steroid receptor function (Ahir et al., 2013; Barr et al., 2009; Bodwell et al., 2004; Davey et al., 2007; Gosse et al., 2014; Hamilton et al., 1998; Kaltreider et al., 2001; Shaw et al., 2007). Many of these studies have utilized cell lines or cultured cells, which provide a great deal of mechanistic information regarding the impact of toxins on specific cellular regulators but precludes the ability to assess the impact of arsenic on developmental programming including the interaction between maternal and fetal physiology.

Our own work using an in vivo mouse model exposed prenatally to 50 ppb arsenic in drinking water has found alterations in the levels of glucocorticoid signaling (Goggin et al., 2012; Martinez-Finley et al., 2009; Martinez et al., 2008), in the hippocampus from adolescent offspring. While it is clear that arsenic, both in vivo and in vitro, produces

significant changes in the glucocorticoid system, the impact of arsenic on the glucocorticoid system in both the developing fetus and the placenta have not been explored. Fetal levels of glucocorticoid are regulated by both the fetal and the placental tissues through the developmentally regulated isozymes, 11 β -hydroxysteroid dehydrogenases (11 β -HSD) 1 and 2, which interconvert active and inactive cortisol (corticosterone). In the mouse, placental expression of 11 β -HSD2 is elevated until about embryonic day 13 (E13), when it serves to inactivate maternal corticosterone and protects the developing fetus from the high glucocorticoid activation. Expression of 11 β -HSD1 begins around E14 and peaks at E18 (Brown et al., 1996; Speirs et al., 2004). 11 β -HSD1 is a reductase, which activates corticosterone and regenerates the glucocorticoid increasing fetal corticosterone levels. The developmental expression and levels of these isozymes are seen as being a critical regulation point for hypothalamic–pituitary–adrenal (HPA) axis negative feedback (Brunton and Russell, 2011; Chapman et al., 2013; Reichardt and Schutz, 1996). Maternal exposure to arsenic may alter the timing of 11 β -HSD isozyme expression levels in an attempt to adapt to the influence of arsenic on GR signaling. Additionally, arsenic readily crosses the placenta (Caumette et al., 2007; Fei et al., 2013; He et al., 2007; Vahter, 2009) and might directly alter expression and levels of the GR signaling complex within the developing fetal brain. Little is known about the impact of arsenic in utero on the developing fetal brain and placenta.

In the present study, we evaluated the effect of 50 ppb (50 μ g/L) arsenic on the glucocorticoid signaling system in both the fetal brain and placenta at two development time-points (E14 and E18) to identify critical shifts in the expression of signaling components which might impact the GR programming and later adult stress responding. The embryonic period from E14 to E18 is critical for the expression shift between 11 β -HSD2 and 11 β -HSD1 and for development of the negative feedback system for the centrally regulated HPA axis. If prenatal arsenic alters the level and/or the timing of the expression of these proteins which regulate the glucocorticoid response, then it may alter the central regulation of HPA axis programming and affect stress responding throughout life.

2. Materials and methods

2.1. Prenatal arsenic exposure paradigm

All procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of New Mexico. Animals were maintained in a 22 °C vivarium on a reverse light/dark cycle with lights on at 2000 and ad libitum access to water and food. Exposure to arsenic was performed as previously described during all three trimesters of development (Martinez et al., 2008; Tyler et al., 2014). Briefly, singly housed female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were acclimated to drinking 50 ppb arsenic-laced water (sodium arsenate, Na₂ HAsO₄·7H₂O Sigma-Aldrich, St. Louis, MO) for seven to ten days prior to mating. Dams were offered ad lib access to TekLad 2020X rodent chow and either 50 ppb arsenic in water or tap water alone during breeding and pregnancy until fetuses and placentas and brains were removed at gestational day (GD) 14 or GD 18. The day of pregnancy was designated as GD1. All tissue was collected early in the lights off period, between 0800 h and 1000 h.

For all of the analyses, there were no conditions where brains or placentas were pooled; only one brain or one placenta was used in the preparation. Male fetuses were selected from within the middle of the uterus. Only one embryo brain or its associated placenta was used from each dam per treatment condition. Due to the different tissue preparations required for protein versus mRNA analyses, different embryo brains and their associated placentas were represented in the Western and the qPCR assays.

2.2. Assessment of As5, As3, MMA and DMA

Levels of arsenic species were determined in whole brain from E14 and E18 arsenic and control fetuses using HPLC–ICP–MS. Samples were taken from 8 separate dams with one embryo per dam analyzed per group. Samples were kept frozen until sample analysis time. The brain tissue was thawed, homogenized and deproteinized by extraction into an equal volume of mobile phase (2 mM octanesulfonic acid and 2 mM malonic acid, pH 7). Control tissue blanks were included in the analysis. Samples were vortexed until dissolved and filtered (0.45 μ m filter). Samples were diluted 1:1 in deionized water and loaded into sealed autosampler vials and maintained at 20 °C. The analytical mobile phase was buffered at pH 4 and brought to volume in 2% HPLC grade methanol. A PerkinElmer Flexar HPLC was coupled with a NexION 300D ICP/MS through injection valve. The ICP/MS mode setting was a Dynamic Reaction Cell (DRC) using oxygen ($O_2 = 0.5$; $q = 0.5$) with a dwell time of 500 ms (2 pts/s). The HPLC separation used a C18 Shiseido Capcell Pak MG (5 μ m $\times$ 4.6 mm $\times$ 25 cm) with a mobile phase of 2 mM octanesulfonic acid +2 mM malonic acid +2% MeOH, pH 4.0 (adjusted with 10% NH_4OH) at a 1.5 mL/min flow rate, 1000–1600 psi, and a column temperature of 50 °C. Samples were injected at a 50 μ L volume. The outlet of the HPLC column was connected directly to an injection valve that is also connected to a glass Meinhard nebulizer leading to the glass cyclonic spray chamber of ICP/MS, allowing continuous transportation of the determinants to the argon plasma of ICP–MS. Retention time for the As species was determined using mixed standards of 10 μ g/L arsenic (III) oxide (iAsIII), arsenic (V) oxide (iAsV), dimethylarsinic acid (DMA) (all from Sigma-Aldrich Chemical Co), and methylarsonic acid (MMA) (Wako Pure Chemical Industries, Ltd). Peaks of different As species were identified by comparison with the retention times of individual standard compounds Calibration standards for Arsenite (As3), Arsenate (As5), Monomethyl Arsenic (MMA), and Dimethyl Arsenic (DMA) were prepared similar to the tissue samples using the buffered mobile phase. The detection limit was 0.010 μ g/L for iAsIII DMA, MMA and iAsV. The system was calibrated using a blank and four point calibration standards. Samples were analyzed in automated mode. Chromatogram retention times were adjusted, peaks were identified and the calibration curves linearity was verified for each species. The analytical data were reprocessed and validated.

