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Genome-wide alteration of histone methylation profiles associated with cognitive changes in response to developmental arsenic exposure in mice

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ABSTRACT

Inorganic arsenic is a xenobiotic entering the body primarily through contaminated drinking water and food. There are defined mechanisms that describe arsenic's association with increased cancer incidence, however mechanisms explaining arsenic exposure and neurodevelopmental or aging disorders are poorly defined. In recent years, arsenic effects on epigenome have become a particular focus. We hypothesize that human relevant arsenic exposure during particular developmental windows, or long-term exposure later in life induce pathophysiological neural changes through epigenomic alterations, in particular histone methylation profile, manifesting as cognitive decline. C57BL/6 wild-type mice were continually exposed to sodium arsenite (100 μg/L) in drinking water prior to mating through weaning of the experimental progeny. A second cohort of aged APP/PS mice were chronically exposed to the same level of arsenic. Cognitive testing, histological examination of brains and genome-wide methylation levels of H3K4me3 and H3K27me3 examined after ChIP-seq were used to determine the effects of arsenic exposure. Developmental arsenic exposure caused significantly diminished cognition in wild-type mice. The analysis of ChIP-seq data and experiments with mouse embryonic stem cells demonstrated that epigenetic changes induced by arsenic exposure translated into gene expression alterations associated with neuronal development and neurological disease. Increased hippocampal amyloid plaques levels of APP/PS mice and cognitive decline provided evidence that arsenic exposure aggravated an existing Alzheimer's disease-like phenotype. We show developmental arsenic exposure significantly impacts histone modifications in brain which remain present into adulthood and provide a potential mechanism by which developmental arsenic exposure influences cognitive functions. We also show that human relevant, chronic arsenic exposure has deleterious effects on adult APP/PS mice and exacerbates existing Alzheimer's disease-like symptoms. The results demonstrate how developmental arsenic exposure impacts the brain epigenome, leading to altered gene expression later in life.

1. Introduction

Inorganic arsenic concentration in the drinking water supply is a primary threat to worldwide human health, especially in rural and developing areas [1]. In addition to naturally occurring arsenic in ground water, arsenic tops the list of toxic substances found at contaminated sites that threaten human health [2]. Although the World Health Organization established 10 μg/L as maximum containment level for arsenic in drinking water, more than 220 million people are exposed to arsenic levels exceeding these guidelines worldwide [3]. Chronic low-level arsenic exposure through drinking water is common in the U. S., especially in the Southwest where 6% of sampled public water-supply systems exceed the 10 μg/L maximum containment level and another 25% exceed 4 μg/L; disproportionately affecting rural and Hispanic communities [4]. Local exposures can be much higher, 21% of water from private wells in Nevada exceeded 100 μg/L [5]; while overall in Maine 18.4% of sampled wells contained arsenic concentrations greater than 10 μg/L, and 4% had arsenic concentrations greater than 50 μg/L [6].

Studies in animals have demonstrated that arsenic levels in brain have a dose–response relationship to drinking water concentrations, showing penetrability of the blood-brain barrier with accumulation in the cortex and hippocampus [7,8]. We have previously shown that low level arsenic exposure in adult mice impaired memory functions [9].

Abbreviations: AD, Alzheimer's disease; ES, Embryonic Stem Cell; NOR, Novel Object Recognition; RWM, Radial Arm Water Maze.

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Wasserman et al. found children in Maine exposed to more than 5 μg/L arsenic in drinking water were at risk for impaired cognition [10–12] and early life exposure to arsenic has been shown to result in impaired learning and memory in rodent models [12–14]. Furthermore, arsenic has been shown to cross the placenta and moderate perinatal exposures can adversely affect learning and memory of the progeny [12,15]. Despite known neurotoxic effects of higher levels of arsenic, much less is known about the underlying molecular and cellular mechanisms of low-level arsenic induced brain pathologies.

Epigenetic events are essential for normal brain development, providing critical modulation of neural gene expression during different developmental stages [16]. Long-term effects of prenatal insults are related to epigenome dysregulation, rendering the central nervous system vulnerable to developmental and aging disorders. Many studies have investigated arsenic effects on epigenetic regulation in liver, lung, muscle and other peripheral organs, but few have focused on the brain. Epigenetic modifications play a major role in arsenic toxicity including changes in DNA methylation, release and abundance of microRNAs and histone modifications [17]. DNA methylation, especially within the CpG dinucleotides, is one of the most widely studied mechanisms of epigenetics [18]. During the metabolism, arsenic is extensively methylated, therefore many studies have focused on altered DNA methylation [19, 20]. While these results were to some extent conflicting, increased methylation of genes involved in neural plasticity was an important finding. Allan et al. observed that developmental arsenic exposure diminished methylation in the SP-1 binding site region of Nr3c1 at embryonic day 14 which was no longer significant at embryonic day 18; furthermore, there was no change in the methylation state at the Egr-1 binging site on Nr3c1 [21]. This suggests that arsenic is likely to produce methylation change through dynamic regulated epigenetic processed that are age, sex and promoter site specific. We were the first to show genome-wide changes in H3K9 acetylation patterns of the offspring from dams exposed prenatally to arsenic [9]. Changes in acetylation patterns were recently confirmed by Solomon et al., where developmental arsenic exposure was associated with H3K9 acetylation of Fkbp5 in male mice and Crh in female which could potentially impact stress and glucocorticoid signaling respectively [22]. It is unclear however how arsenic induced epigenetic reprogramming regulates neuronal function impacting cognition or if arsenic induced alterations in gene expression associated with normal development and aging result in long-term consequences for brain structure, function and degeneration.

There is an increased focus on understating how a person's exposome, or measure of all exposures in a lifetime, relates to healthy aging. Environmental factors and gene-by-environment interactions are poorly understood in healthy brain aging and development of neurogenerative disease, such as Alzheimer's disease (AD). There is sufficient evidence to hypothesize a relationship between chronic low-level arsenic exposure over a broad range of ages and exasperated phenotypes of neurodegenerative diseases. For example, low-level arsenic exposure was associated with increased gene transcription for amyloid precursor protein (APP) [23], hyperphosphorylation of tau protein [24], oxidative stress [25], and inflammation [26,27], all of which are associated with cognitive decline and pathological mechanisms of AD. Epidemiological data demonstrate that environmental arsenic over a broad range (7–18 μg/L) in topsoil was associated with prevalence of dementia [28]. Analysis of 434 participants from the Project FRONTIER demonstrated a correlation between long-term low-level arsenic exposure from groundwater to poor executive function, visuospatial skills and language [29]; this is important since these cognitive domains are the earliest impacted in AD. Recently, Li et al. demonstrated in mainland China a soil arsenic concentration dependent increase in AD related deaths which provides evidence for a possible association between low-level arsenic exposure and AD mortality risk [30].

We hypothesize that low-level arsenic exposures during important developmental windows or long-term exposures later in life induce pathophysiological neural changes through epigenomic alterations, triggering impaired brain development and healthy aging. We have examined the effects of a developmental (preconception until weaning) exposure to arsenic, at human-relevant level, on changes in histone methylation profile and cognitive function in mouse progeny. Chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq) with antibodies for methylated lysine 4 of histone 3 (H3K4me3), as well as methylated lysine 27 of histone 3 (H3K27me3), were used to evaluate the differences in enrichment and determine differences in Gene Ontology terms between arsenic exposed and control groups. We also determined changes in cognitive function and pathological phenotype of AD model mice exposed to the same low-level of arsenic.

