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Developmental arsenic exposure is associated with sex differences in the epigenetic regulation of stress genes in the adult mouse frontal cortex

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Abstract

Previously, we reported sex-dependent changes to global histone modifications and impairment of executive function in adult mice exposed to moderate levels of arsenic during early development. The present study investigated the effects of developmental arsenic exposure (DAE) on gene specific histone 3 lysine 9 acetylation (H3K9ac) and tri-methylation (H3K9me3) in the frontal cortex of male and female adult C57BL/6J mice. Dams were exposed to 50ppb arsenic in drinking water prior to and throughout gestation, and until pup weaning; once weened, the pups were given tap water (< 5ppb arsenic) and raised to postnatal day 70. Using chromatin immunoprecipitation and qPCR, we assessed changes in H3K9 regulatory markers and mRNA expression levels of the stress signaling genes, Nr3c1, Crh, Crhr1, Hsd11b1, and Fkbp5. In female DAE mice, H3K9ac association with the Crh promoter was increased and this corresponded with increased Crh mRNA expression. Crhr1 mRNA expression was increased in female DAE mice, while expression was reduced in male DAE mice. DAE resulted in chromatin and mRNA changes associated with decreased glucocorticoid signaling in male but not female frontal cortex: increased H3K9ac association with Fkbp5 and a corresponding increase in Fkbp5 mRNA expression. In addition, there was a decrease of Hsd11b1 mRNA expression in DAE males. These results suggest a sex-dependent response to arsenic during embryonic development leading to different patterns of gene regulation and expression.

Keywords

Arsenic; Frontal cortex; Sex differences; Epigenetics; Stress

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Declaration of Competing Interest

The authors declare the research was conducted in the absence of any conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2020.114920.

1. Introduction

In 2001, the US Environmental Protection Agency (EPA) set a new standard of 10 ppb arsenic in public drinking water, meeting the 1993 World Health Organization's recommendation (United States Environmental Protection Agency, 2002; World Health Organization, 2011). This policy change applied to public water supplies; rural communities reliant on private ground water wells are still at risk of exposure to elevated arsenic levels (Credo et al., 2019; Sinha and Prasad, 2019). Chronic exposure to arsenic has been associated with an increased frequency of a broad range of health problems (Huang et al., 2019; Moon et al., 2012; Davey et al., 2007; Zhou and Xi, 2018; Suntararuks et al., 2019; Thomas et al., 2019; Farkhondeh et al., 2019; Chung et al., 2019), while developmental exposure to arsenic has been associated with impaired neurological development, potentially leading to cognitive deficits later in life (Rodríguez-Barranco et al., 2013; Yorifuji et al., 2016; Rodrigues et al., 2016; Wasserman et al., 2004; Von Ehrenstein et al., 2007; Rosado et al., 2007; Aung et al., 2016; Htway et al., 2019; Wang et al., 2018; Freire et al., 2018). Developmental toxin exposure can lead to compensatory changes in gene expression within the fetal-placental unit in an effort to accommodate for the presence of the toxin (McMullen and Mostyn, 2009; Wadhwa et al., 2009; Gluckman et al., 2007; Appleton et al., 2017; Winterbottom et al., 2019; Kaushal and et al., 2017). The theory of the developmental origins of health and disease predicts that these compensatory changes may ultimately lead to compromised health and increased incidence of disease later in life (McMullen and Mostyn, 2009; Wadhwa et al., 2009; Gluckman et al., 2007).

Early life disruption of glucocorticoid and hypothalamic-pituitary-adrenal (HPA) axis signaling has been associated with impaired neurological development and cognition (Rodriguez et al., 2011; Tyler and Allan, 2014; Maccari et al., 2014; Wolf et al., 2016; Kino, 2015). Sex differences in stress response have been reported, however this area of research is far from being completely understood (Segni et al., 2019; Buban and et al., 2020). In previous publications, our lab has shown that developmental arsenic exposure (DAE) alters the expression of key stress genes (GR, Hsd11b1, Hsd11b2, Fkbp5, Crhr1) essential to HPA axis functioning in a sex-dependent manner at key embryonic time points (Allan et al., 2015; Caldwell et al., 2015), and that these sexually dimorphic changes carry on into adulthood (Caldwell et al., 2018; Goggin et al., 2012; Martinez et al., 2008). In the current studies, we are seeking to identify epigenetic changes that might prove to be the mechanism for the gene and protein modifications we have already identified in our DAE model.

Throughout life, genes are dynamically regulated via epigenetic mechanisms that are cell type/location-, sex-, and developmental stage-specific, and are modified by environmental factors (Morgan et al., 2005; Morey and Avner, 2010). Many publications focus on global changes to epigenetic markers and modifying proteins caused by recent arsenic exposure (Cronican et al., 2013; Zhou et al., 2008), with few exploring if this epigenetic dysregulation is sex-dependent (Winterbottom et al., 2019; Howe et al., 2017; Bommarito et al., 2017). In our past work we have found that perinatal arsenic alters levels of histone deacetylase (HDAC) 1, 2, and 5 in male but not female adult frontal cortex (Tyler et al., 2018). In the same study, we found that males, but not females, showed impairment in executive function (Y-Maze alternation task). In another study, we previously observed that DAE decreased

global H3K9ac and the histone modifying proteins, GCN5 and PCAF, in male but not female frontal cortex (Tyler et al., 2015).