2.3. Maternal plasma corticosterone levels

Pregnant dams at GD14 and GD18 were sacrificed by decapitation and trunk blood was collected using Safe-T-Fill EDTA capillary tubes (Ram Scientific Inc. #077051) and plasma was separated by centrifugation at 1000 $\times$ g for 10 min at 4 °C, and stored at –80 °C until use in the corticosterone assay. Corticosterone (CORT) levels in plasma were determined using the DetectX Corticosterone EIA Kit (Arbor Assays, Ann Arbor, MI; #K014-H1) per the

manufacturer's instructions. Optical density absorbance was determined by an Infinite M200 instrument and data analyzed using 4PLC ReaderFit online software.

2.4. Nucleic acid purification

gDNA and mRNA were co-purified using the Qiagen RNeasy Plus Mini Kit (cat: 74134; Qiagen Inc., Valencia, CA) and the Qiagen DNeasy Blood and Tissue Kit (cat: 69504) following a user-defined method obtained from the manufacturer. Briefly, frozen whole placentas or fetal brains were ground with a mortar and pestle on liquid nitrogen and lysed in the Buffer RLT Plus. 200–600 µL of tissue lysate was passed through a QIAshredder column (cat: 97654, Qiagen Inc.) to homogenize the sample. Tissue homogenate was placed directly onto the RNeasy spin column, omitting the gDNA Elimination column step, and processed using the standard RNeasy manual method. Tissue homogenate and Buffer RW1 flow-through were saved and divided into 2 equal volumes. 250 µL of 100% ethanol was added to each tube and tubes were stored for at least 2 h at –20 °C. The solution was then centrifuged for 15 min at 10,000 rpm, supernatant was removed, and the pellets were processed with the standard DNeasy manual method starting at the addition of Proteinase K and Buffer ATL. gDNA and mRNA were quantified on a NanoDrop 1000 Spectrophotometer (cat: NC9904842; Thermo Fisher Scientific, Wilmington, DE) with 260/280 ratios greater than 1.8 and 1.9, respectively. gDNA was stored at –20 °C and mRNA was stored at –80 °C. Any remaining tissue homogenate was stored at –80 °C.

2.5. mRNA reverse transcription and qPCR

1 µg of mRNA was reverse transcribed using the Qiagen QuantiTect Reverse Transcription Kit (cat: 205313) following the manual method. cDNA was quantified on a NanoDrop with 260/280 ratio greater than 1.8 and temporarily stored at –20 °C.

cDNA was run in triplicate on an ABI 7300 Real-Time PCR System (Life Technologies, Grand Island, NY) using the Roche FastStart Universal SYBR Green Master (ROX) (cat: 04913914001; Roche Applied Sciences, Indianapolis, IN). Primer efficiencies were established to be between 90% and 110% with endogenous control efficiencies within 5% of target efficiencies. Hprt endogenous control primer sequences (set A forward: TGACACTGGCAAACAATGCA and reverse: GGTCCTTTTCACCAGCAAGCT and set B forward: AAGACTTGCTCGAGATGTCATGAA and reverse: ATCCAGCAGGTCAGCAAAGAA) as described (Caldwell et al., 2008; Gilsbach et al., 2006). Target gene primer sequences for Fkbp4 (forward: CCTCTCGAAGGAGTGGACATC and reverse: TCCCCGATCATGGGTGTCT), Fkbp5 (forward: TGAGGGCACCAGTAACAATGG and reverse: CAACATCCCTTTGTAGTGGACAT), Hsd11b1 (forward: CAGAAATGCTCCAGGGAAAGAA and reverse: GCAGTCAATACCACATGGGC), Hsd11b2 (forward: GGTTGTGACACTGGTTTTGGC and reverse: AGAACACGGCTGATGTCCTCT), and Nr3c1 (forward: AGCTCCCCCTGGTAGAGAC and reverse: GGTGAAGACGCAGAAACCTTG) were from PrimerBank (Spandidos et al., 2008; Spandidos et al., 2010; Wang and Seed, 2003). Custom primers were purchased from either Invitrogen (cat: A15612; Life Technologies) or Sigma-Aldrich (cat: VC00021; Sigma-Aldrich Co. LLC., St. Louis, MO). No template and reverse transcription negative

controls were included and verified to be negative. All sample Ct values were less than 35 cycles and dissociation curves were analyzed to verify that only a single target was amplified. Gene target fold expression values were obtained using the Comparative CT method and statistical analysis was conducted on GraphPad Prism 6 Software, version 6.03 (GraphPad Software, San Diego, CA).

2.6. Sex determination qPCR

Due to low expression levels at early embryonic ages and dependent upon tissue type, gDNA was analyzed instead of mRNA for the Sry gene for sample sex determination. Sry primer sequences forward: GCTGGGATGCAGGTGGAAAA; reverse: CCCTCCGATGAGGCTGATATT (PrimerBank ID: 6755761a1) were purchased from Invitrogen (cat: A15612). Samples were run in duplicate using Roche FastStart Universal SYBR Green Master (ROX) according to standard methods. Sex was determined by raw Ct values: Male 22, Female 29. This method was verified with known samples.

2.7. Tissue preparation for protein quantification

Frozen (−80 °C) mouse placentas were homogenized on ice with a 5 mL conical Pyrex Tissue Grinder (26307-608; VWR) in 700 mL cold homogenization buffer (40 mM Tris-HCl, 4 mM CHAPS, 1:1000 Sigma Protease Inhibitor Cocktail, 200 μM sodium orthovanadate, pH = 7.5 at +4 °C brought up with NaOH). During homogenization, SDS was added for a final concentration of 1% v/v. The homogenate was split into 2 micro centrifuge tubes and vortexed at high speed for 20 s. Samples were then sonicated for 3, 15 second intervals and kept on ice between sonications. Cellular debris was removed by centrifugation at 15,000 ×g for 5 min at +4 °C. Supernatant was collected and stored at −80 °C.

