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Transgenerational Effects of Arsenic Exposure on Learning and Memory in Rats: Crosstalk between Arsenic Methylation, Hippocampal Metabolism, and Histone Modifications

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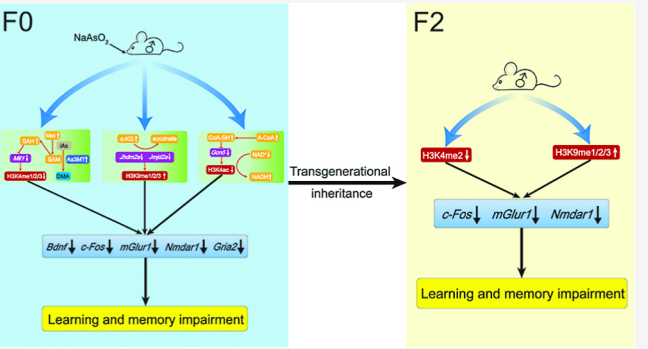
ABSTRACT: Arsenic (As) is widely present in the natural environment, and exposure to it can lead to learning and memory impairment. However, the underlying epigenetic mechanisms are still largely unclear. This study aimed to reveal the role of histone modifications in environmental levels of arsenic (sodium arsenite) exposure-induced learning and memory dysfunction in male rats, and the inter/transgenerational effects of paternal arsenic exposure were also investigated. It was found that arsenic exposure impaired the learning and memory ability of F0 rats and down-regulated the expression of cognition-related genes *Bdnf*, *c-Fos*, *mGlur1*, *Nmdar1*, and *Gria2* in the hippocampus. We also observed that inorganic arsenite was methylated to DMA and histone modification-related metabolites were altered, contributing to the dysregulation of H3K4me1/2/3, H3K9me1/2/3, and H3K4ac in rat hippocampus after exposure. Therefore, it is suggested that arsenic methylation and hippocampal metabolism changes attenuated H3K4me1/2/3 and H3K4ac while enhancing H3K9me1/2/3, which repressed the key gene expressions, leading to cognitive impairment in rats exposed to arsenic. In addition, paternal arsenic exposure induced transgenerational effects of learning and memory disorder in F2 male rats through the regulation of H3K4me2 and H3K9me1/2/3, which inhibited *c-Fos*, *mGlur1*, and *Nmdar1* expression. These results provide novel insights into the molecular mechanism of arsenic-induced neurotoxicity and highlight the risk of neurological deficits in offspring with paternal exposure to arsenic.



KEYWORDS: *arsenic, learning and memory, histone modifications, transgenerational inheritance, epigenetic mechanism*

INTRODUCTION

Arsenic (As) is a naturally occurring metalloid widespread in the environment.[1] It was estimated that 94−220 million people are potentially exposed to high concentrations of arsenic via consumption of groundwater globally,[2] which poses a significant threat to human health. As a highly toxic substance, arsenic exposure has been demonstrated to cause a variety of adverse health outcomes, including neurotoxicity.[3,4] Epidemiological studies have shown an association between chronic arsenic exposure and poor cognitive performance in both children and adults.[5] In addition, many rodent studies revealed that arsenic could induce neural dysfunction by altering epigenetics, hippocampal function, glucocorticoid and hypothalamus−pituitary−adrenal axis pathway, as well as glutamatergic, cholinergic, and monoaminergic signaling.[6] The hippocampus is a crucial brain structure in the process of learning and memory, and its lesions can destroy spatial learning and working memory.[7] Experimental studies have shown that exposure to arsenic induces hippocampus-dependent behavioral deficits characterized by impaired spatial, working, long-term, and short-term memory.[6] Moreover, it was found that arsenic exposure can induce significant learning and memory impairment in rodents by down-regulating the expression of cognition-related genes (*Nmdar*, *Ampars*, *CamkII*, *Bdnf*, and *Nr2b*) in the hippocampus.[8−10]

Histone post-translational modifications refer to a variety of chemical modifications, such as methylation, acetylation, phosphorylation, and ubiquitination that occur on the N-terminal amino acid residues of core histones.[11] Studies have shown that histone modifications play a key role in the transcription of genes that regulate synaptic plasticity and memory formation.[12] For instance, *MLL2* mutant mice showed specific loss of H3K4me2 and H3K4me3 in the hippocampus,

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which may be the cause of learning and memory impairment.[13] It has been confirmed that histone H3K4me2/3-specific demethylase KDM5C-knockout mice exhibit memory deficits.[14] H3K9 methyltransferase EHMT1-insufficient mice also exhibit intellectual disabilities and autistic phenotypes.[15] In addition, overexpressed histone deacetylase HDAC2 in neurons impaired dendritic synaptic plasticity and memory formation, while *Hdac2* deletion caused memory facilitation.[16] H3K9 acetylation of hippocampal *Cdk5* promoter attenuated fear memory retrieval,[17] and H3K23ac reduction repressed learning-related gene expression and resulted in courtship learning defect.[18] However, the role of histone modifications in arsenic-induced cognitive impairment is still rarely mentioned.[6]

It has been documented that the histone modification status can be disrupted by arsenic,[19,20] but the involved mechanisms remain largely unknown. Inorganic trivalent arsenic (iAs^{III}) can be metabolized and methylated to organic monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) by arsenite methyltransferase (AS3MT), in which methyl groups are transferred from *S*-adenosylmethionine (SAM) to arsenic.[21] In addition, histone modification is dynamically regulated by specific modifying enzymes whose activities require metabolites that either serve as substrates or act as activators/inhibitors. Like AS3MT, histone methyltransferases also use SAM as the methyl donor for the process of methylation, while histone demethylases (JMJC family) utilize α-ketoglutarate (α-KG) as a substrate to catalyze the demethylation reaction.[22] Therefore, through the competition of the methyl donor SAM, arsenic methylation may affect histone methylation. Histone acetylation is catalyzed by various histone acetyltransferases (HATs), which transfer the acetyl group from Acetyl-CoA to histone tails. On the contrary, sirtuins can deacetylate histones by using NAD^+ as a cosubstrate.[22] Overall, arsenic methylation and the altered concentrations of relevant metabolites could influence histone modification levels. In view of the relationship between arsenic methylation, body metabolism, and histone modifications, we hypothesized that arsenic methylation in the rat hippocampus and the hippocampal metabolism changed by arsenic may alter specific histone modifications to dysregulate the expression of learning and memory-related genes, thereby affecting cognitive ability.