The interaction between the frontal cortex and the HPA axis is poorly understood. Though there is evidence that the frontal cortex is capable of indirectly activating and repressing this known stress pathway, the means by which it does this have yet to be completely elucidated (Herman, 2009). Known for its role in executive function, the frontal cortex is able to predict the consequences of behaviors before they are acted out, thus assisting in the organism's ability to choose the best outcome for survival (Uribe-Mariño et al., 2016). Mental health disorders, such as anxiety, depression, schizophrenia, and bipolar disorder, are known disruptors of executive function and are also associated with altered HPA axis signaling (Sinclair et al., 2013; Wetherell et al., 2017). HPA axis dysregulation has been found to impair executive function, with Corticotrophin Releasing Hormone (Crh) and glucocorticoids identified as likely mediators in this process (Uribe-Mariño et al., 2016; Harms et al., 2017; Butler et al., 2017). However, the roles these ligands play in frontal cortex development are not very clear. Crh has been identified as a neuro-protector and as a crucial factor in neurogenesis (Bayatti et al., 2003; Koutmani et al., 2013). Thus, it is likely that Crh could be utilized to resist assaults to neurological development.

Here, we assessed the effects of DAE on the epigenetic landscape of key stress pathway genes in the frontal cortex of adult male and female mice on postnatal day 70, (PN70), long after the toxin has been removed from the mouse's environment. In these studies, we measured the levels of H3K9ac and H3K9me3 associated with promoters in the genes encoding FK506-binding protein 51 (Fkbp5), 11β-hydroxysteroid dehydrogenase 1 (Hsd11b1), Crh, corticotropin releasing hormone receptor 1 (Crhr1) and glucocorticoid receptor (Nr3c1). We present evidence that DAE produced sex-dependent alterations in epigenetic regulation patterns of key HPA axis genes and suggest that these changes are an attempt to compensate for developmental damage caused by arsenic exposure.

2. Methods

2.1. Chemical hazards

Arsenic is classified as a human co-carcinogen; all arsenicals were handled with caution in accordance with MSDS standards.

2.2. Developmental arsenic exposure paradigm

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of New Mexico (UNM) and followed recommendations made in “Recognition and Alleviation of Pain and Distress in Laboratory Animals” (Recognition and Alleviation of Pain and Distress in Laboratory Animals, 1991). C57BL/6 J mice purchased from Jackson Labs were kept on a reverse light/dark cycle (lights off at 0800, lights on at 2000), with ad libitum access to food and water in the Animal Resource Facility at UNM. Arsenic exposure was performed as previously described (Tyler and Allan, 2014; Tyler et al., 2015). 10 days prior to mating, single housed female mice were acclimated to drinking 50 parts-per-billion arsenic water. Arsenic water was prepared by dissolving Sodium Arsenate

(10048–95-0, Sigma Aldrich) in a 50/50 mix of tap water and Milli-Q Water. Control mice were given standard UNM tap water, which did not exceed 5 ppb arsenic and control tissue levels were undetectable (Tyler and Allan, 2014; Tyler et al., 2015). Mating occurred over 5 days. Dams drank arsenic water or tap water throughout mating, gestation and until the pups were weaned into same sex, littermate cages on PD23. Once weaned, the pups were provided ad libitum access to food and standard UNM tap water. At post-delivery day 70 (PD70), animals were euthanized via rapid decapitation. The frontal cortex from each mouse was rapidly micro-dissected, snap frozen in liquid nitrogen, and stored at −80 °C until analysis. Female mice were exposed to male bedding and then later confirmed via vaginal cytology to be in either the diestrus or proestrus phase of their cycle at the time of tissue collection. Each assay assessed tissue from at least 6 different litters for each experimental group to avoid litter effects. Additional dams, exposed to the same arsenic model, were euthanized via rapid decapitation at gestational day 14 (GD14), the fetal brains and bodies were snap frozen in liquid nitrogen and stored at −80 °C (Caldwell et al., 2015). Sex determination of fetal tissue was done by qPCR detection of the Sry gene.

2.3. Evaluation of mRNA expression using quantitative PCR

Briefly, total RNA, including microRNA, was isolated from tissue using the Invitrogen mirVana miRNA isolation kit (A1561, ThermoFisher) following the manufacturer's instructions. Total RNA was analyzed using a NanoDrop 1000 to ensure appropriate 260/280 and 260/230 ratios. The total RNA concentration was measured using a Qubit 2.0 fluorometer (ThermoFisher). Reverse transcription with gDNA digestion was performed using 0.5–1.0 μg total RNA with the QuantiTect RT Kit (205,313, Qiagen) following the manufacturer's instructions. An RT Negative control was used to confirm the elimination of gDNA. All qPCR assays were run with a LightCycler96 (Roche) with FastStart Essential DNA Green Master (Roche) following the manufacturer's instructions for cycling parameters. Fold expression of mRNA transcripts was determined by the comparative C_T method (Livak and Schmittgen, 2001).

2.4. Primer design and validation

All primers were tested for efficiency; only primers between 95%–105% efficient were used. The house keeping gene, hydorxyxanthine phosphoribosyltransferase (Hprt), was used as an endogenous control for all mRNA assays. Hprt was not altered by prenatal exposure to arsenic, making it an acceptable housekeeping gene (Tyler et al., 2018). The variation in efficiency between Hprt and target primers was below 5%. Primer sequences were obtained from PrimerBank, past publications, or designed by the authors, [Suppl. Table 1]. All mRNA primers span an exon-exon boundary. For ChIP-qPCR assays, primers were designed to target the promoter sequence of the target gene, as histone modifications are reported to have clear regulation at these sites (Black et al., 2012). All new primers were validated by melt curve and electrophoresis on agarose gel treated with ethidium bromide. All qPCR assays were run and reported in accordance with the Minimum Information for Publication of qPCR Experiments (MIQE) guidelines (Bustin et al., 2009).

