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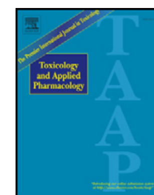

Developmental exposure to 50 parts-per-billion arsenic influences histone modifications and associated epigenetic machinery in a region- and sex-specific manner in the adult mouse brain

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Epidemiological studies report that arsenic exposure *via* drinking water adversely impacts cognitive development in children and, in adults, can lead to greater psychiatric disease susceptibility, among other conditions. While it is known that arsenic toxicity has a profound effect on the epigenetic landscape, very few studies have investigated its effects on chromatin architecture in the brain. We have previously demonstrated that exposure to a low level of arsenic (50 ppb) during all three trimesters of fetal/neonatal development induces deficits in adult hippocampal neurogenesis in the dentate gyrus (DG), depressive-like symptoms, and alterations in gene expression in the adult mouse brain. As epigenetic processes control these outcomes, here we assess the impact of our developmental arsenic exposure (DAE) paradigm on global histone posttranslational modifications and associated chromatin-modifying proteins in the dentate gyrus and frontal cortex (FC) of adult male and female mice. DAE influenced histone 3 K4 trimethylation with increased levels in the male DG and FC and decreased levels in the female DG (no change in female FC). The histone methyltransferase MLL exhibited a similar sex- and region-specific expression profile as H3K4me3 levels, while histone demethylase KDM5B expression trended in the opposite direction. DAE increased histone 3 K9 acetylation levels in the male DG along with histone acetyltransferase (HAT) expression of GCN5 and decreased H3K9ac levels in the male FC along with decreased HAT expression of GCN5 and PCAF. DAE decreased expression of histone deacetylase enzymes HDAC1 and HDAC2, which were concurrent with increased H3K9ac levels but only in the female DG. Levels of H3 and H3K9me3 were not influenced by DAE in either brain region of either sex. These findings suggest that exposure to a low, environmentally relevant level of arsenic during development leads to long-lasting changes in histone methylation and acetylation in the adult brain due to aberrant expression of epigenetic machinery based on region and sex.

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Introduction

Arsenic exposure is a worldwide health concern as several millions of people are exposed to this environmental toxicant *via* natural and anthropogenic sources each year (Naujokas et al., 2013). Efforts to minimize exposure have resulted in allowance of 10 μg/L (parts-per-billion, ppb) arsenic in water as stipulated by the Environmental Protection Agency (EPA) and World Health Organization (WHO); however, in several countries (including in the U.S. prior to 2006), 50 ppb arsenic remains the standard allotment (WHO, 2011). Additionally, there are places where access to drinking water containing arsenic within the WHO limits is simply not possible, and populations within these regions are exposed to excessive arsenic (in the parts-per-million range) resulting in damage to almost every organ system, including the brain (Jiang et al., 2013; Bustaffa et al., 2014). Epidemiological studies have demonstrated that even low levels of arsenic exposure can negatively impact the body, including increasing the propensity toward developing psychiatric disorders and cognitive dysfunction (Zierold et al., 2004; Brinkel et al., 2009). Importantly, *in utero* and developmental arsenic exposure results in learning and memory deficits in children and may underlie long-lasting susceptibility to disease later in life (reviewed in Tyler and Allan, 2014). However, relatively little is known about the long-term influence of low levels of arsenic exposure, particularly in the brain.

Research over the past decade has provided evidence that arsenic alters the epigenetic landscape in various cell types. The epigenome consists of DNA methylation and histone modifications that collectively constitute chromatin structure and ultimately chromatin function, conferring regulation of gene expression (Kouzarides, 2007). Of particular interest are studies on histone posttranslational modifications (HPTM), as histone modifications can be dynamic in response to the extrinsic environment and are paramount for proper neurogenesis and

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differentiation of neural stem cells in the brain (Hsieh and Eisch, 2010; Day and Sweatt, 2011). Indeed, epigenetic dysregulation of HPTMs has been postulated as a molecular mechanism underpinning psychiatric disorders such as depression (Mateus-Pinheiro et al., 2011; Sun et al., 2013). However, the impact of arsenic on the epigenetic status of the brain has not been thoroughly investigated, particularly in the context of developmental exposure. To date, there have been three studies on the effects of developmental exposure to arsenic in the brain, suggesting an impact of arsenic on histone acetylation and DNA methylation with concurrent deficits in proteins that may underlie learning and memory deficits (Zarazua et al., 2010; Martinez et al., 2011; Cronican et al., 2013). Conversely, the literature on the effect of arsenic on the epigenome is quite extensive in the context of cancer (Ray et al., 2014). *In vitro* studies have demonstrated arsenic exposure influences histone methylation, acetylation, and phosphorylation along with the protein expression of chromatin modifying enzymes that impart these modifications in human carcinoma cell lines (Zhou et al., 2008; Ren et al., 2011; Chervona et al., 2012a). Further, *in vitro* arsenic exposure in human PBMCs influences canonical H3 protein expression potentially underlying arsenic-induced sensitivity to DNA damage (Brocato et al., 2014). While very few epidemiological studies have assessed the effect of arsenic exposure (via drinking water) on the epigenome, those that have report a particularly strong correlation between arsenic exposure and altered histone methylation and acetylation, with a differential influence of arsenic dependent on sex (Chervona et al., 2012b). To our knowledge, there have been no reports on the long-term epigenetic consequences of developmental arsenic exposure in the brain, particularly of HPTMs and their associated chromatin modifying enzymes.