Frozen (−80 °C) mouse fetal brains were homogenized following established protocols (Buckley and Caldwell, 2003) with the following modifications. Brains were homogenized in 100 μL of ice cold homogenization buffer (HB) (20 mM Tris-HCl, pH = 7.4, 1 mM EDTA, 320 mM sucrose, 1:1000 protease inhibitor cocktail (Sigma P8340), 200 μM sodium orthovanadate). Nuclei was separated by centrifugation at 1000 ×g for 6 min, supernatant was collected, pellet was resuspended in HB and spun again at 1000 ×g for 10 min, and the supernatant was combined with previous and stored at −80 °C as the post nuclear lysate (PNL, containing cytosolic and membrane proteins). Nuclei containing pellet was resuspended in extraction buffer (homogenization buffer with 75 mM NaCl, 75 mM KCl, and 1% v/v Triton X-100). Nuclei were lysed via sonication in 2, 15 s intervals. After resting 20 min on ice, cellular debris was removed by centrifugation at 15,000 ×g for 10 min at +4 °C. Supernatant containing nuclear proteins was collected and stored at −80 °C. Purity of nuclear (Nuc) and PNL fractions was verified by Western blotting probing for CREB and NR1 subunit of NMDA receptor.

2.8. Protein expression quantification via immunoblotting

Immunoblotting was conducted using established protocols (Caldwell et al., 2014) and adapted for fluorescent detection. Total loaded protein and antibody concentrations were optimized to be in the linear range of signal detection. Target proteins were assessed in the

E14 placental homogenate, E14 brain nuclear fraction, E14 brain PNL fraction, E18 placental homogenate, E18 brain nuclear fraction, and E18 brain PNL fraction. Samples (n = 12 per group) were run on the same blots for each target protein. To avoid membrane autofluorescence, we used the Immobilon-FL Transfer Membrane (Millipore; IPFL07810). Membranes were blocked in LI-COR Blocking Buffer (LI-COR 927–40000) for 1 h at room temperature. Membranes were incubated in primary antibody solution, overnight, at +4 °C. See table 1 for the total protein loaded and primary antibody concentrations and product info. Antibodies were diluted in PBS-T (10× PBS (Life Technologies; 70011-044), ddH₂O, 1% v/v Tween-20) Membranes were incubated for 45 min in corresponding LI-COR fluorophore labeled secondary antibodies: IRDye® 680 Donkey anti-Rabbit IgG (LI-COR; 926-68073) and IRDye® 800CW Donkey anti-Goat (926-32214), diluted 1:15,000 in PBS-T. Imaging of fluorophore labeled antibodies was performed using two-channel infrared detection Odyssey® Infrared Imaging System (LI-COR; LIC-9201-00) with Image Studio version 3.1 (LI-COR Biosciences). Quantification of band was conducted through Image Studio version 3.1. All blots were normalized by Coomassie staining (Perrone-Bizzozero et al., 1996) using IRDye® Blue Protein Stain (LI-COR; #3343C056). Total protein concentrations used were demonstrated to be within the linear range of this Coomassie staining protocol. All blots were corrected to their corresponding total protein Coomassie stains for quantification.

2.9. Statistical analysis

Data from mRNA and protein expression studies were analyzed using independent Student's *t*-test. Comparisons of protein changes across treatment conditions and developmental age were analyzed using a two-way ANOVA (SPSS v.22). Data are presented as mean ± SEM with n = 7–12 and are reported for each assay. For all experiments, the reported n refers to the number of litters and only one animal per litter is represented in each determination.

3. Results

3.1. Levels of arsenic species (As⁵, MMA and DMA) were elevated in the brains from arsenic exposed fetuses relative to controls

Both the reduced form of inorganic arsenic, trivalent (As (III)) and the oxidized pentavalent (As (V)) form, accumulate in tissues. Metabolism of arsenic by oxidative methylation leads to the formation of monomethylarsenicals (MMA III and MMA V) and dimethylated arsenicals (DMA III and DMA V). The levels of the trioxide form, arsenite, were not significantly different between the two exposure groups (Table 2).

3.2. Prenatal arsenic did not alter the number of fetuses, crown to rump length or placental weight at the specific gestational time points tested

While our earlier work did not observe differences in the number of live pups or in pup growth at PN7 (Martinez et al., 2008), it was important to evaluate the impact of prenatal arsenic on both the number of fetuses and their apparent developmental age (crown to rump length) in the present study. Additionally, there has been reported association between placental weight and the expression of 11β-HSD2 (Wyrwoll et al., 2009), thus we also measured wet weight of the placentas associated with the fetuses. There were no significant

differences in the number of apparent live normally developing fetuses between the two treatment conditions. The average number of live fetuses from dams ($n = 6-9$) at gestational days 14 and 18 exposed to arsenic or control water are presented in Supplemental Fig. 1A. Further, there were no differences in the number of nonviable fetuses between the treatment groups (data not shown). Since all dams were mated during a defined 12 hour period, all fetuses would be expected to be of the same apparent gestational age based on crown to rump length. The crown-to-rump lengths, measured at the time of removal, were not different at either gestational time point between the treatment conditions (Supplemental Fig. 1B). Finally, while placental weight did increase from E14 to E18, there were no differences between arsenic and controls at either of the two developmental time points (Supplemental Fig. 1C).

3.3. Prenatal arsenic did not alter maternal plasma corticosterone levels

Levels of corticosterone in plasma were determined at the time of euthanasia from 7 dams in each of the prenatal treatment conditions. There were no significant differences between the treatment groups for plasma corticosterone levels at GD14 or GD18 indicating the arsenic exposure condition did not alter maternal circulating levels of corticosterone (Table 3). Maternal corticosterone levels did increase from GD 14 to GD 18 as the time of parturition approached.

3.4. Impact of prenatal arsenic at E14 in placenta and fetal brain protein and mRNA

Our previous work in adolescent offspring of arsenic exposed dams revealed a dysregulation of central control of the HPA axis (Goggin et al., 2012). To explore the origins of this dysregulation, we evaluated the impact of prenatal arsenic on the GR pathway in both the placenta and fetal brain at E14 and E18. Fold mRNA expression for Nr3c1 (GR), FKBP5 (FKBP51), HSD11B1 (11b-hydroxysteroid dehydrogenase 1), and HSD11B2 (11b-hydroxysteroid dehydrogenase 2) were not different between control and arsenic exposed E14 placenta (supplemental Fig. 2). However, assessment of E14 placental protein revealed a significant increase in 11 β -HSD2 in the arsenic exposed group (Fig. 1 [t(13) = 2.4, $p = .04$, $r = 0.52$]) but there were no differences in the protein levels of GR, FKBP51 or 11 β -HSD1.