2.5. Chromatin immunoprecipitation (ChIP) of H3K9me3 and H3K9ac chromatin

The ChIP protocol and reagents were as described in Cotney and Noonan, 2015 (Cotney and Noonan, 2015), using Magna ChIP Protein A + G magnetic beads (cat. 16–663, EMD Millipore), anti-H3K9ac antibody (9649S Cell Signaling), anti-H3K9me3 antibody (13969S, Cell Signaling), and Rabbit IgG (sc-2027×, SCBT). Frozen tissue was weighed and then placed in ice-cold PBS and homogenized using sterile Biomasher disposable micro homogenizers (KT749625-0030, VWR). Formaldehyde was added to each homogenate to a final concentration of 1% (*w/v*) and incubated at room temperature for 15 min with constant rotation. A final concentration of 150 mM glycine was added to quench the fixing reaction. The homogenate was centrifuged at 1000 x g_{max} for 6 min and the pellet was re-suspended in cell lysis buffer, then spun again to isolate nuclei. The nuclear pellet was washed once with cell lysis buffer, re-suspended in nuclear lysis buffer and sonicated with a BioruptorPLUS (Diagenode) for 12 cycles of 30s ON and 90s OFF at low power in a water bath maintained at a constant +4 °C. 5 μL of sonicated nuclear lysate was reverse cross-linked, then run on a 1% agarose gel with ethidium bromide to confirm shearing of chromatin into 400-600 bp fragments. Antibodies were bound to magnetic beads following cited protocol and manufacturer's instructions. 7 μL of antibody were used per ChIP reaction. For H3K9, the acetylated and tri-methylated targets were immunoprecipitated using the same sonicated nuclear lysate and sharing one input sample. Each IP reaction had an initial chromatin concentration of 4.32 ng/μL; the Input for each sample received a factor of 10 less chromatin than the IP reactions. A pool of all sonicated nuclear lysate samples was used for the *IgG* and *No Antibody* negative controls; an input sample of the pool was run as well. After the ChIP reaction, DNA was isolated from each sample with the DNeasy Kit (69581, Qiagen) following manufacturer's instructions starting at the filter column loading step. DNA concentration was measured using Qubit 2.0 fluorometer. The H3K9me3 antibody yielded an averaged DNA concentration of 5.54 ng/μL with a range from 2.78 to 7.48 ng/μL. The H3K9ac antibody yielded an average DNA concentration of 1.60 ng/μL with a range from 0.73 to 2.74 ng/μL. The input samples yielded an average DNA concentration of 2.81 ng/μL with a range from 0.96 to 4.32 ng/μL. Each acetylated IP, tri-methylated IP, and Input sample was run in triplicate on a LightCycler96 machine (Roche). A constant amount, 1.25 ng, of immunoprecipitated (IP) and input (IN) chromatin was used for each qPCR reaction.

2.6. ChIP quantitative PCR data analysis

There are no MIQE guidelines for publishing ChIP-qPCR data, and, unfortunately, there are several published methods that fail to accurately describe the method of analysis used (Shen et al., 2018; Li et al., 2015; Zhang et al., 1979; Divoux et al., 2018). In some studies, the Comparative C_T method has been used to analyze ChIP-qPCR data. The protocol for the Imprint Chromatin Immunoprecipitation Kit (CHP1, Millipore Sigma) and the paper Wang and Lindås, (2018) both use the comparative C_T method and both methods provide different and incorrect solutions. Both also cite the same two papers that only cover mRNA expression Comparative C_T analysis (Livak and Schmittgen, 2001; Yuan et al., 2006).

The most common method for ChIP-qPCR analysis is the % Input method with Cq data obtained from running qPCR with a constant volume of ChIP DNA (Akkers et al., 2012;

Asp, 2018). Here, we present a calculation adaptation that allows us to calculate the % Input from Cq values obtained by qPCR reactions with a constant amount of chromatin present in the qPCR assay. A constant amount was used to reduce variation in the concentration of DNA in the qPCR reactions, which would result in a variation in the amplification efficiency. Since a constant amount and not a constant volume was used, we could not use the Input Dilution Factor (DF) value of 10, based on the ChIP dilution difference between input and IP samples. Instead, we calculated the appropriate Input DF using Eq. (1). This alternative dilution factor reflects the actual yielded amounts from each ChIP reaction, and provides a correction in the variation between samples caused by user error. This allows for each sample to have a unique dilution factor that accurately reflects the yielded amount from each ChIP reaction.

$$\textit{Input DF} = \frac{\textit{Initial amount IP}\,(ng)}{\textit{Initial amount IN}\,(ng)} \times \frac{\textit{Final amount IN}\,(ng)}{\textit{Final amount IP}\,(ng)} \quad (1)$$

Then, the % Input is calculated using Eq. (2), the common % Input equation, with the Input DF from Eq. (1).

$$\%\ \textit{Input} = 2^{Cq(IP) - (Cq(IN) - Log_2(\textit{Input DF}))} \quad (2)$$

Background levels were estimated by calculating the % Input of the pooled negative IP control (IP IgG) compared to the Pooled Input fraction (IN Pool). The % Input values were corrected for background by subtracting the % Background from each % Input. The average % Background we calculated was 0.99% with a range from 0.11% to 3.89% [Suppl. Table 2.].

2.7. Statistical analysis

Male and female samples were run on separate plates and data was analyzed separately. Outliers were identified using Cohen's D and omitted from the analysis. All data are presented as mean ± SEM and a p value of < 0.05 was the threshold for statistical significance. All data analysis was conducted using t-tests run in GraphPad Prism 8 software (GraphPad, San Diego, CA). The number of litters used for each experiment is reported in the figure legends, only one animal per litter was used, with a minimum of $n = 6$ per assay.

3. Results

3.1. DAE did not alter H3K9 tri-methylation levels

To determine if DAE created lasting alterations in the levels of H3K9me3, a repressive posttranslational histone modification, on specific genes, we assessed the levels of stress signaling genes, Fkbp5, Hsd11b1, Crh, Crhr1 and Nr3c1 associated with anti-H3K9me3 antibody immunoprecipitates. This antibody was tested by microarray (Active Motif, cat#13005) and showed a primary specificity for H3K9me3 (28.6%), with weaker non-specific binding to H3K9me2 (12%) and H3K36me3 (11.5%) [Suppl. Fig. 1]. Of the promoter regions tested, no significant change to the tri-methylation of H3K9 was found

between arsenic and control groups of frontal cortex from either sex. [Suppl. Fig. 2]. The remainder of the Results section will focus on H3Kac and mRNA levels.