2. Materials and methods

2.1. Animals

All reagents were from Fisher Scientific unless documented in the text. Animal studies followed guidelines described in the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh; ensuring mice were treated humanely and regarding alleviation of suffering. Animals were housed in a temperature and humidity-controlled facility with a 12 h light/dark cycle and ad libitum access to food and water. Randomly assigned C57BL/6 wild-type (WT, Hilltop Laboratories) dams of the parental generation were assigned to either control of arsenic exposure group. For the arsenic exposure, 100 μg/L of sodium arsenite was introduced in the drinking water starting 7 days prior to timed mating with a WT sire. Harem breeding (1 male and 2 females) was utilized for timed mating. The WT experimental progeny and parents were continually exposed to arsenic in the drinking water from birth until weaning (total of 46 days of arsenic exposure); at which time they were placed on normal drinking water (Fig. 1). The mice in the control group were provided normal drinking water throughout the experiment timeline. Three sets of harem breeding were utilized for each exposure group with two males and two females chosen at random from the first timed litters used for the WT experimental progeny. The initial weights (control 1.46 ± 0.03; arsenic 1.44 ± 0.04), average number of pups per litter (control 7.6; arsenic 9.3) and preweaning mortality (control 3.3%; arsenic 1.7%) of the experimental pups was not significantly different between groups.

A second experimental cohort included APP/PS1ΔE9 mice on a C57BL/6 J background [B6. Cg-Tg (APPsw, PSEN1ΔE9)85Dbo/J] from The Jackson Laboratory (referred to as APP/PS). APP/PS mice express familial human amyloid precursor protein (APP) with Swedish mutation (APPsw), and human PS1 (presenilin 1) exhibiting deletion in exon 9 (PS1ΔE9). APP/PS mice between 6 and 7 months of age were randomly assigned to the control or arsenic exposure group. This age was chosen because this represents a timepoint when this mouse model has

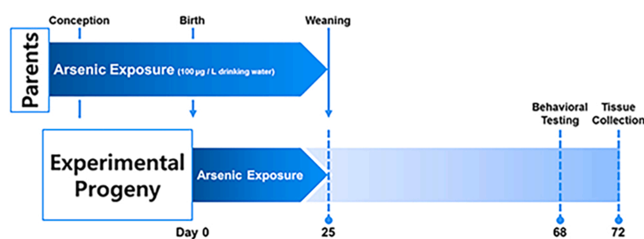

Fig. 1. Timeline for developmental arsenic exposure in experimental progeny. WT dams assigned to the arsenic group were exposed to 100 μg/L sodium arsenite in the drinking water starting before conception until the time of weaning (day 46 after arsenic exposure). Experimental progeny underwent novel object recognition and fear conditioning for changes in cognitive function at 2–3 months of age. Tissues were collected for ChIP-seq analysis following behavioral testing.

developing amyloid plaque pathology. The arsenic group was exposed to 100 μg/L of sodium arsenite in the drinking water for 3 weeks prior to and continually until behavioral testing and tissue collection was completed. The control group was giving normal drinking water throughout the study design. Control and arsenic treated water were prepared and changed every other day for all experiments. Equal sexes were used in each experimental group throughout the study. All efforts were made to minimize the number of animals used.

2.2. Behavioral testing

All behavioral paradigms were completed during the light phase; always started 3 h following the transition from dark to light phase to minimize the impact of circadian rhythms. Mice were acclimated to the behavioral suite 1 h prior to testing each day. The external cues during each phase of testing were maintained for all experimental groups and a curtain was used to shield the examiner. To minimize stress of bright light, the behavioral suite utilized diffuse low light with the center of each apparatus illuminated at 20 lux. Temperature and humidity of the behavioral suite were the same as housing conditions. Sound levels were minimized during testing and maintained around 80 dB which is equivalent to the housing conditions. To thoroughly remove odor cues the apparatus and objects were cleaned with 70% ethanol followed by a final wash with water. Changes in cognition in the developmentally exposed WT mice were evaluated at 2–3 months of age, while the APP/PS mice were assessed between 6 and 7 months of age.

2.2.1. Novel object recognition (NOR)

Changes in long term memory associated with arsenic exposure were examined using the novel object recognition paradigm [31]. First, mice were acclimated to the behavioral paradigm box (40 cm × 40 cm X 30 cm tall- white plastic box) for 5 min 24 h later mice were lowered into the box with two objects (Lego structure) that were the same and allowed to explore both objects during a 5 min trial. During this stage of training the trial was repeated including a 5 min intra-trial interval. 24 h following the last training trial, an object was substituted for the novel object (similar sized metal bolt) and mice were allowed to explore both objects during a 10 min trial. Exploratory visits were defined as mice touching or within a 1 cm radius while oriented toward the object. Animals were always placed in the center of the paradigm box during all stages of testing. Testing was recorded with AnyMaze software (Stoelting Co.) and decreased percentage of total time exploring the novel object (time with novel object / total time exploring × 100) denotes decline in long-term memory retention associated with arsenic exposure.

2.2.2. Contextual cued fear conditioning

Fear conditioning was performed as described previously [31]. Fear conditioning was started 24 h following the end of NOR. Mice were first acclimated to the chamber for 2 min prior to starting the conditioned stimulus (Tone of a 30 s duration, 85 dB and 2800 Hz). In the final 2 s of the tone, mice were administered a 0.7 mA foot shock in the barred floor, and this stage was repeated. After the last cycle, mice were left in the chamber for 30 s then returned to housing cages. 24 h later contextual fear conditioning was performed by assessing freezing behavior during a 5 min trial in the original conditioning chamber. Cued fear conditioning was performed 24 h later by introducing mice to a new chamber where the grey walls were replaced with black and white striped. Mice were acclimated to the novel chamber for 2 min prior to the start of the original conditioned stimulus for 3 min, with freezing behavior measured. Freezing was defined as no movement except breathing and was scored with AnyMaze software.

2.2.3. Radial arm water maze (RWM)

Changes in spatial learning and memory were assessed utilizing a two-day radial arm water maze protocol as performed before [32]. RWM was a six-arm paradigm (arms measured 20 cm wide, 40 cm long, and 8 cm high walls above the water level) and a central area (30 cm in diameter), filled with opaque water (21 ± 1 °C) to 1 cm above a hidden platform (diameter of 10 cm). Following handling, animals were acclimated to the RWM for a 5 min trial without the hidden platform. Two days of training contained two 6 trial blocks followed by a 3-trial block (15 total trials per day). Between trail blocks animals were allowed to rest for 30 min. During training day 1, a visible platform (flag 6 cm above the water level) was used in trials 1, 3, 5, 7, 9, and 11. All other trials on day 1 and all trails on day 2 utilized a hidden platform. Animals were placed into an arm and swam to find the platform for 60 s. Once on the platform they were allowed to rest there for 20 s to familiarize themselves with the spatial cues. Mice that did not find the platform were guided to the platform and allowed to rest there for 20 s. Animals were run in groups of four providing a 5 min inter-trial interval. The end of a unique arm was the start location for each trial with the platform location changed between groups to avoid location bias. AnyMaze software was used to record the animals swim path. Time errors and total number of incorrect arm entries were added together for the overall trial performance. A time error was defined as no arm entries after 15 s. An incorrect arm entry was identified once 50% of an animal's body entered an arm without the platform. For the 15 daily training trials, performance during 3 consecutive trials was averaged for a total of 5 blocks per day and 10 blocks for the entire RWM for each animal.