In whole E14 fetal brain, arsenic exposure produced a significant decrease in Nr3c1 (Fig. 2 [t(15) = 3.1, $p = .007$, $r = 0.8$]), HSD11B1 (Fig. 2 [t(10) = 2.6, $p = .03$, $r = 0.58$]) and HSD11B2 (Fig. 2 [t(14) = 2.1, $p = .05$, $r = 0.54$]) mRNA expression and had no effect on FKBP5 mRNA expression. Similarly, GR protein levels in both the post nuclear lysate (a membrane and cytosolic fraction, t(12) = 2.4, $p = .04$, $r = 0.57$) and nuclear fraction (t(12) = 2.8, $p = .02$, $r = 0.63$) were reduced in arsenic exposed E14 brain (Fig. 3). Levels of FKBP51 in the nuclear fraction were slightly higher in the arsenic E14 brain (Fig. 3 [t(14) = 2.3, $p = .04$, $r = 0.52$]) but not different in the post nuclear lysate fraction. 11 β -Hydroxysteroid dehydrogenases are membrane associated enzymes and thus, were assessed only in the post nuclear lysate fraction. Arsenic decreased expression 11 β -HSD1 (Fig. 3 [t(12) = 2.3, $p = .04$, $r = 0.55$]) and increased 11 β -HSD2 (Fig. 3 [t(12) = 2.6, $p = .03$, $r = 0.60$]) protein compared to controls.

3.5. Impact of prenatal arsenic at E18 in placenta and fetal brain protein and mRNA

Prenatal arsenic (50 ppb) did not significantly alter the E18 placental mRNA expression of HSD11B2 (11 β -hydroxysteroid dehydrogenase 2), Nr3c1 (GR), FKBP5 (FKBP51) or HSD11B1 (11 β -hydroxysteroid dehydrogenase 1, Supplemental Fig. 3). However, protein levels of GR (Fig. 4 [t (11) = 2.7, p = .02, r = 0.63]) were decreased in the E18 placentas taken from prenatal arsenic dams compared to controls. Placental levels of FKBP51, 11 β -HSD2 and 11 β -HSD1 proteins were not significantly altered by prenatal exposure (Fig. 4).

In E18 brain, prenatal arsenic exposure did not alter the mRNA expression of Nr3c1, FKBP5, HSD11B2 or HSD11B1. However, there was a trend for the mRNA expression of HSD11 β 1 to be lower in arsenic exposed E18 brains (Fig. 5, r = 0.42). Nuclear expression of GR protein was significantly decreased in prenatal arsenic E18 brain (Fig. 6 [t(12) = 2.3, p = .04, r = 0.55]), and no difference in nuclear localization of FKBP51 (Fig. 6). There were no changes in the post nuclear lysate fraction in either GR or FKBP51. Prenatal arsenic reduced the levels of 11 β -HSD1 (Fig. 6 [t(12) = 2.2, p = .04, r = 0.54]) while increasing 11 β -HSD2 [t(11) = 3.9, p = .003, r = 0.76] compared to control. Data are mean $\pm$ SEM, n = 7–12. The intensity of the specific antibody was corrected to within blot Coomassie stain as described in methods section.

3.6. Comparison of 11 β -HSD1, 11 β -HSD2, nuclear and post-nuclear lysate GR in E14 and E18 brain

In order to assess across the ages on the most significant and persistent changes in the brain proteins, a two way ANOVA was conducted with prenatal exposure and embryonic age as between group factors for: 11 β -HSD1, 11 β -HSD2, GR in the nuclear and post nuclear lysate fractions. For both 11 β -HSD 1 and 2 there were significant prenatal exposure effects [F(1,23) = 8.6, p = .007 and F(1,22) = 13.6, p = .001, respectively] and significant developmental time-point effects [F(1,23) = 155.4, p < .0001 and F(1,22) = 44.9, p < .0001] respectively and no significant interaction between prenatal treatment and embryonic age. This finding suggests that while the levels differed due to prenatal treatment, there were no differential effects on developmental trajectory of the expression levels of either isozyme. The analysis comparing both nuclear and post nuclear lysate fractions of GR observed a significant prenatal treatment effect [F(1,21) = 13.8, p = .001 and F(1,21) = 7.1, p = .015, respectively] but no effect of embryonic age or interaction between embryonic age and prenatal exposure. This is in agreement with the observation that expression of GR is not significantly altered by embryonic age. Moreover, the data indicate that GR expression levels, while altered by the prenatal treatment condition were not differentially altered across the two developmental time-points tested.

3.7. Impact of prenatal arsenic on expression of the mineralocorticoid receptor (MR) in nuclear and post-nuclear lysate in E14 and E18 brain

Changes in the levels of mineralocorticoid in brain have been associated with learning and memory (Sase et al., 2014) and have been proposed to play a key role in cognitive benefits of 11 β -HSD1 deficits (Yau et al., 2011). Our previous work using the adolescent perinatal arsenic mice, we observed a decrease in MR translocation to the nucleus upon activation suggesting a reduction in MR signaling (Martinez-Finley et al., 2011). Thus, it was

important to assess changes in MR protein expression in the embryonic brain. Prenatal arsenic exposure did not alter the expression levels of MR in either the nuclear or post nuclear lysate fractions from E14 and E18 fetal brain (supplemental Fig. 4).

3.8. Impact of prenatal arsenic on protein levels of corticotrophin releasing hormone (CRH)

CRH protein is expressed throughout the brain (Abou-Seif et al., 2012) but is enriched in the hypothalamus and hippocampus where it plays a role in negative feedback control. CRH levels were assessed in the brain post nuclear lysate at E14 and E18. Prenatal arsenic exposure lead to a decrease in CRH protein at E14 but there was no difference from controls at E18 (Fig. 7 [t(12)] = 3.0, p = .01). CRH levels in placenta were not different at either time point (data not shown). Thus while lower levels of CRH at E14 could explain the reduction in GR nuclear levels the normalized levels at E18 do not correspond to the continued decrease in nuclear GR seen at E18.

4. Discussion

Several researchers have identified the GR as a key target for arsenic effects (Ahir et al., 2013; Barr et al., 2009; Bodwell et al., 2004; Gosse et al., 2014; Simons et al., 1990; Stancato et al., 1993). The GR pathway sits at a critical interaction and integration point to play a role in all of the key arsenic associated effects from: inflammation to cognition, and from immunity to neoplasia. Using a comparative toxicogenomics data base, Ahir and colleagues (Ahir et al., 2013) identified the GR pathway as the top affected pathway for arsenic, along with several other thiol reactive metals. The GR pathways were affected by as little as 7.5 ppb arsenic. Despite significant evidence that GR is altered by arsenic, few studies have evaluated the developmental changes within the GR system and the potential impact of these developmental changes on later adult programming of the GR system.