3.2. DAE alters H3K9ac association with the promoter of Fkbp5 in a manner consistent with measured mRNA expression in male but not female PN70 frontal cortex

To determine if DAE created lasting alterations in the levels of H3K9ac, an activating posttranslational histone modification, on specific genes, we analyzed gene levels following chromatin immunoprecipitation using an anti-H3K9ac antibody. Immune complexes were isolated from the same lysate used to analyze H3K9me3. The anti-H3K9ac antibody was also tested by microarray and showed a primary specificity for H3K9ac (81.8%), with less than 5% binding reactivity to nonspecific histone modifications [Suppl. Fig. 1]. In the frontal cortex of arsenic-exposed male PN70 mice, we found a significant ($p = .0161$) increase in H3K9ac associated with the promoter of Fkbp5 [Fig. 1A]. Consistent with this increase of an activating histone modification we also measured a significant ($p = .0211$) increase in the mRNA expression of Fkbp5 in arsenic-exposed PN70 male frontal cortex [Fig. 1B]. In arsenic-exposed PN70 female frontal cortex, we measured a modest but not significant increase in H3K9ac associated with the Fkbp5 promoter and Fkbp5 mRNA expression [Fig. 1C, D].

3.3. DAE did not alter the level of the Hsd11b1 promoter bound to H3K9ac, but did alter Hsd11b1 mRNA levels in male PN70 frontal cortex

Neither male nor female DAE PN70 frontal cortex showed a change in H3K9ac associated with the promoter region of Hsd11b1 [Fig. 2A, C]. Levels of Hsd11b1 mRNA in DAE PN70 male frontal cortex were found to be significantly ($p = .0413$) decreased [Fig. 2B], while female Hsd11b1 mRNA levels were not altered by DAE [Fig. 2D].

3.4. DAE did not alter the level of H3K9ac nor H3K9me3 associated with the Nr3c1 promoter nor did DAE alter levels of Nr3c1 mRNA

In order to ensure a detailed analysis of stress pathway genes, we subjected Nr3c1 to our battery of epigenetic and mRNA assays. DAE caused no significant changes to Nr3c1 promoter H3K9 acetylation, nor did we measure any changes in Nr3c1 mRNA levels in male and female PN70 frontal cortex. [Suppl. Fig. 3].

3.5. DAE altered Crh promoter H3K9 acetylation and Crh mRNA expression in female but not male PN70 frontal cortex

In the frontal cortex of arsenic-exposed female PN70 mice, there was a significant ($p = .0062$) 2.3-fold increase in H3K9ac bound to the Crh promoter [Fig. 3C]. Consistent with an increase in this activating histone posttranslational modification, we also measured a significant ($p = .0212$) increase in Crh mRNA expression in female PN70 frontal cortex [Fig. 3D]. No significant changes were measured in arsenic-exposed male PN70 frontal cortex [Fig. 3A, B].

3.6. Male and female PN70 frontal cortex showed opposite Crhr1 mRNA expression resulting from DAE

We measured a significant decrease ($p = .0383$) in Crhr1 mRNA expression in arsenic-exposed male PN70 frontal cortex [Fig. 4B]. Conversely, we measured a significant increase ($p = .0325$) in Crhr1 mRNA expression in female PN70 frontal cortex [Fig. 4D]. Neither male nor female arsenic-exposed PN70 frontal cortex showed a change in H3K9ac bound to the promoter region of Crhr1 [Fig. 4A, C].

4. Discussion

The frontal cortex is well known for its role in executive function. In this study we show a glimpse of the different epigenetic changes male and female mice make in response to damage caused by perinatal arsenic exposure. In our previous publications we show that DAE caused severe executive dysfunction in male but not female adult (PN70) mice, tested in a Y-Maze alternation task (Tyler et al., 2018). In the present study, we focused on the H3K9 modification states on key stress regulatory genes. DAE did not produce a detectable change in H3K9me3 association levels with any of the tested genes, however this result may be due to the broad cross-reactivity of the antibody used [Suppl. Fig. 1]. We observed sex-specific changes in H3K9ac association with the Fkbp5 and Crh genes that correspond with changes in mRNA expression. The observed patterns of epigenetic and mRNA expression changes are consistent with sex-specific effects of DAE on stress response and executive function, and suggest that there are neuroprotective mechanisms that reduce the effects of DAE in females (Fig. 5).

The only H3K9 modification we found altered by DAE in the adult male frontal cortex was an increase in the level of H3K9ac associated with the Fkbp5 gene [Fig. 1A]. This is in contrast to our previously reported global decrease in H3K9ac levels in the PD70 male frontal cortex (Tyler et al., 2015). As expected with an activating marker, the mRNA levels of Fkbp5 were also increased in the adult male frontal cortex. The observed Fkbp5 gene-specific increase in H3K9ac, and corresponding increase in Fkbp5 mRNA, in tissue in which there is a global decrease in H3K9ac indicates that our DAE model exerts specific effects, rather than global toxicity, on the development of males.

An increase in Fkbp5 mRNA is predicted to lead to an upregulation of Fkbp51 protein, which will reduce the overall sensitivity to glucocorticoid signaling (Guy et al., 2016). In DAE male frontal cortex, this is likely to be enhanced by the observed reduction in Hsd11b1 mRNA expression [Fig. 2B], which is predicted to lead to reduced 11β-hydroxysteroid dehydrogenase 1 protein and reduced intracellular generation of active glucocorticoids (Chapman et al., 2013). We also found a significant decrease in the mRNA expression of Crhr1 in the PN70 male frontal cortex [Fig. 4B]. Though not directly involved in glucocorticoid signaling, Crhr1 will be activated during a stress response (Uribe-Mariño et al., 2016).