2.3. Animal tissue processing

Following behavioral testing, mice were anesthetized using Avertin (intraperitoneal injection, 250 mg/kg), blood collected using EDTA coated syringes and perfused through the heart with 20 ml of cold PBS. There was no significant difference in the weight of the WT (control 21.8 ± 0.4; arsenic 20.9 ± 0.6) or APP/PS (control 29.7 ± 1.1; arsenic 28.8 ± 1.5) experimental groups at the time of perfusion. After removal, brains were cut into two hemispheres with one hemisphere being dropped fixed for 48 h in 4% paraformaldehyde followed by storage in 30% sucrose at 4ºC, for histological analysis. The hippocampus and cortex from the other hemisphere were removed and snap frozen on dry ice, to be used for ChIP-seq analysis. Frozen samples were stored at − 80ºC before processing.

2.4. Histology

All procedures were as previously published [31]. One brain hemisphere was embedded in OCT medium. 30 μm coronal sections were cut starting at 150 μm posterior to the beginning of the dentate gyrus with six serial sections approximately 700 μm apart. Sections were stored in glycol-based cryoprotectant at − 20 °C until staining.

2.4.1. X-34 staining

Serial sections were mounted on slides and washed 10 min with PBS. Sections were stained for 10 min with 1,4-bis(3-carboxy-4-hydroxyphenylethenyl)-benzene (X-34) (100 μm) followed by 5 × 1 s dips in PBS. Sections were destained for 2 min with 0.2% NaOH in 80% unbuffered ethanol; followed by 5 × 1 s dips in PBS and a final 10 min PBS wash.

2.4.2. GFAP staining

Serial free-floating sections were stained with rabbit anti-GFAP antibody as previously [31]. Sections were washed three times with PBS and blocked for 60 min using 3% normal goat serum in PBS with 0.2 Triton-X for permeabilization. Sections were incubated in anti-GFAP antibody (Z033429–2, Agilent; 1:1000) for 3 h. Following three 10 min PBS washes, sections were incubated with goat anti-rabbit 594-labeled antibody (DI-1594, Vector Biolabs; 1:250) for 1 h. Following three 10 min PBS washes, sections were mounted on slides. All staining steps were performed at room temperature on a shaker.

Imaging was performed with the Nikon Eclipse 80i microscope (40X

total magnification) and MetaMorph 7.0 software (Molecular Devices) was used to define the percent area coverage for X-34 and GFAP staining.

2.5. Chromatin immunoprecipitation and ChIP-seq

Brain tissue from WT mice in the arsenic and control groups was processed for Chromatin Immunoprecipitation and sequencing. Brain homogenates were sonicated for 3 pulses (15 s) at a 30-amplitude, a 120 s pause and 3 pulses (15 s) at a 40-amplitude using a Sonic Dismembrator (Model 705, Fisher Scientific) to generate fragments 200–600 base pairs (bp) in size. We used rabbit polyclonal antibodies that are anti-trimethyl Lys4 Histone H3 (H3K4me3, CS200580, Millipore) and anti-trimethyl Lys27 Histone H3 (H3K27me3, 07–449, Millipore) for immunoprecipitation. For ChIP validation, ChIP-QPCR with mouse Glucagon (*Gcg*) used as a negative control was performed to determine normalized fold enrichment to input of *Gcg*. TruSeq ChIP library preparation kit (Illumina) was utilized to generate the ChIP libraries following the protocol provided by the manufacturer. During each step, AMPure XP beads (Beckman Coulter) were used to purify the samples. Adapter-ligated samples were separated using a Pippin Prep (Sage Science) with 2% agarose gel to isolate 250–300 bp DNA fragments and to exclude un-ligated adapters. Library validation was performed with Agilent Technologies 2100 Bioanalyzer to determine purity, size and concentration.

Validated libraries were sequenced on an Illumina hiSeq2000 instrument at the Next-Generation Sequencing Core at the University of Pennsylvania (Philadelphia, PA; https://ngsc.med.upenn.edu/#/). The following programs were used in sequencing data processing: Subread (http://subread.sourceforge.net) to align sequencing reads to the mouse genome (mm9); Homer pipeline and Bedtools (http://bedtools.readthedocs.org/en/latest/) for processing of the BAM/BED files, peak calling, and annotation.

2.6. Gene ontology analysis

To identify biological processes associated with significantly affected genes, Gene Ontology (GO) analysis was performed with Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7; [33,34]). GO analysis was performed on the top 3000 unique genes that were present in the control and arsenic groups for both H3K27me3 and H3K4me3. Exclusion was based on the distance from nearest transcription start site with $\leq$ 90 kb being the cut point. Ingenuity Pathway Analysis (IPA) was used to identify common biological functions in both arsenic and control groups. Span scores for the common genes in these pathways were utilized to generate heatmaps.

2.7. Embryonic stem cell (ES) arsenic exposure

Mouse embryonic stem cells (ES, line R1 (ATCC)) were grown on T75 flasks coated with gelatin (0.1%) utilizing normal GMEM ES culture medium supplemented with 10% FBS, sodium pyruvate (1 mM), L-glutamine (2 mM), 2-mercaptoethanol (0.1 mM), nonessential amino acids (0.1 mM), 100 units/ml mouse leukemia inhibitory factor and antibiotics. Neuronal differentiation was performed using a previously published protocol [35]. ES were plated on dishes in medium without mLIF to induce embryonic body creation. Embryonic bodies were then treated with 5 μM retinoic acid, dissolved in DMSO. DMSO alone was used as a negative control. Following 4 days of treatment embryonic bodies were trypsinized and plated on poly-L-ornithine/laminin-coated plates containing N2B27 medium (DMEM supplemented with glucose, N2A and B27). At this point, the cells were treated with 500 nM Arsenic or PBS as control. RNA was isolated 6 days after plating and treatment with As, corresponding to 10 days total following retinoic acid treatment. The effect of arsenic on the expression of *Tubb*, *Vglut* and *App* was examined by RT-qPCR.

2.8. Statistical analysis

Performance in the novel object recognition and fear conditioning were analyzed by unpaired t-test. One-way ANOVA followed by Tukey post hoc test was used to determine significance for multiple comparisons. Statistical significance for the RWM was determined by two-way repeated measures ANOVA (General Linear Model/RM-ANOVA) with exposure and learning trial as factors. GraphPad Prism (version 4.0) was used for all statistical analyses unless otherwise mentioned in the text. All results were reported as means $\pm$ SEM and differences were considered significant when $p < 0.05$.

3. Results

3.1. Developmental arsenic exposure significantly diminishes cognitive performance in wild-type mice

Previously, we reported that arsenic exposure (100 μg/L in drinking water) in mature 6-month-old WT mice significantly diminished cognitive performance [9]. In the current study, we wanted to determine the effect of developmental arsenic exposure on learning and memory function (see Fig. 1 for experimental timeline). To examine if a developmental arsenic exposure has the same effect as exposure in mature mice, we exposed WT dams to 100 μg/L arsenic for seven days prior to mating, during pregnancy and until weaning of offspring. The experimental progeny was exposed until they were weaned and then placed on normal tap water for the rest of the experiment. Memory deficits were evaluated by contextual fear conditioning and novel object recognition (NOR) paradigms at 2–3 months of age.