The studies presented here represent an initial effort to determine if arsenic induced changes within the GR signaling pathways laid down in utero result in altered GR programming in the adult. Our current data are in agreement with our earlier published work on arsenic exposed offspring at postnatal day 35 (Goggin et al., 2012; Martinez-Finley et al., 2009; Martinez et al., 2008). In these studies, we found lower brain levels of GR protein and mRNA in the arsenic-exposed mice. Additionally, arsenic exposure altered the developmentally expressed ratio of the 11 β HSD isozymes. Proper levels and temporal expression of the 11 β -HSD1 is required for development of negative feedback regulation (Harris and Seckl, 2011; Harris et al., 2001). In both the neonate and the adult, negative feedback control over the HPA is established by activity in centrally located GRs within the hypothalamus, hippocampus, amygdala and frontal cortex (Arnett et al., 2011; Herman et al., 2012; Herman et al., 1989; Sapolsky et al., 1984; Sapolsky et al., 1991). In the developing fetus the negative feedback pathways are laid down around E16.5 and are modulated by intrauterine levels of corticosteroid; levels which are regulated by the 11 β -HSD isozymes. Placental expression of 11 β -HSD2 plays a key role in programming fetal tissue for sensitivity to glucocorticoids into adulthood, as it determines the level of maternal cortisol to which the fetal tissue is exposed. In the rodent, placental mRNA expression of 11 β -HSD2 is high up to E15.5, to exclude maternal circulating corticosterone from

interacting with the fetus and to prevent premature maturation. At E16.5 the mRNA expression of 11 β -HSD2 significantly decreases (Brown et al., 1996; Thompson et al., 2002) and the expression of 11 β -HSD1 isozyme increases; permitting an elevation of intrauterine corticosterone to assist in the maturation of organ systems (Thompson et al., 2002). In the present study, placental levels of 11 β HSD2 protein were elevated from the arsenic exposed dams at E14 but not at E18. At E18 arsenic reduced the levels of placental GR protein. While it is accepted that the placental 11 β HSD isozymes play a role in determining the amount and timing of intra-uterine glucocorticoid exposure, they are assumed to play a role in the activation of placental GR (Chapman et al., 2013). However, studies on the expression patterns of placental GR have identified an increase in expression up to E14 and then a more gradual increase and a small reduction in GR expression from E16 until birth (Speirs et al., 2004). Thus far, studies have failed to observe a change in GR expression relative to the pronounced developmental changes in the 11 β -HSD isozymes (Thompson et al., 2002). It remains unclear what role the placental GR expression plays with regard to fetal development. Most work in this area has focused on glucocorticoid effects on fetal growth and not on the impact of altered GR expression on the placenta. There is evidence that placental GR is involved in placental growth regulation (Gennari-Moser et al., 2011), but since there were no differences between the arsenic and controls in placental weights, it is unlikely that the reduced levels of placental GR had a significant impact on placental size.

In the present study, protein and mRNA expression was decreased for GR, while 11 β -HSD2 protein was increased at E14 and continued to be elevated in E18 brain, suggesting a possible maturational delay in the prenatal arsenic mice. Further there was a decrease in 11 β -HSD1 protein levels at both E14 and E18 brains indicating a bias away from corticosterone and activation of GR, which is further supported by decreased GR protein levels at E14 and E18 brains. An increase in 11 β -HSD1 is necessary for maturation and development of the negative feedback regulatory system. These findings, along with our previous work (Goggin et al., 2012), suggest that prenatal arsenic has damaged the regulators of neuronal glucocorticoid feedback regulation and altered the programming of the GR system.

Within the brain, areas of high levels of 11 β -HSD1 expression include areas (hypothalamus, hippocampus, amygdala and frontal cortex) which play a critical role in glucocorticoid negative feedback regulation (Chapman et al., 2013; Reichardt and Schutz, 1996). This enzyme serves to regenerate active glucocorticoids from their inactive 11-keto form. Negative feedback control over the HPA axis is established, in part, by levels of fetal corticosterone which can come from both fetal and maternal sources (Huang et al., 2012; Reichardt and Schutz, 1996). Normal levels of circulating corticosterone are seen in the 11 β -HSD1^{-/-} mice, but they have an impaired negative feedback demonstrating that 11 β -HSD1 activity is required for this process (Harris et al., 2001). A series of studies assessing 11 β -HSD1 expression following stress and glucocorticoid exposures suggests that changes in enzyme activity represented a key mechanism for turning up or down the negative feedback system (Jamieson et al., 1997). In our study we observed a decrease in 11 β -HSD1 at E14 and E18 and an increase in 11 β -HSD2 expression in brain. This profile would lower the level of GR activation and attenuate the proper development of the GR negative feedback system. This conclusion is supported by the reduction in GR within the nuclear fraction of

the fetal brain (Figs. 3 and 6). Moreover, in our work on adolescent offspring exposed in utero to arsenic, we observed a blunted corticosterone response to predatory scent stress, indicating that the failure of the negative feedback process in the arsenic exposed mice is initiated early in development and continued into postnatal life (Goggin et al., 2012).