Our past work shows that arsenic has a severe impact on frontal cortex development in males, leading to executive dysfunction in adulthood. Our current research expands these findings and indicates that, in adult males, there is an overall reduction in stress pathway

function. It is possible that this is an attempt to limit the negative impact that stress signaling can have on executive functioning caused by acute and chronic stress (Arnsten, 2015; Marko and Riečanský, 2018).

As expected from our previous research (Allan et al., 2015; Caldwell et al., 2018; Tyler et al., 2018; Tyler et al., 2015), our findings in the adult female frontal cortex were, in some cases, different from what we found in the males. Fkbp5 [Fig. 1C, D], Hsd11b1 [Fig. 2C, D], and Nr3c1 [Suppl. Fig. 3D-F] histone modifications and mRNA levels were not altered in DAE females, indicating that DAE does not change GR signaling in females. Crh mRNA levels were significantly elevated in the PN70 DAE female frontal cortex [Fig. 3D]. This corresponds with a significant increase of H3K9 acetylation on the Crh gene [Fig. 3C]. While elevated Crh signaling in the frontal cortex has been found to impair executive function (Uribe-Mariño et al., 2016), we have shown that arsenic-exposed females display no executive dysfunction (Tyler et al., 2018). We postulate that the finding of elevated Crh mRNA levels in the female frontal cortex originates as an embryonic response to arsenic exposure and persists into adulthood. In support of this proposal, Crh mRNA levels are significantly increased in DAE female pups at gestational day 14 (GD14) [Suppl. Fig. 4]. Similarly, we have previously reported that Crh mRNA levels are reduced in GD14 male pup brain (Caldwell et al., 2015) and in the present study we found that Crh mRNA levels are reduced in the PN70 male frontal cortex.

Crh has been found to have neuroprotective qualities, preventing apoptosis and maintaining healthy neuron development (Bayatti et al., 2003; Koutmani et al., 2013). We propose that the elevation in Crh levels in females acts as a neuroprotective mechanism during development, and this mechanism persists into adulthood. Conversely, the reduction in Crh in males leads to neuronal damage during development and increased vulnerability to neuronal insults in adulthood. Finally, whereas males significantly decreased Crhr1 mRNA expression [Fig. 4B], females significantly increased it [Fig. 4D], with no change of H3K9 modifications [Fig. 4A, C]. The elevation in Crhr1 levels in females is expected to further enhance Crh signaling in the PN70 DAE female frontal cortex.

H3K9 tri-methylation and H3K9 acetylation are only two small units of the greater histone code. Post-translational histone modifications themselves are only part of the myriad of regulatory pathways that control gene expression. H3K9 modifications did not produce the observed reduction in Hsd11b1 mRNA expression [Fig. 2B] in males, and the opposing Crhr1 mRNA expression between males [Fig. 4B] and females [Fig. 4D]. It is possible that these changes were orchestrated by other histone modifications, DNA methylation, or even indirect regulation of transcription factors. To fully grasp the impact of DAE on mature stress pathways we must continue to methodically study other avenues of gene regulation.

5. Conclusion

We have uncovered sex differences in the epigenetic regulation of stress genes in the frontal cortex of adult mice that were exposed to arsenic throughout gestational and early postnatal development. We have previously found that, while males appear more vulnerable to the damaging effects of arsenic, females are more protected (Tyler et al., 2018). Our current

findings support this idea and indicate that these vulnerability/resilience effects persist into adulthood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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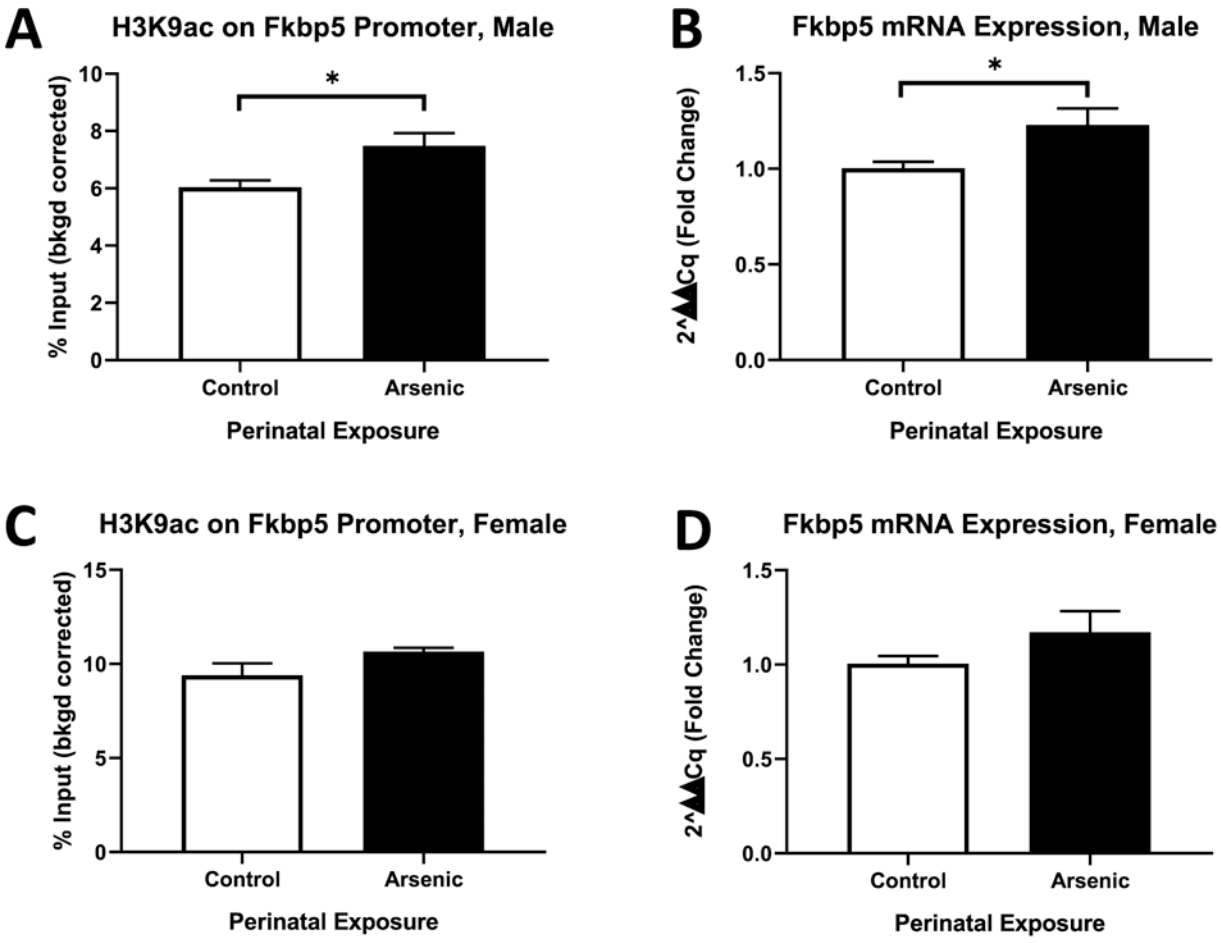