As seen on Fig. 2 A, developmental arsenic exposure significantly impaired performance during the contextual phase of fear conditioning when compared to animals on normal drinking water. No differences in the level of freezing was observed when both groups were placed in a novel environment suggesting the significant differences observed were not due to changes in normal activity and movement (Fig. 2B). Furthermore, we found no effect of arsenic exposure on performance during the cued phase of testing (Fig. 2 C). It is well established that contextual phase of testing is hippocampal dependent while the cued phase is dependent on the amygdala and utilization of different brain regions could explain differences observed. Fig. 2D showed the performance of the same mice in NOR which tests long-term memory deficits. We found that developmental arsenic exposure caused a significant decrease in NOR performance. The effects of arsenic exposure on cognitive performance was equally observed in males and females (Supplement Fig. 1). The conclusion from these studies was that developmental arsenic exposure significantly diminished learning and memory function similarly to those observed in mice exposed to arsenic later in life [9].

3.2. Developmental arsenic exposure modifies the epigenetic landscape in brain of mice

To determine the epigenetic changes induced by developmental arsenic exposure we performed ChIP-seq assays to map genome-wide changes in the enrichment of two histone marks – H3K4me3, a marker of active promoters, and H3K27me3 – associated with Polycomb repressive complex and a strong marker of repressed promoters [36]. We found that the arsenic-exposed and control groups had 7515 common genomic sites for H3K4me3. There were 328 unique sites for arsenic exposed and 2274 unique sites for control mice (Fig. 3A). Furthermore, arsenic exposure affected H3K4me3 enrichment genome wide, increasing the binding to intragenic sites (46.3% vs 43% in control) (Fig. 3B). To identify GO functional biological processes affected by arsenic exposure we used DAVID. We found that the most significant GO Biological Process terms corresponding to the unique genomic sites for H3K4me3 in arsenic exposed group were regulation of steroid synthesis,

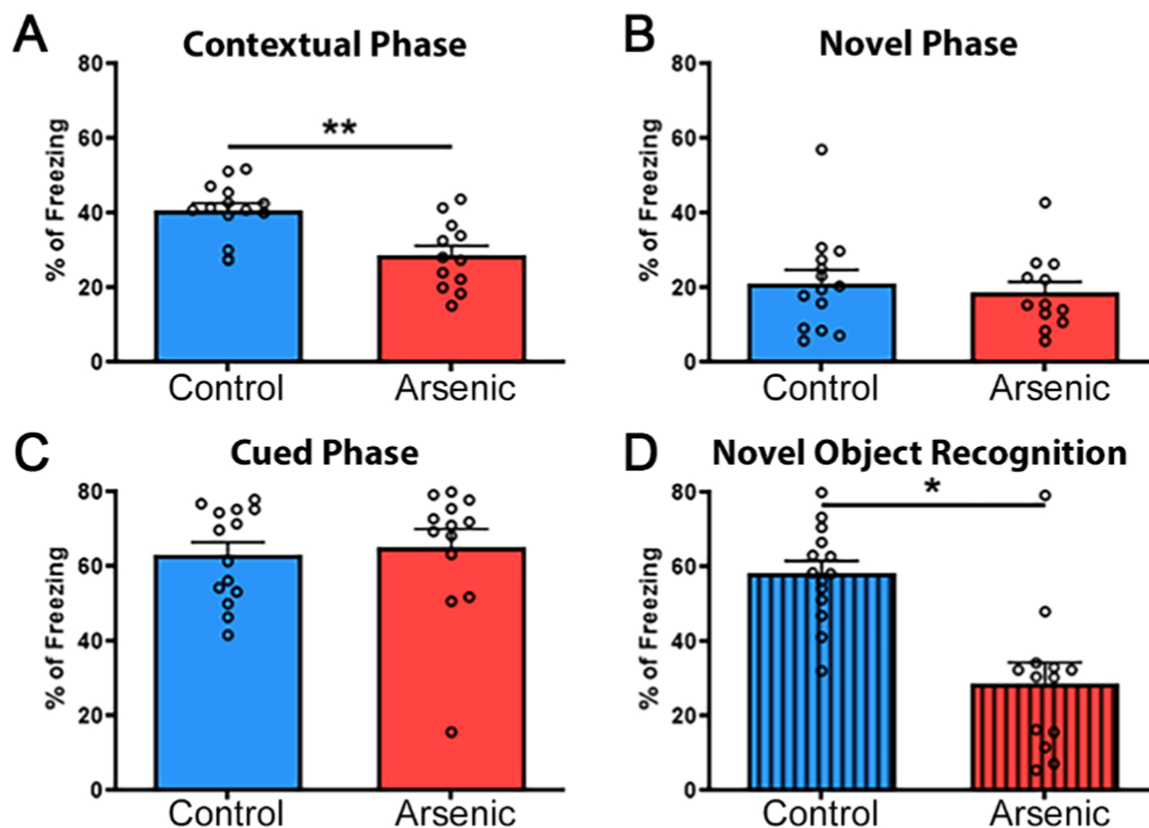

Fig. 2. Effect of developmental arsenic exposure on cognitive function of mice. A. Developmental arsenic exposure (100 μg/L in drinking water) significantly impaired the performance of 2-month-old mice during the contextual phase of fear conditioning. All mice exhibited comparable performance during the novel phase (B) and cued phase (C) of testing. D. The deleterious effect of developmental arsenic exposure on cognitive function was confirmed utilizing the novel object recognition paradigm. Control mice spend significantly more time exploring the novel object compared to arsenic exposed mice. Significance determined by unpaired t-test. n = 12 mice per group with equal sexes. Bars represent mean ± SEM. * * = $p < 0.01$; * = $p < 0.05$.

mitosis, and nuclear division. For example, we observed increased enrichment in arsenic exposure group around promoter of *Srebf1* (Sterol regulatory element binding transcription factor 1) and *Nr3c1* (Glucocorticoid receptor) suggesting that the transcription of these genes may be affected by developmental arsenic exposure. Previous data demonstrated that *Srebf1* expression was affected by exposure to low levels of arsenic [37]. Also, studies suggest that glucocorticoid hormones affect target genes such as brain-derived neurotrophic factor (*Bdnf*), critical for neuronal function and behavior [38]. The most significant GO Biological Process terms for H3K4me3 sites unique to the control group were cellular response to stress, chromatin organization, and cytoskeleton organization. Using Ingenuity Pathway Analysis (IPA) software we found that following arsenic exposure the H3K4me3 enrichment was associated with phagocytosis, and autophagy signaling as the most significant canonical pathways. Interestingly, several genes associated with neurotransmission such as *Slc17A7* (also called *Vglut1*) and *Syt1* or development such as *Psen1* (presenilin 1) lost their H3K4me3 mark close to the transcriptional start site.

Using IPA, we also analyzed significantly enriched over the corresponding background common H3K4me3 genomic sites in arsenic exposed and control groups and generated heatmaps to cluster high and low H3K4me3 enrichment scores. Within the number of common binding sites in the proximal promoters, identified in both conditions, we found differential H3K4me3 enrichment for 184 genes critical to nervous system development and function; 109 of those showed higher scores in arsenic exposed mice implying most genes affected by arsenic were associated with nervous system development (Fig. 4 A). We performed the same analysis for genes associated with neurological disease and found 50 out of 79 common genes with higher enrichment scores in arsenic exposed compared to control group (Fig. 4B). A more precise analysis of sequencing data derived from samples subjected to sequential IP for H3K4me3 and H3K27me3 in parallel to transcriptome / RNA-seq data is necessary to reveal correlation between histone modifications, gene expression levels and arsenic exposure effects.