We are interested in the mechanisms that mediate the long-lasting toxicity of developmental arsenic exposure into adulthood when the presence of arsenic is quite low. Using a perinatal exposure paradigm to 50 ppb sodium arsenate through all three trimesters of fetal/neonatal development, we have previously demonstrated that arsenic reduces adult hippocampal neurogenesis, particularly differentiation of neural progenitor cells, leading to greater susceptibility to developing stress-induced depressive-like symptoms in adult male mice (Tyler and Allan, 2013; Tyler et al., 2014). Adult neurogenesis is comprised of several processes that include the continual mitotic activity of dentate gyrus neural progenitor cells and their subsequent maturation and integration into the hippocampal circuitry (Ming and Song, 2005). These processes are finely orchestrated by several epigenetic factors, including histone modifications (Ma et al., 2010). We have provided evidence that developmental arsenic exposure (DAE) alters the expression of many neurogenesis-related genes in the adult dentate gyrus (Tyler and Allan, 2013); however, the mechanism by which arsenic induces this damage is currently not known. Based on the extensive literature investigating the effects of arsenic on the epigenome in relation to cancer, we hypothesize that DAE leads to altered epigenetic processes, specifically histone modifications, in the adult mouse brain.

Here, we report an influence of DAE on levels of trimethylation of histone 3 lysine 4 (H3K4me3) and acetylation of histone 3 lysine 9 (H3K9ac) and on associated chromatin-modifying proteins including MLL, KDM5B, GCN5, PCAF, HDAC1, and HDAC2 in a sex- and region-dependent manner. No changes in levels of total H3 or H3K9me3 levels in either tissue of either sex were observed. This is the first report to demonstrate that low-level arsenic exposure during development influences the epigenetic landscape of the brain in adulthood long after arsenic exposure has diminished.

Materials and methods

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

Developmental arsenic exposure paradigm. The Institutional Animal Care and Use Committee at the University of New Mexico (UNM) approved the animal protocols, including the arsenic exposure paradigm, used in this study. C57BL/6 mice obtained from Jackson Labs were maintained on a reverse light/dark cycle (lights off at 0800) with *ad libitum* access to food and water in the Animal Resource Facility at UNM. Arsenic exposure was performed as previously described (Tyler et al., 2014), as depicted in Fig. 1. Briefly, singly-housed female mice aged 55 days were acclimated to drinking 50 parts-per-billion arsenic water (sodium arsenate, Sigma Aldrich) for 10 days prior to mating. Arsenic water was prepared weekly using standard tap and MilliQ water. Control mice were administered tap water from UNM, which contains approximately 2–5 ppb arsenic. Mating occurred for five days; dams continued to drink arsenic-laced water throughout pregnancy until offspring were weaned at postnatal day (PD) 23. Offspring were group housed separately by sex, four per cage, with *ad libitum* access to food and tap water. At PD70, animals were euthanized via rapid decapitation, and the frontal cortex and dentate gyrus from both male and female animals were microdissected and snap frozen and stored at −80 °C until further analysis. Female animals were exposed to male bedding prior to euthanizing. Synchronicity of cycle was confirmed via vaginal cytology, and only brain tissue from females in the proestrus phase was used, as some biochemical and behavioral measures are impacted by the phase of the estrous cycle (Warren and Juraska, 1997). Multiple rounds of breeding were performed for sufficient numbers of litters for arsenic or control groups. For each experiment, at least 6 different litters from different dams were used; *e.g.* $n = 6$ represents the number of different litters used with one animal per litter to avoid litter effects.

Histone extraction. For preparation of extracted histones, microdissected tissue, either the dentate gyrus or the frontal cortex, derived from one animal, was homogenized in a Biomasher II disposable microhomogenizer (Kimble Chase) using PBS buffer containing 0.5% Triton-X 100 (v/v), 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (w/v) sodium azide (NaN_3), 5 mM sodium butyrate (NaB), and 1 μg/μl protease inhibitor cocktail (Sigma, #P8340). Tissue homogenates were centrifuged at 6500 ×*g* for 10 min at 4 °C; nuclei were washed and centrifuged as before. The pellet was resuspended in 1 N HCl and acid extraction was allowed to occur overnight at 4 °C; 1 N NaOH was added the following day to neutralize the acid. Lysates were centrifuged as before and the supernatant saved for protein quantification. Bradford assays were performed to determine the concentration of histone protein. Aliquots were stored at −80 °C until further use.

Histone modification assessment. Approximately 8–12 μg histone protein, determined by antibody optimization, was separated using NUPAGE 10% bis–tris gels (Invitrogen, NP0316) and transferred to a PVDF membrane (Millipore Corporation, IPFL00010). Membranes were incubated overnight at 4 °C using the following primary antibodies diluted in PBS-T: H3 (1:1000 for DG and FC; Cell Signaling, 3638), H3K4 trimethyl (1:1000 for DG and FC; Abcam, ab8580), H3K9 trimethyl (1:2000 for DG; 1:1000 for FC; Epigentek, A-4036), and H3K9 acetyl (1:500 for DG and FC; Epigentek, A-4022). Membranes were incubated for 45 min in their respective secondary antibodies (1:15,000) from LiCOR: rabbit IRDye 680RD and mouse IRDye 800CW. Quantification of protein expression was performed using Image Studio, and values are expressed as each individual histone mark normalized to H3 for individual immunoblots, after it was determined that H3 was not altered by DAE. Evaluation of each histone modification was performed on separate gels and blots to avoid confounds. The $n = 6–10$ litters depending on histone mark and will be expressed for each individual blot in the results.

Evaluation of chromatin modifying proteins. Immunoblotting for chromatin-modifying proteins was conducted essentially as previously described using our established protocols (Goggin et al., 2012; Tyler et al., 2014). Dentate gyrus or frontal cortex tissue lysates from one

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