Fig. 1.

H3K9ac association with, and mRNA expression of, Fkbp5. (A & C) Background corrected % Input levels of Fkbp5 Promoter enriched by ChIP using anti-H3K9ac antibody, quantified by qPCR, in male frontal cortex (A) DAE significantly *(P = .0161, df = 10, n = 6) increased H3K9ac association with the Fkbp5 Promoter, no change was seen in female DAE mice (df = 10, n = 6) (C). (B & D) Fold Change of Fkbp5 mRNA Expression, quantified by two-step RT-qPCR, in male frontal cortex (B) DAE significantly *(P = .0211, df = 10, n = 6) increased levels of Fkbp5 mRNA transcripts relative to endogenous control, no change was seen in female DAE mice (df = 13, n = 7–8) (D).

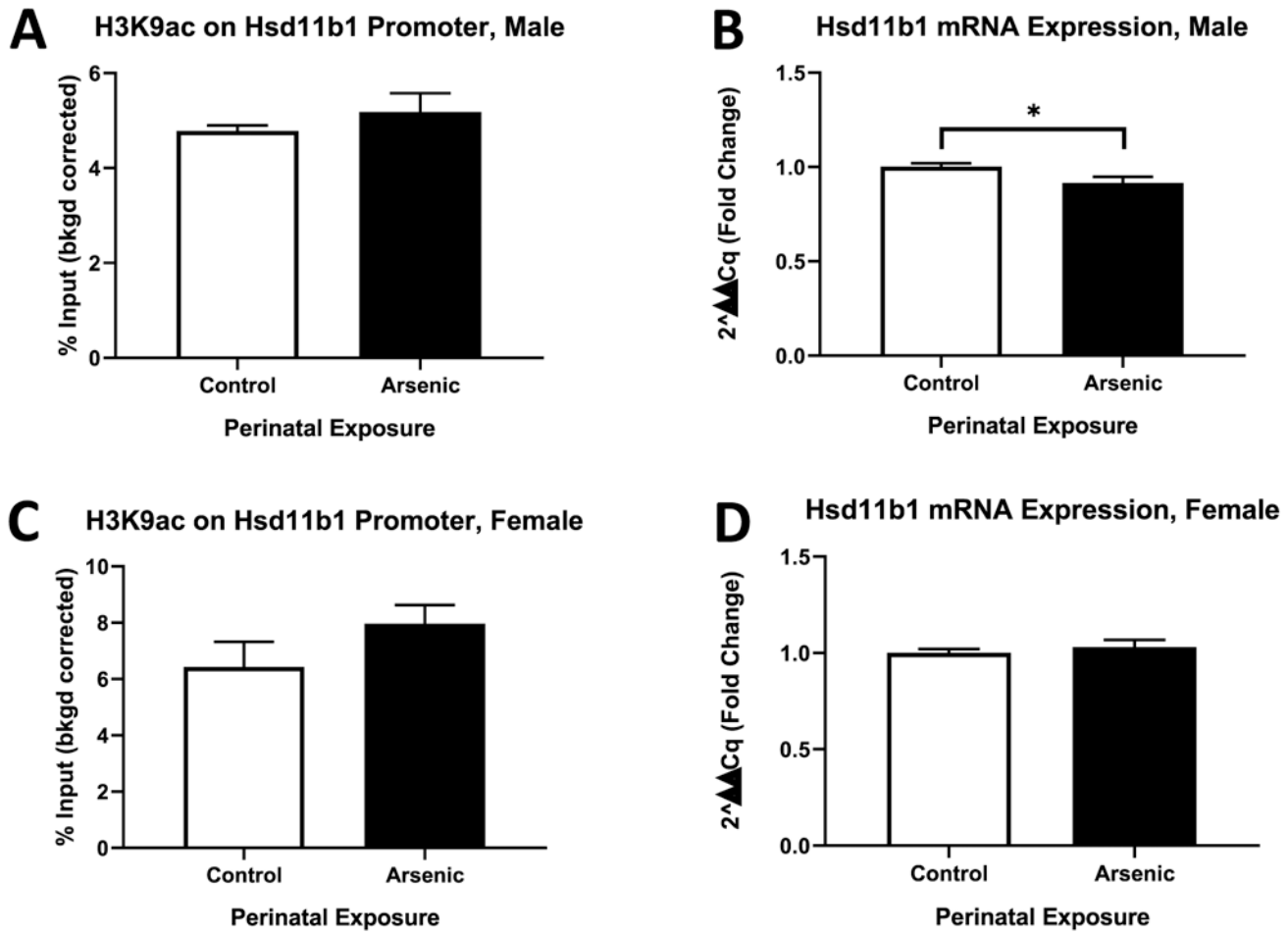

Fig. 2.