The analysis of H3K27me3 enrichment scores (a repressive mark) revealed 1633 common binding sites for the arsenic exposure and control groups. We identified 793 sites unique to arsenic, and 4307 sites unique to the control group (Fig. 5A). Using DAVID, we found that the most highly significant GO Biological Process terms for H3K27me3 sites in the arsenic exposed group were neuron development, neuron projections morphogenesis, embryonic organ development, and axon guidance (Fig. 5B). The most significant category affected in the arsenic exposure group was axon guidance, while most significantly affected in the control group were categories in cell death and regulation of transcription. The enrichment of the H3K27me3 mark at genomic sites associated with neuronal development suggests that arsenic could have a repressive effect on several pathways within these categories. The conclusion drawn from the Chip-seq analysis was that exposure to arsenic significantly affected the epigenetic regulation of several genes associated with neuronal fate and development thereby strengthening the concept of the deleterious effects of a developmental exposure to arsenic in relation to cognition.

3.3. Exposure of mouse embryonic stem cells (ES) to arsenic decreases expression of neuronal differentiation gene-markers

To determine the effect of arsenic on neuronal differentiation ES were treated at a stage of embryonic bodies (Fig. 6A) with retinoic acid to induce the conversion of pluripotent ES to neuronal cell progenitors [35]. Retinoic acid containing media were replaced after 4 days and

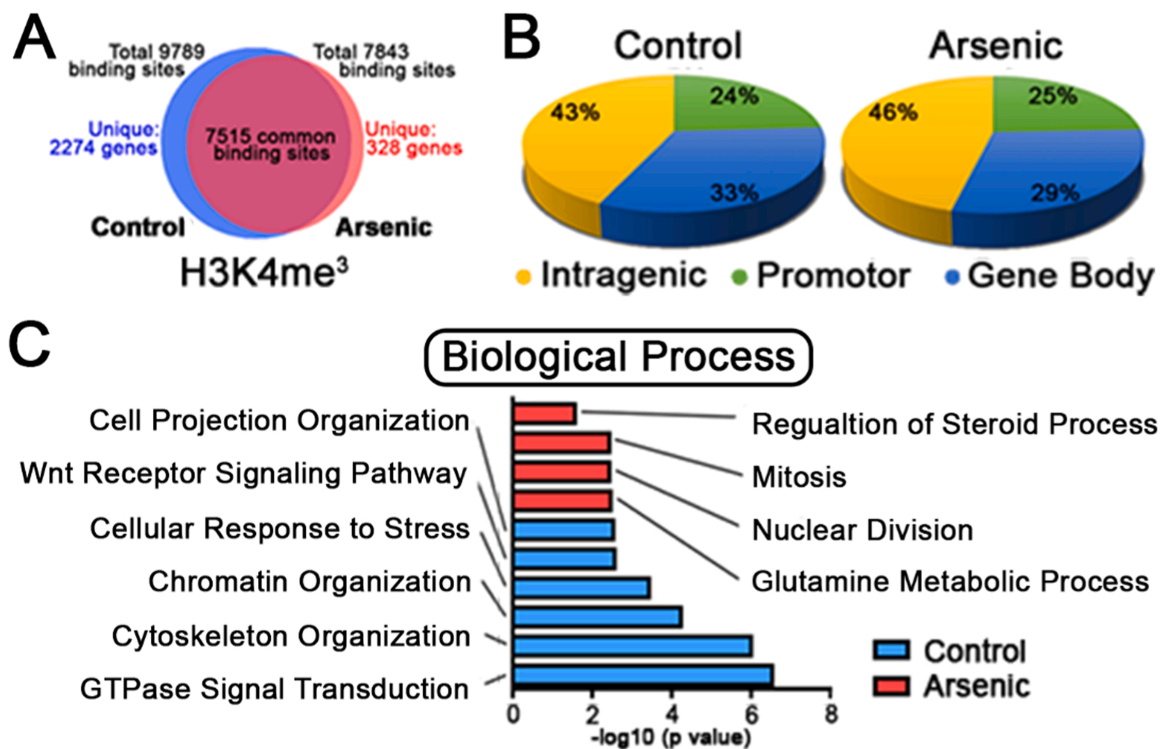

Fig. 3. ChIP-seq data shows trimethylation at H3K4 in response to developmental arsenic exposure. A. Venn Diagram presenting numbers of common and unique peaks found using Homer for control (blue) and arsenic (red) groups. B. Genome-wide distribution of histone modification sites for H3K4me3 in control and arsenic exposed mice. C. Most significant GO Biological Process categories (-log10 p value) revealed by ChIP-seq following developmental arsenic exposure identified with DAVID.

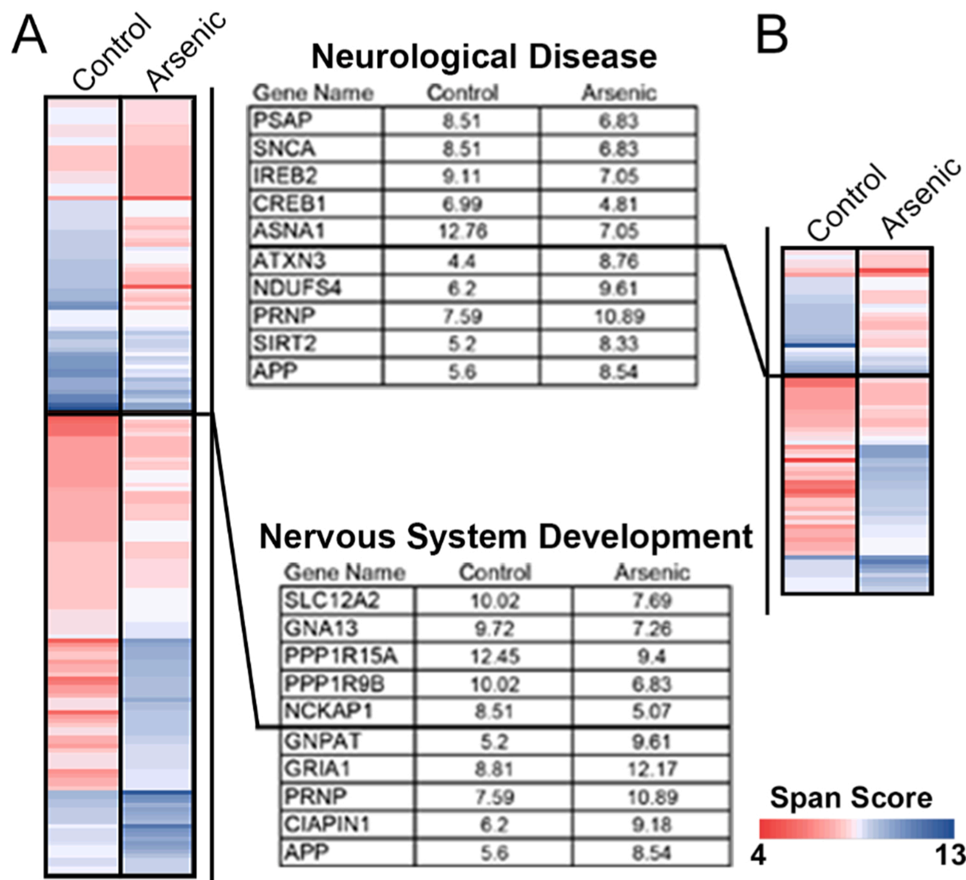

Fig. 4. Heat map generated from span scores for common genes H3K4me3 in nervous system development and function and neurological disease (IPA). For each biological process the top five span score differences are shown down the middle with the genes higher in arsenic than controls on the bottom portion of each heatmap. A. 184 common genes were found to be involved in nervous system development and function and were significantly enriched over the corresponding background (109 genes were significantly up regulated and 75 genes were significantly down regulated). B. 79 common genes were found to be involved in neurological disease (50 genes were significantly up regulated, and 29 genes were significantly down regulated).