In studies on adult rodents, HSD11B1 global knockout (Yau et al., 2011; Yau and Seckl, 2012) or partial pharmacological inhibition of 11 β -HSD1 (Sooy et al., 2010) have been shown to improve cognitive performance in several tasks. However, even though exposure to arsenic reduced levels of 11 β -HSD1 both the fetal and adolescent mouse brain, we observed a reduction in learning and memory performance in the prenatal arsenic mice (Martinez-Finley et al., 2009; Martinez et al., 2008; Tyler and Allan, 2013). There are several possible reasons for the apparent disagreement between these two sets of findings. Prenatal arsenic produced a small decrease in 11 β -HSD1 and an increase in 11 β -HSD2 in the embryonic brain. The ratio of the 11 β -HSD1 and 11 β -HSD2 is critical to establish the glucocorticoid negative feedback system (Holmes and Seckl, 2006; Huang et al., 2012). We believe that prenatal arsenic exposure, in altering this ratio of 11 β -HSD types during a critical period, results in a shift of the set point for central regulation of the HPA, resulting in a blunted feedback system. In studies using the 11 β -HSD1 knock out mice, it is unclear if there is an effect on the central HPA feedback system, but peripheral negative feedback inhibition appears to be reduced with an upregulation of the HPA axis and increase in adrenal output depending upon the background strain (Carter et al., 2009; Harris et al., 2001). Moreover, it is not clear in the 11 β -HSD1^{-/-} mice that there is a shift in the developmental expression of 11 β -HSD2 as seen in the prenatal arsenic exposed mice. Thus, the impact of in utero arsenic exposure on the glucocorticoid pathway may be more complex than in the global 11 β -HSD1 knockout or acute pharmacological inhibition. In the KO mice there appears to be a shift away from GR towards MR regulation (Yau et al., 2011). In human studies, patients with congenital deficiency in 11 β -HSD1, mineralocorticoid activity is abnormally high (Milford et al., 1995), supporting the observation that a genetic decrease in 11 β -HSD1 results in a shift towards increased MR signaling. Mineralocorticoid receptors in the brain are critical for memory formation and inhibition of the MR has been shown to attenuate learning (de Kloet et al., 2008; Harris et al., 2013; Joels et al., 2012; Pavlides and McEwen, 1999; Stranahan et al., 2010). However, MR brain levels in prenatal arsenic exposed E14 and E18 in both the post nuclear lysate and nuclear fractions were not different from those in control (Supplemental Fig. 4). Moreover, we have previously observed a decrease in MR activity in our prenatal arsenic mice (Martinez-Finley et al., 2009), suggesting that both MR signaling and GR signaling are deficient in the adolescent. This combined deficit in MR and GR may explain why we observe cognitive deficits despite the decrease in 11 β -HSD1 in the prenatal exposed arsenic mice.

In general, all of the mRNA expression findings are in agreement with the observed protein expression data, suggesting that there are no obvious effects of prenatal arsenic on protein translation, although in some assessments, the mRNA trended in the same direction as the protein but failed to achieve significance. An exception to this is that the mRNA levels for Nr3c1 were not reduced in the E18 brain from arsenic-exposed mice, but we did observe a lower nuclear level of GR within this tissue. In our mouse model, prenatal arsenic decreased

GR expression beginning at E14 and maintained to E18 in the brain from the exposed fetuses.

Glucocorticoids have been shown to regulate their own transcription (autoregulation) with elevations in glucocorticoid activity producing a decrease in GR mRNA levels (Burnstein et al., 1991; Govindan et al., 1991; Kalinyak et al., 1987). Counter to this, in our data we observed a decrease of 11 β -HSD1 as well as a decrease of GR in the E14 and E18 brains in the arsenic treated mice. This raises the question as to whether negative regulation of GR by glucocorticoids is fully developed at this embryonic time point. The ability of GR to bind to GRE has been shown to increase with gestational age (Jaskoll et al., 1996) with a 20 fold increase seen between E13 and E17 in the mouse. A study by Erdeljan et al. (2001), found that corticosterone decreased the levels of GR mRNA in cultured fetal hippocampi cells, indicating that negative autoregulation is possible in fetal cells. However, several in vivo studies, using exogenously applied glucocorticoids, have either failed to show an impact on GR expression (Kalinyak et al., 1987; Rosewicz et al., 1988) or have reported an increase in GR expression (Dean and Matthews, 1999; Sloboda et al., 2008). Further, it is commonly observed that as the expression of 11 β -HSD1 increases from mid gestation (E14) in the rodent, there is a corresponding increase in the levels of GR mRNA (Speirs et al., 2004). Within the fetal rodent brain, glucocorticoid and GR levels increase during development in parallel suggesting that the autoregulation seen in the adult is not as simple in the developing fetus. Thus, negative autoregulation of GR expression by glucocorticoids with the fetus appears to be more complex than in the adult.

Under basal conditions, the fraction of GR within the nucleus is a small portion of the total GR (Wang et al., 2002). Additionally, FKBP51 regulates the transport of GR into the nucleus (Zhang et al., 2008) and we observed an increase of nuclear FKBP51 in the arsenic exposed E14 brain. This suggests that transport of GR was not the cause of the lower nuclear levels of GR in the arsenic tissue. The lower nuclear level seen in our study is likely reflective of the reduced 11 β -HSD1 levels within the brain, as reduced intracellular formation of corticosterone is predicted to reduce the translocation of GR into the nuclear fraction. Reduced levels of GR within the nucleus would negatively affect gene transcription. In our previous work on using this developmental arsenic exposure model we observed a decrease in several transcriptional targets which are known to be regulated by GR (Martinez-Finley et al., 2011).

Reduction of the pentoxide form to the trioxide primarily utilizes glutathione (GSH). The dams were exposed to arsenate (As V) and the elevated levels of As V are indicative of the treatment but the fact that the levels of As III were not significantly elevated suggests that there is a limit to the reductive capacity in the fetuses. The conversion of the trivalent arsenic to methylated metabolites is through the activity of arsenic (3+) methyltransferase (Thompson et al., 2002). Methylation of the arsenic to MMA and DMA primarily depends on methyltransferase reactions utilizing S-adenosylmethionine (SAM) (Chen et al., 2009). Activity of the methyltransferases is critical for metabolic activity beginning at the blastocyst stage (Jin et al., 2010), thus it is likely that the methylation of As III is occurring rapidly in the arsenic exposed condition leading to low As (III) levels and higher MMA and DMA in the arsenic exposed fetal tissue. In our study there was a significant increase in the

concentration of As (III), MMA and DMA associated with the older embryonic age. It is possible that this is due to the increase in accumulation due to maturation of the brain between E14 and E18 as the brain size and cellular differentiation is greatly enhanced.

Several studies using cell lines have indicated that arsenic itself and, in particular, MMA forms of arsenic can inhibit the interaction of GR with glucocorticoid receptor response elements (GREs) in gene promoters. However, the DMA species failed to affect GR–GRE binding (Gosse et al., 2014). In the present study, we assessed the levels in fetal brain of both MMA and DMA and found them to be modestly elevated in the arsenic exposed fetuses, with the largest effect on the DMA levels. In distribution studies using in vivo exposure in rodents, levels of total arsenic are typically lowest in the brain relative to other organs and the DMA species is the most prevalent (Li et al., 2013; Lu et al., 2014). In studies using a maternal arsenic exposure model and assessing the offspring, the transfer of arsenic species into the developing fetal brain was highly efficient. Again, the DMA species of arsenic were the most prevalent form seen (Jin et al., 2010; Xi et al., 2010). Additionally, several others (Hughes et al., 2003; Kitchin et al., 1999; Styblo et al., 1995) have reported that a majority of arsenite is methylated to dimethyl arsenic, suggesting that the methylation reaction is highly effective even early in gestation. Our findings agree with these previous studies although our exposure levels are significantly lower and correspondingly, the total levels of arsenic seen in fetal brain are lower. Thus, while DMA did not directly interfere with GR–GRE binding in the cell culture model (Gosse et al., 2014), it may play a role in reducing the levels of GR and reducing GR activation as a consequence of the reduction of 11 β -HSD1 within the brain.