H3K9ac association with, and mRNA expression of, Hsd11b1. (A & C) Background corrected % Input levels of Hsd11b1 Promoter enriched by ChIP using anti-H3K9ac antibody, quantified by qPCR, in male (df = 10, n = 6) and female (df = 11, n = 7–6) DAE frontal cortex no significant change was measured in the association of H3K9ac with the Hsd11b1 Promoter. (B & D) Fold Change of Hsd11b1 mRNA Expression, quantified by two-step RT-qPCR, in male frontal cortex (B) DAE significantly *(P = .0413, df = 12, n = 7) decreased levels of Hsd11b1 mRNA transcripts relative to endogenous control, no change was seen in female DAE mice (df = 11, n = 7–6) (D).

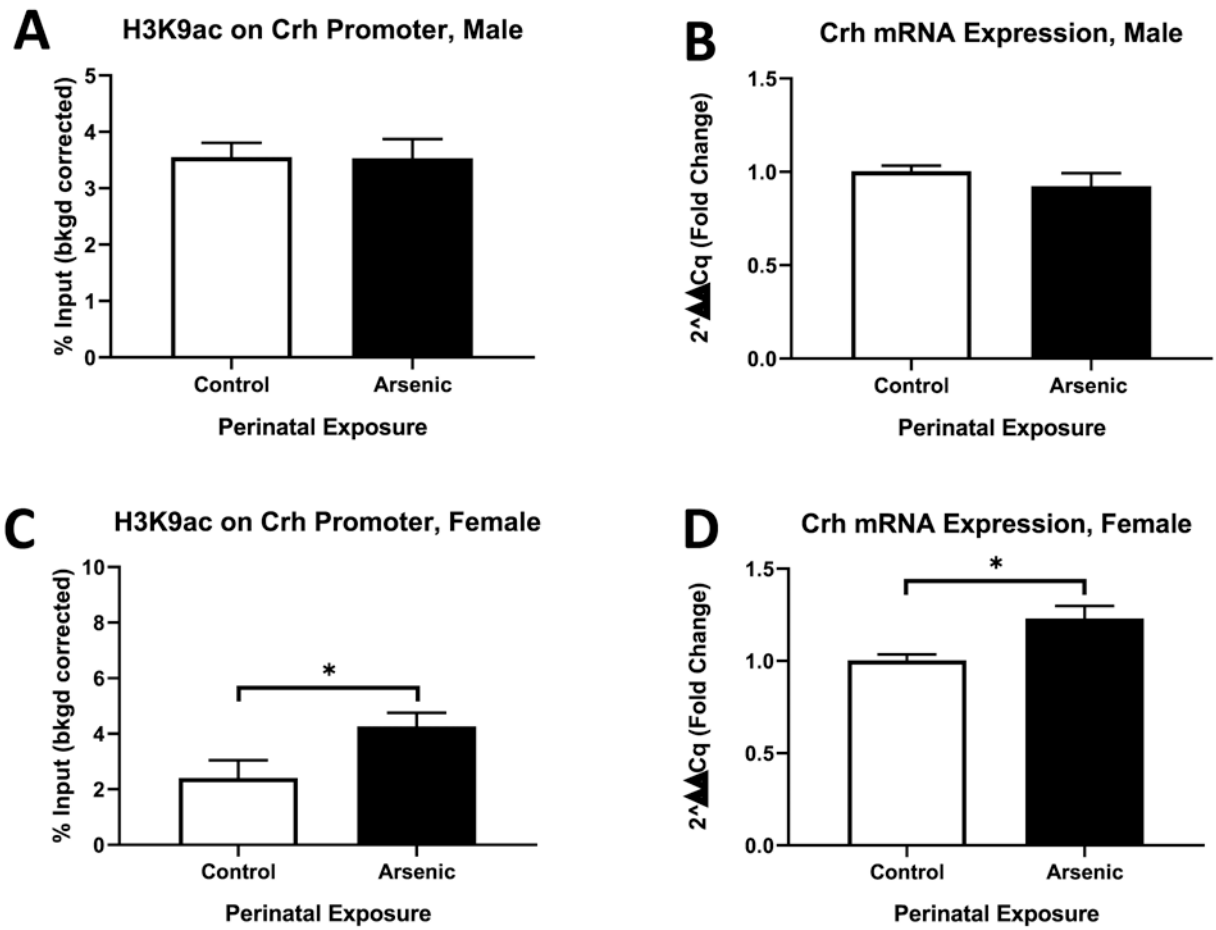

Fig. 3.

H3K9ac association with, and mRNA expression of, Crh. (A & C) Background corrected % Input levels of Crh Promoter enriched by ChIP using anti-H3K9ac antibody, quantified by qPCR, in female frontal cortex (C) DAE significantly *(P = .0456, df = 10, n = 6) increased H3K9ac association with the Crh Promoter, no change was seen in male DAE mice (df = 10, n = 6) (A). (B & D) Fold Change of Crh mRNA Expression, quantified by two-step RT-qPCR, in female frontal cortex (D) arsenic exposure significantly *(P = .0212, df = 12, n = 7) increased levels of Crh mRNA transcripts relative to endogenous control, no change was seen in male DAE mice (df = 11, n = 7–6) (B).