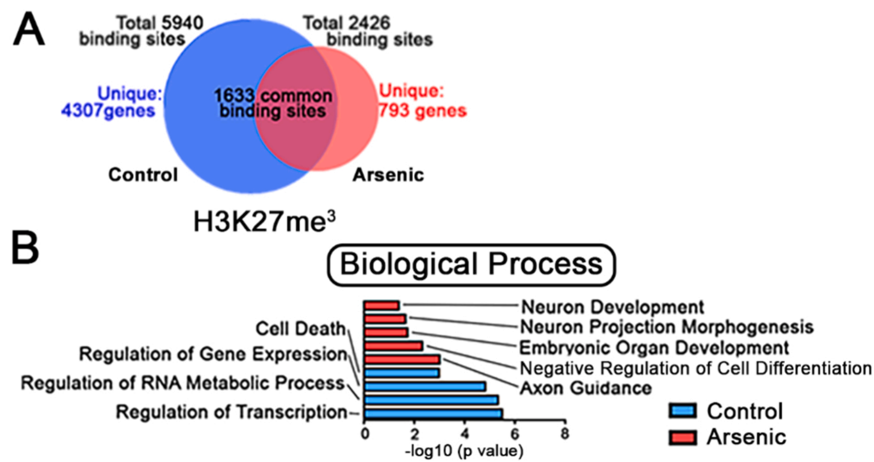

Fig. 5. ChIP-seq data reveals trimethylation at H3K27 in response to developmental arsenic exposure. A. Venn Diagram presenting numbers of common and unique peaks found using Homer for control (blue) and arsenic (red) groups. B. Most significant Biological Process categories (-log10 p value) attributed to arsenic exposure identified by DAVID.

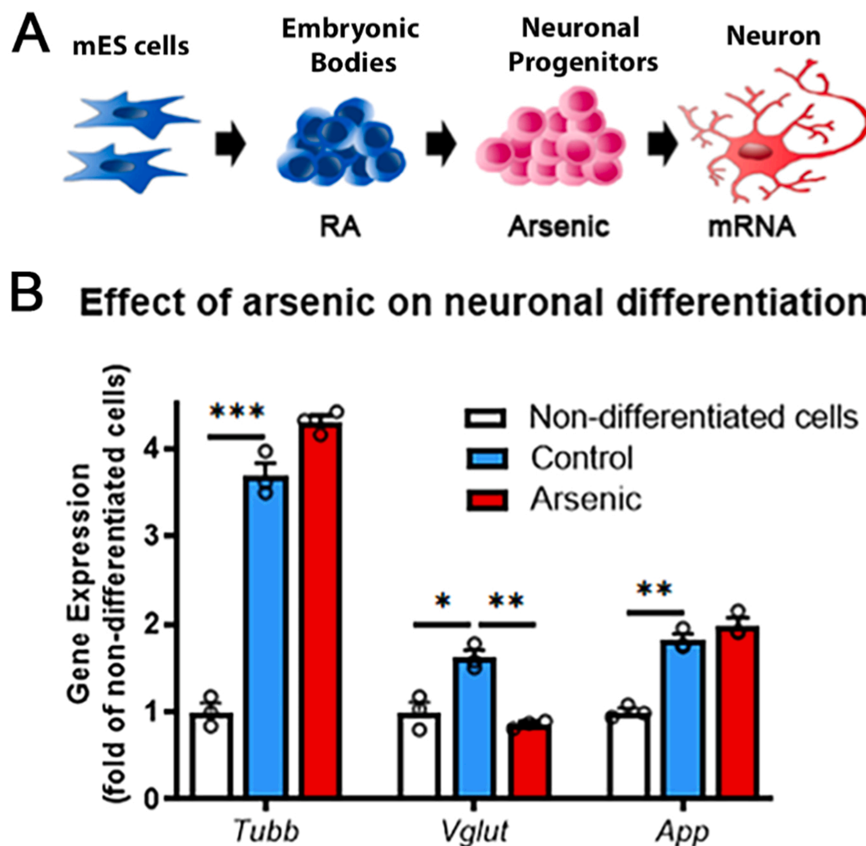

Fig. 6. The effects of arsenic exposure on expression of neuronal differentiation gene-markers in ES. ES were treated with 5 μM retinoic acid for 4 days and plated for neuronal differentiation for 6 days in medium with 500 nM arsenic or PBS (control). A. Stages of embryonic stem cells, from undifferentiated embryonic stem cells to embryonic bodies, to neuronal progenitors, and finally neurons. B. Graph showing mRNA levels in 3 different neuron related genes assessed by fold change of non-differentiated cells (control = neuronal progenitors not treated with arsenic, arsenic = neuronal progenitors treated with arsenic). Analysis by one-way ANOVA followed by Tukey's multiple comparison test. Data represents gene expression from three independent ES culture experiments. Bars represent mean ± SEM. * $p < 0.05$; * * $p < 0.01$; * ** $p < 0.001$.

neuronal progenitors were treated with arsenic (500 nM) or PBS (control) for 6 days. mRNA level was compared in cells stimulated and non-stimulated with retinoic acid (non-differentiated cells). To confirm the conversion of ES into neurons we examined gene expression levels of neuronal differentiation markers such as *Tubulin β* class 1 (*Tubb*), *Slc17a7* (also known as *Vglut1*) and *App* [39,40]. As shown on Fig. 6B, only cells treated with retinoic acid converted to neurons (compare white to red columns). Interestingly, arsenic suppressed mRNA expression level of *Vglut1* which correlates to the absence of H3K4me3 mark in our ChIP-seq data. These results strongly support the conclusion that epigenetic changes induced by arsenic exposure could translate into changes in gene expression associated with neuronal development and differentiation.

3.4. Exposure of adult APP/PS mice to arsenic exacerbates memory deficits and amyloid pathology

We next examined the cognitive effects of arsenic exposure in 6–7-month-old APP/PS mice. The arsenic exposure group was provided drinking water with arsenic for 3 weeks prior to testing, while the control APP/PS and WT littermates were provided normal drinking water. We utilized the radial water maze (RWM) to assess working

memory and spatial learning. Fig. 7A shows RWM data for the three experimental groups, both APP/PS groups preformed worse than the WT mice throughout the entirety of the experiment. In the last two trial blocks of RWM (Fig. 7B), the APP/PS mice exposed to arsenic had a higher number of errors than the APP/PS exposed to normal drinking water; emphasizing the negative impact arsenic exposure had on learning and memory function.