In adolescent offspring exposed to arsenic prenatally we saw a similar pattern of decreased levels of GR and 11 β -HSD1 in the hippocampus. We suggested that the decreased levels might be a means to compensate for elevated corticosterone in the offspring. However, given the present findings, it appears that the decreased GR and 11 β -HSD1 levels may not be a compensatory response, but represent an actual effect of the arsenic exposure during development which is maintained into adolescence (Goggin et al., 2012). The effect of the reduced GR signaling within the brain would result in a diminished negative feedback system.

Brain expression of CRH is evident as early as E13.5 in the mouse (Grino et al., 1989). It is widely distributed throughout the brain (Muglia et al., 1994) and plays many roles other than regulation of neuroendocrine regulation. Work with the *Crh*^{-/-} mice indicate that CRH is not required for maternal corticosterone secretion during pregnancy (Venihaki et al., 2000). CRH levels were lower in brain PNL at E14 in the arsenic exposed but were normalized by E18. Although the levels of circulating fetal corticosterone were not assessed in our study due to limited fetal blood, the lower levels of CRH at E14 would be predicted to reduce fetal corticosterone levels. This, along with increased 11 β HSD2 and decreased GR at E14, would add to the reduced GR activity in the fetal brain. Work by Huang et al. (Huang et al., 2012) using the *Cyp11a1* knockout mouse, observed that if the fetus fails to produce corticosterone the offspring have a blunted HPA negative feedback response. In our work on the adolescent offspring of arsenic exposed dams we observed a continued decrease in 11 β -HSD1 levels in brain, but unlike the present data, there was an increase in hypothalamic CRH levels

(Goggin et al., 2012). Despite the increase in hypothalamic CRH, prenatal arsenic exposed mice failed to display an increase in either ACTH levels or Pomc mRNA indicating either reduced or desensitized CRH receptors. In support of this, prenatal arsenic decreased hippocampal CRH receptor levels compared to control mice (Martinez et al., 2008). Thus, while there is a decrease in CRH levels in the fetal brain at E14 this did not result in an upregulation of CRH receptor in the adolescent brain. In the mouse CRH is first detected at E13.5 and then rises until E18, then decreases at birth and rises again into adulthood (Keegan et al., 1994; Schmidt et al., 2003). In our study, the levels are not different at E18 in the prenatal arsenic mice and this might suggest a developmental delay in expression that is caught up by E18 but then fails to recover postnatally.

Maternal corticosterone levels have been shown to play an important role in fetal growth (Xu et al., 2011) and in transplacental transmission of nutrients (Vaughan et al., 2012). However, in our study, fetal numbers and crown to rump lengths at the two gestational ages were not altered by arsenic. Moreover, maternal corticosterone levels were not different between the arsenic and control exposure conditions suggesting that the arsenic exposure is not producing a measurable stress response in the dams. Thus, the differences we have seen in the 11 β -HSD isozymes, brain CRH and GR levels are not due to differences in maternal circulating corticosterone levels between the two treatment conditions.

Maternal plasma corticosterone was elevated at gestational day 18 compared with day 14 (table 3). This is a common response in dams as parturition approaches (Barlow et al., 1975). While dam plasma corticosterone levels increase in pregnancy, the pregnant dam is hypo-responsive to stress (Douglas et al., 2003). Increased cortisol concentrations during maturation of the fetal hypothalamic–pituitary–adrenal (HPA) axis in the last weeks of pregnancy induce hyperactivity of the HPA axis due to permanent desensitization of glucocorticoid receptors in the hippocampus and a subsequent decrease in negative feedback regulation of the HPA axis.

5. Conclusions

In our previous work assessing the impact of perinatal arsenic exposures on the function of the central and peripheral HPA system, we found significant support to conclude that arsenic exposure in utero resulted in a suppression of the glucocorticoid negative feedback system due to a decrease in GR and 11 HSD1 in the hippocampus. It was unclear if this deficit in HPA function was due to an indirect effect of arsenic resulting in a compensatory response to altered fetal HPA development or if the changes seen in the adolescent mouse were reflective of the fetal changes. Whether the changes or accommodations in the GR pathway are in response to a direct effect of arsenic or are an indirect effect of arsenic on another system which intersects with the GR pathway is not clear from the present studies. What is indicated by these findings is that prenatal arsenic modifies the GR signaling pathway such that the system is set with a lower level of GR activity and that there is a deficit in the proteins needed to establish a normal negative feedback system in the brain. This altered GR set point and defective negative feedback system is maintained into the adolescent period and may be an important mediator of the behavioral deficits we have seen using this model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GR	glucocorticoid receptor
HSD	hydroxysteroid dehydrogenase
E14	embryonic day 14
E18	embryonic day 18
GD	gestational day
MMA	methylarsonic acid
DMA	dimethylarsinic acid
PNL	post nuclear lysate
Nuc	nuclear fraction

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Fig. 1.

Prenatal arsenic (50 ppb) significantly increased E14 placental protein expression of 11β-HSD2 in the arsenic exposed group compared to control, but did not alter levels of GR, FKBP51 or 11β-HSD1. Data are mean ± SEM, n = 8 *p = .04. The intensity of the specific antibody was corrected to within blot Coomassie stain as described in the Materials and methods section. Representative blot images are presented below the figure. Looking from left to right, the first 3 bands are control and last 3 bands are prenatal arsenic.

Fig. 2.

Prenatal arsenic (50 ppb) significantly reduced the level of mRNA expression of Nr3c1 (GR), HSD11B1 (11b-hydroxysteroid dehydrogenase 1) and HSD11B2 (11b-hydroxysteroid dehydrogenase 2). The E14 brain. No effect on the levels of FKBP5 (FKBP51) was observed. Fold expression of the target genes were acquired by the Ct (Comparative CT) method. Data presented are mean $\pm$ SEM n = 7–11, *p = .05, **p = .02.

Fig. 3.