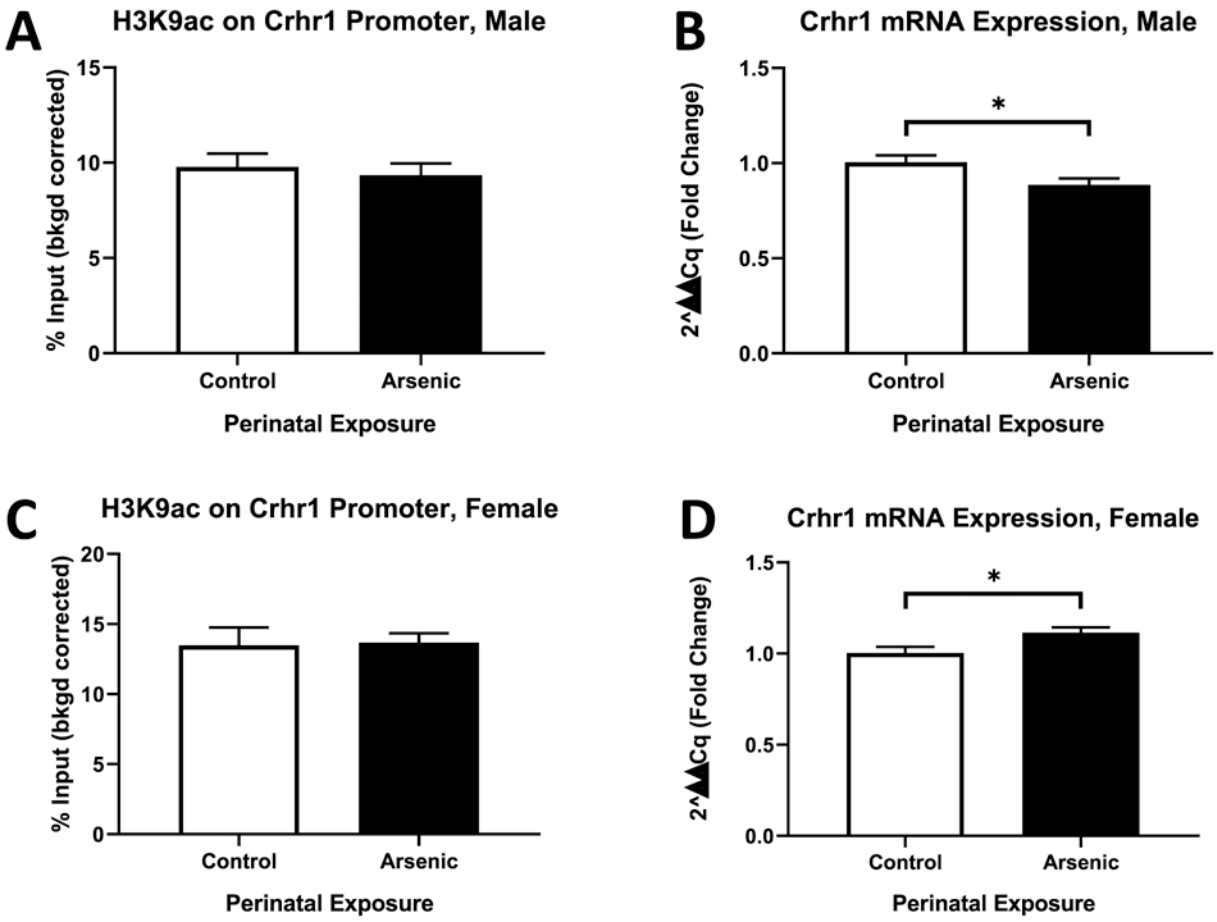

Fig. 4.

H3K9ac association with, and mRNA expression of, Crhr1. (A & C) Background corrected % Input levels of Crhr1 Promoter enriched by ChIP using anti-H3K9ac antibody, quantified by qPCR, in male (df = 12, n = 7) (A) and female (df = 10, n = 6) (C) DAE frontal cortex no significant change was measured in the association of H3K9ac with the Crhr1 Promoter. (B & D) Fold Change of Crhr1 mRNA Expression, quantified by two-step RT-qPCR, male frontal cortex (B) DAE significantly *(P = .0383, df = 11, n = 7–6) increased levels of Crhr1 mRNA transcripts relative to endogenous control, female frontal cortex (D) DAE significantly *(P = .0325, df = 12, n = 7) decreased levels of Crhr1 mRNA transcripts relative to endogenous control.

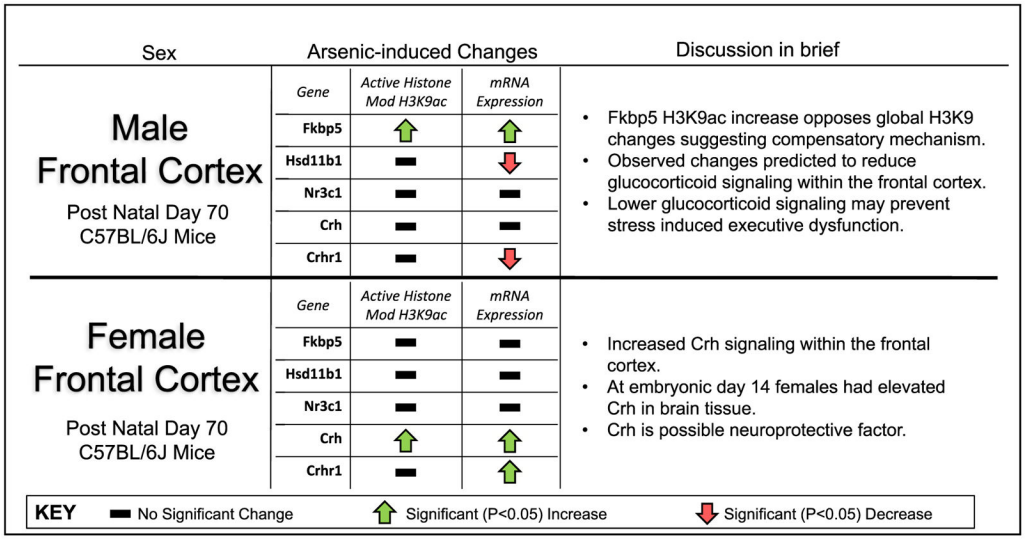

Fig. 5.
Graphic summary of results and discussion.