We then determined how exposure to arsenic impacts amyloid pathology and glial function in this APP/PS mouse model. X-34 staining was used to visualize amyloid plaques in the cortex and hippocampus. Arsenic exposure significantly increased the level of amyloid plaques in the hippocampus of APP/PS mice compared to control (Fig. 8 A). There was also a trend of arsenic exposure increasing the amyloid plaque levels in the cortex, however this did not reach significance. We also found a significant increase in the percent of the tissue stained with GFAP positive astrocytes in the hippocampus, and comparable trend, but insignificant, in the cortex following arsenic exposure in the APP/PS mice (Fig. 8B). Male and female APP/PS mice demonstrated similar responses to arsenic exposure in both cognitive and histological endpoints assessed (Supplement Fig. 2).

4. Discussion

Arsenic is known to be a toxicant at high levels and can be found both naturally as well as anthropogenically and acute arsenic toxicity is only one avenue of arsenic exposure leading to serious health consequences. The global presence of arsenic present in drinking water at chronic, low levels is becoming a growing area of concern in many regions. The 10 μg/L standard established by the World Health Organization is regularly exceeded in many places, and arsenic exposure has been correlated with complications in nearly all systems of the body including immunological effects and central nervous system dysfunction [41]. In addition, the 10 ug/L standard may not be sufficient to protect against neural developmental and cognitive effects of arsenic [10].

We have previously reported on changes in enrichment levels and effects of H3K9ac under similar exposure conditions [9]. The aim of the study was to extend our understanding of epigenetic changes – abundance of H3K4me3 and H3K27me3 marks and their association with a developmental arsenic exposure. H3K4me3 is a well-known symbol of active genes and is distributed along the transcription start site as well as the promotor regions. H3K27me3 was selected because it is the most well-characterized repressive mark and is important in the repression of key transcriptional regulators during many developmental stages [42]. Between these two marks there is a wider range of epigenetic changes to look at and thus a stronger base for understanding biological significance of gene regulation.

When comparing conditions and looking at histone modifications as an endpoint, the lack of gene presence is often as important as the presence of a certain gene. In our analysis of H3K4me3 genomic sites, there was a large amount of overlap between the arsenic exposed and control groups, but also significant changes in some important genes for neural function. Two genes, *Slc17A7* and *Psen1* were present in the controls as expected but absent in the arsenic treated mice. *Psen1* is one of the three genes (*APP*, *Psen1*, and *Psen2*) that regulate APP catabolic processes and Aβ formation and associated with AD [43]. *Slc17A7* (also known as *Vglut1*) is a gene that is strongly associated with neurotransmission and significantly disrupted by the developmental arsenic exposure. Alterations in *Slc17A7* were further validated with our in vitro model system, where *Vglut1* showed a reduction of expression in the arsenic treated mice which overlaps with the Chip-seq data from the H3K4me3 where *Vglut1* was not present in the analysis. The absence of genes involved in neurotransmission and neurodevelopment such as these in the arsenic exposed group is indicative of the overall epigenetic changes associated with arsenic exposure.

To explore the relevant biological processes of developmental arsenic exposure, we performed IPA analysis on processes of neurological disease and nervous system development. In both processes the Amyloid precursor protein (*App*) gene was found to be higher in the arsenic group, which confirms what was observed prior by other studies [23]. Knowing that H3K4me3 is an activation marker the higher span score is indicative of higher gene expression in the arsenic group. One of the genes in the Neurological disease process that was found to have a lower span score in the arsenic exposed group was the cAMP response element-binding protein (*Creb*) gene. *CREB* activity is considered vital

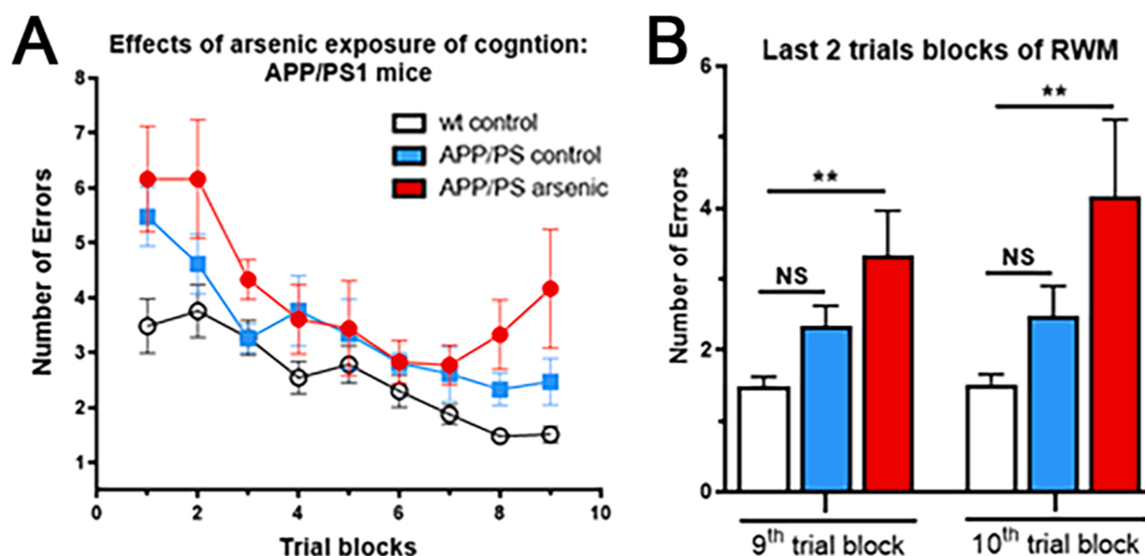

Fig. 7. The effect of arsenic exposure on cognitive function of adult APP/PS mice. APP/PS mice (6–7 month-old) were exposed to 100 μg/L of arsenic in drinking water for 3 weeks. Age and gender matched APP/PS littermates and WT mice were given normal drinking water and used as controls. A. Learning curves representing RWM performance of APP/PS mice after arsenic or control exposure. Two-way repeated measures ANOVA was used to analyze the data, with a significant influence of arsenic exposure $F(1168)= 8.62$; $p < 0.001$. n = 10 mice per group with equal sexes. Points represent mean ± SEM. B. The last two trial blocks of RWM demonstrate that APP/PS mice exposed to arsenic learned the task at a significantly slower rate as exemplified by their increased number of errors. One-way ANOVA followed by Tukey's multiple comparison test was utilized to analyze each trial. n = 10 mice per group with equal sexes. Bars represent mean ± SEM. * * = $p < 0.01$; NS = no significance.