Prenatal arsenic (50 ppb) significantly decreased E14 brain protein expression of GR in both the post nuclear lysate (* $p = .04$) and nuclear fractions (** $p = .02$). There was an increase in nuclear localization (* $p = .04$) and no change in the post nuclear lysate FKBP51. Prenatal arsenic reduced the levels of 11 β -HSD1 (* $p = .04$) while increasing 11 β -HSD2 (** $p = .03$) compared to control. Data are mean $\pm$ SEM, $n = 7$. The intensity of the specific antibody was corrected to within blot Coomassie stain as described in the Materials and methods section. Representative blot images are presented below the figure. Looking from left to right, the first 3 bands are control and last 3 bands are prenatal arsenic.

Fig. 4.

Prenatal arsenic (50 ppb) significantly decreased E18 placental protein expression of GR (* $p = .02$) in the arsenic exposed group compared to control, but did not alter levels of, FKBP51, 11 β -HSD2 or 11 β -HSD1. Data are mean $\pm$ SEM, $n = 7-10$. The intensity of the specific antibody was corrected to within blot Coomassie stain as described in the Materials and methods section. Representative blot images are presented below the figure. Looking from left to right, the first 3 bands are control and last 3 bands are prenatal arsenic.

Fig. 5.

Prenatal arsenic (50 ppb) did not significantly alter the level of mRNA expression of Nr3c1 (GR), FKBP5 (FKBP51), HSD11B1 (11b-hydroxysteroid dehydrogenase 1) or HSD11B2 (11b-hydroxysteroid dehydrogenase 2) in the E18 brain. Fold expression of the target genes were acquired by the $\Delta\Delta C_t$ (Comparative C_t) method. Data presented are mean $\pm$ SEM n = 7–11.

Fig. 6.

Prenatal arsenic (50 ppb) significantly decreased E18 brain protein expression of GR in nuclear fractions (** $p = .02$). Prenatal arsenic reduced the levels of 11 β -HSD1 (* $p = .05$) while increasing 11 β -HSD2 (** $p = .003$) compared to control. Data are mean $\pm$ SEM, $n = 7-10$. The intensity of the specific antibody was corrected to within blot Coomassie stain as described in the Materials and methods section. Representative blot images are presented below the figure. Looking from left to right, the first 3 bands are control and last 3 bands are prenatal arsenic.

Fig. 7.

Prenatal arsenic (50 ppb) significantly decreased E14 brain protein expression of CRF in the post nuclear lysate (** $p = .01$) compared to control. There was no difference in CRF levels seen in the brains of E18 fetuses. Data are mean $\pm$ SEM, $n = 7-10$. The intensity of the specific antibody was corrected to within blot Coomassie stain as described in the Materials and methods section. Representative blot images are presented below the figure. Looking from left to right, the first 3 bands are control and last 3 bands are prenatal arsenic.

Table 1

Antibodies, sources and concentrations used for the various tissues and developmental time-points.

Immunoblot protein and antibody information					
Age/fraction/tissue	Total protein	Antibody concentration	Target protein	Product code	Company
E14 whole homogenate of placenta	10 µg	[1:1000]	GR	sc-1004	Santa Cruz
	10 µg	[1:1000]	HSD11b2	sc-20176	Santa Cruz
	18 µg	[1:1000]	HSD11b1	sc-19259	Santa Cruz
	18 µg	[1:1000]	FKBP51	ab-2901	Abcam
E14 post nuclear lysate of brain	10 µg	[1:1000]	GR	sc-1004	Santa Cruz
	10 µg	[1:1000]	HSD11b2	sc-20176	Santa Cruz
	18 µg	[1:1000]	HSD11b1	sc-19259	Santa Cruz
	18 µg	[1:500]	FKBP51	sc-13983	Santa Cruz
	18 µg	[1:500]	MR	sc-11412	Santa Cruz
E14 nuclear lysate of brain	18 µg	[1:500]	HSD11b1	sc-19259	Santa Cruz
	18 µg	[1:500]	FKBP51	sc-13983	Santa Cruz
	10 µg	[1:1000]	GR	sc-1004	Santa Cruz
	18 µg	[1:500]	MR	sc-11412	Santa Cruz
E18 whole homogenate of placenta	15 µg	[1:500]	HSD11b1	sc-19259	Santa Cruz
	15 µg	[1:1000]	FKBP51	ab-2901	Abcam
	15 µg	[1:500]	HSD11b2	sc-20176	Santa Cruz
	15 µg	[1:1000]	GR	sc-1004	Santa Cruz
E18 post nuclear lysate of brain	20 µg	[1:1000]	HSD11b1	sc-19259	Santa Cruz
	20 µg	[1:1000]	FKBP51	ab-2901	Abcam
	15 µg	[1:1000]	HSD11b2	sc-20176	Santa Cruz
	15 µg	[1:1000]	GR	sc-1004	Santa Cruz
	15 µg	[1:500]	MR	sc-11412	Santa Cruz
E18 nuclear lysate of brain	20 µg	[1:500]	HSD11b1	sc-19259	Santa Cruz
	20 µg	[1:1000]	FKBP51	ab-2901	Abcam
	20 µg	[1:500]	HSD11b2	sc-20176	Santa Cruz
	20 µg	[1:500]	GR	sc-1004	Santa Cruz
	15 µg	[1:1000]	MR	sc-11412	Santa Cruz

Table 2

Levels of arsenic, arsenate, MMA and DMA in the brain tissue from embryonic day E14 and E18 mice.

	<u>As (V)</u>		<u>As (III)</u>		<u>MMA (III)</u>		<u>DMA (III)</u>	
	E14	E18	E14	E18	E14	E18	E14	E18
Control	1.18 (±0.016)	0.013 (±0.002)	ND	0.3 (±0.1)	0.021 (±0.002)	0.7 (±0.006)	0.0125 (±0.003)	0.5 (±0.1)
Arsenic	2.34(±0.08) ***	2 (± 0.9) **	ND	2 (± 0.9)	.024 (±0.004)	1.0 (±0.007) ***	0.026 (±0.001) *	1.4 (±0.1) ***

Data are mean ug/kg (±SEM), ND not detectable.

*
p = .003.

**
p = .001.

p = .0001.

Table 3

Levels of plasma corticosterone in mouse dams taken at gestational day 14 and 18.

	Gestational day 14	Gestational day 18
Control	348 ($\pm$ 41)	744 ($\pm$ 85)
Arsenic	348 ($\pm$ 18)	741 ($\pm$ 73)

Data are mean ng/mL ($\pm$ SEM), n = 7.