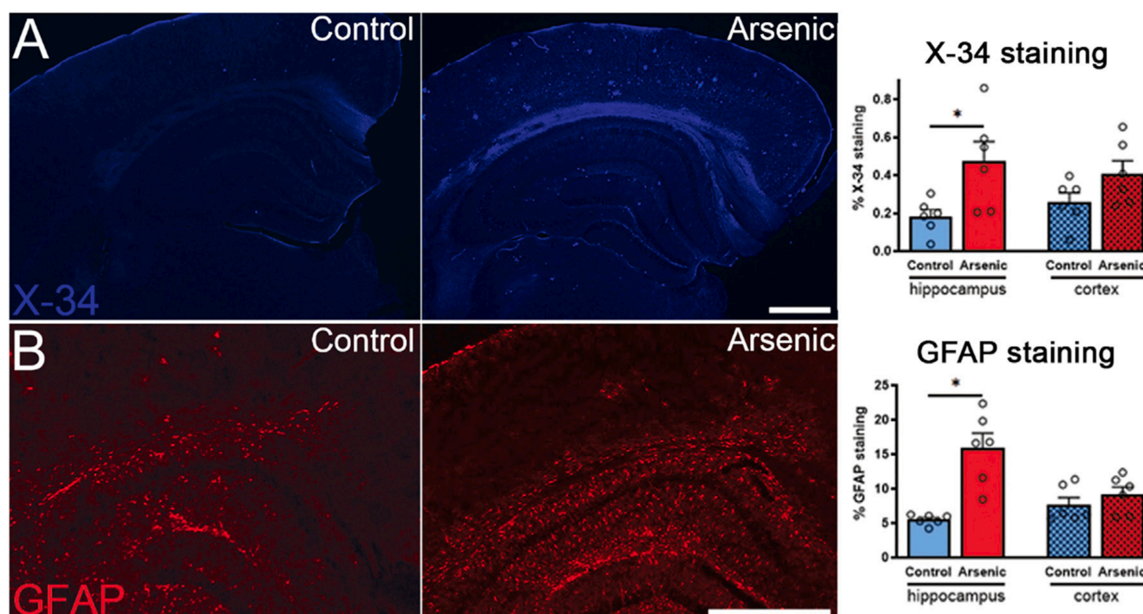

Fig. 8. The effect of arsenic exposure on the levels of amyloid plaques and GFAP positive astrocytes in adult APP/PS mice. A. 6–7-month-old APP/PS mice exposed to arsenic (100 μg/L in drinking water) had significantly more X-34 staining, which labels compact amyloid plaques, in hippocampus. There was a similar, but insignificant trend, for arsenic to increase the amyloid plaques in the cortex ($p = 0.1252$). Representative X-34 staining images are shown. Bar = 500 μm. B. These same APP/PS mice had significant more GFAP staining in the hippocampus and again a similar, but insignificant trend, for arsenic to increase GFAP staining in the cortex. Representative GFAP staining images from hippocampus are shown. Bar = 500 μm. Significance determined by unpaired t-test. n = 6 mice per group with equal sexes. Bars represent mean ± SEM. * = $p < 0.05$.

for proper neuronal function and has been shown to regulate neuronal survival and synaptic plasticity; both important to long-term memory formation [44]. It is known that a total loss of *CREB* and the corresponding pathways is lethal [45], furthermore partial disturbance is associated with several neurological disorders such as Parkinson's disease, Huntington's disease and AD [46–48]. Our data would suggest that a developmental exposure to arsenic can increase the expression of the *App* gene, while disrupting *Creb* gene expression could contribute to many of the neurological deficits associated with arsenic exposure.

Using immunoblotting technique, Tyler et al. previously demonstrated that a similar developmental arsenic exposure at 50 μg/L influenced H3K4me3 in a sex and region-specific manner in the adult mouse brain. Specifically, they showed increased H3K4me3 in both the dentate gyrus and cortex of male mice exposed to arsenic; with no change in cortex and decreased H3K4me3 in dentate gyrus of female mice exposed to arsenic compared to control [49]. This group using the same developmental arsenic exposure and ChIP followed by qPCR did not observe any differences in H3K4me3 in the frontal cortex following arsenic exposure for the promoter regions tested which were limited to Fkbp5, Hsd11b1, Crh and Crhr1. They did observe increased H3k4ac in female mice on the Crh promoter which was associated with stress signaling and increased H3k4ac in male mice on the DAE promoter which was associated with glucocorticoid signaling [22]. In our current study we utilized a slightly higher, yet still human relevant, arsenic exposure and a more advanced technique of ChIP followed by next generation sequencing analysis (ChIP-Seq). This more advanced technique allows for peak calling associated with histone modifications to better understand the epigenetic processes involved in the control of gene transcription which can modify biological processes. These studies show that human relevant low-level arsenic exposure during development causes long-lasting alterations in histone methylation in brain changing the epigenetic landscape which could impact brain development and healthy brain aging. While the analysis of the sequencing data as conducted does not allow segregation of males and females, the lack of differences in cognitive performance within the groups before and after exposure to arsenic presumably indicates no sex differences in histone modifications within the same groups.

H3K27me3 is a repressive mark and the analysis of ChIP-seq data confirmed our hypothesis that arsenic negatively affects neuronal development. The most highly significant biological processes found to be repressed in the arsenic group were nearly all categories related to neuron development. These results complement nicely the results of H3K4me3 analyses, and all together provide a potential molecular mechanism by which developmental arsenic exposure impacts memory functions assessed in the fear conditioning and novel object behavioral paradigms.

A leading hypothesis about AD pathogenesis states that amyloid-β overproduction and tau hyperphosphorylation results in plaque and tangle formation, respectively, in brain and this drives AD symptoms [50]. Studies demonstrate that arsenic exposure causes neuronal apoptosis and ferroptosis [51,52], as well as transcriptional upregulation of the amyloid precursor protein (APP) gene [23]. Exposure to arsenic in drinking water at environmentally relevant concentrations causes ultrastructural changes also in rat brains [13,53,54]. Therefore, arsenic exposure may participate in at least some of the pathophysiological steps of AD and development of AD symptoms.

The results of this study allow a connection between adult and developmental arsenic exposure, which builds on our previous finds that during embryonic life low-level arsenic exposure triggered global H3K9 hypo-acetylation and impaired cognitive function in adult mice [9]. ChIP-seq data demonstrate that genome-wide enrichment of H3K2me3 marks exacerbates neurological disease symptoms. In the current study we observed impaired cognitive function in both WT and APP/PS mice; and accelerated amyloid plaque pathology and astrogliosis in APP/PS mice. Thus, important biological processes affecting histone modifications were related to neuron fate and development. In an AD mouse model that has an existing neurological impairment and predefined deposition of amyloid plaques, an arsenic exposure seems to exacerbate the preexisting symptoms. The cumulative insult of arsenic on the brain could result in impaired APP processing and increased amyloid deposition explaining why AD occurs at an advanced age as well as the progressive nature of the disease. If a concrete link can be formed

between arsenic exposure and AD pathogenesis, then we could aim at decreasing the global AD burden by simply increasing the water purification and food consumption standards.

In conclusion, we provided evidence that a developmental arsenic exposure alters biologically significant histone modifications in brain which remain present into adulthood and provide a potential mechanism by which developmental arsenic exposure impacts cognitive functions. The repressive mark H3K27me3 showed higher levels of suppression in many biological processes related to neuron fate and development in the arsenic group and specifically in the transcription start site of several genes associated with neurotransmission such as *Psen1* and *Slc17A7*. We have also shown that human relevant, chronic arsenic exposure in drinking water has deleterious effects on adult APP/PS mice and exacerbates the existing AD like symptoms of the mice. The findings presented here provide the first steps towards determining the effect of a developmental arsenic exposure on the epigenetic landscape of the brain and how exposure early in life can alter gene expression later in life.

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CRediT authorship contribution statement

NFF, RK, IL: Conceptualization, Methodology, Data curation, Visualization, Writing – original draft preparation; **AB:** Writing – reviewing and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Graphical abstract created with BioRender.com.

Data sharing

Upon the acceptance of the manuscript for publication ChIP-seq data will be submitted and made available through NCBI GEO.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.03.008.

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