As an important branch of epigenetic modifications, histone modification can cause stable and heritable changes in biological traits and then be transmitted to offspring without changing the DNA sequence.[23] Exposure to arsenic during pregnancy in mice can reduce H3K9ac levels in the hippocampus and cortex of offspring, which is associated with cognitive impairment in adulthood.[24] Another study found that arsenic exposure altered motor activity and increased anxiety-like behaviors in F0 and F2 generations of zebrafish by increasing H3K4me3 in the nervous system of both generations.[25] However, there are still few studies investigating the arsenic exposure-induced paternal transmission of learning and memory disorder across generations via the mechanism of histone modification.

Therefore, in this study, the male rat models were exposed to environmentally relevant doses of inorganic arsenic, and the changes in learning and memory ability, cognition-related gene expressions, histone modifications, arsenic methylation, and key metabolite levels in rat hippocampus were investigated to explore the role of histone modifications in arsenic-induced cognitive impairment. Furthermore, the intergenerational (F1) and transgenerational (F2) effects of paternal arsenic exposure on learning and memory via histone modification reprogramming were also studied. These results will aid us in better understanding the epigenetic mechanism of neurotoxicity and the risk in offspring induced by environmental arsenic exposure.

MATERIALS AND METHODS

Rat Arsenic Exposure and Breeding. Eighty male Sprague−Dawley (SD) rats (4 weeks old, F0) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All animals were maintained under a 12 h light−dark cycle and had free access to water and pellets. After one week of acclimation, the rats were exposed to 0.1, 1, and 10 mg/L of sodium arsenite ($NaAsO_2$) via drinking water for 60 days (n = 20/group), and the rats in the control group received deionized water.

After arsenic exposure, 10 rats of each group were used for the Morris water maze (MWM) test and then sacrificed to collect serum and hippocampus tissue for further assays, and the remaining 10 male rats in the control or 10 mg/L group were mated with nonexposed females (at a ratio of 1:2) to produce the F1 generation, respectively. After sexual maturity, 10 F1 male rats from each group were mated with nonexposed females to produce the F2 generation (Figure S1). At the age of 3 months, the male rats (10/group) of the F1 and F2 generation were subjected to MWM tests and then sacrificed to collect hippocampus tissue for further analyses. The animal experiment scheme was approved by the Laboratory Animal Ethics Review Committee of the Institute of Urban Environment, Chinese Academy of Sciences.

It has been reported that human populations could be exposed to arsenic in natural waters at a level ranging from <0.0005 to >5 mg/L globally.[20,26] In this study, the highest dose of $NaAsO_2$ for rat exposure (10 mg/L) was equivalent to 5.77 mg/L arsenic, which can be translated to 0.93 mg/L for humans (within the range of human exposure level), according to the dose conversion algorithm from rat to human.[27] Additionally, 10 mg/L $NaAsO_2$ in drinking water corresponds to 1.4 mg/kg body weight (bw), which is about 1/30 of LD_{50} (41 mg/kg) of $NaAsO_2$ for rats.[28] Therefore, the $NaAsO_2$ exposure doses selected for this study are considered to be environmentally relevant and toxicologically significant.

MWM Test. MWM is a classical test to detect learning and memory ability, which contains two stages: positioning navigation experiment and space exploration experiment. The positioning navigation experiment was designed to train the rats to learn and remember the position of the platform, and the space exploration experiment was to test the learning and memory ability of the rats.[29] In brief, the experimental apparatus consists of a water tank, and a black escape platform was placed at one of the four pool quadrants and submerged approximately 1 cm below the water surface. After the positioning navigation experiment was repeated for 6 days, the space exploration experiment was carried out on the seventh day when the platform was removed. The movement track of the rats within 1 min, the time of first swimming to the platform (the first incubation period), the time and distance of swimming in the target quadrant where the platform was located, and the number of times crossing the platform were recorded. The MWM test was performed on a Noldus EthoVision XT system (Ugo Basile, Italy).

Histopathological Analysis of Rat Brain Tissue. Rat brain tissue was fixed in paraformaldehyde at room temper-

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ature for 24 h. The fixed brain tissue was then dehydrated to make it transparent and permeated. Afterward, the brain was embedded with paraffin and sectioned by a microtome. The slices of tissue were stained using the hematoxylin and eosin (HE) and Nissl staining kit, respectively. The slides were scanned using a digital slide microscope scanning system (Olympus, Japan). The number of Nissl-positive neurons in the hippocampal CA1 region was analyzed using ImageJ software.

Total Arsenic Quantification in Rat Serum and Hippocampus. Total arsenic was quantified as reported previously.[29] Rat serum (500 μL) or hippocampal tissue (30 mg) was mixed with 2 mL of 65% nitric acid (Merck, Germany) and kept at room temperature overnight. Then, 6 mL of 30% hydrogen peroxide was added, and the mixture was digested using a Microwave Digestion System (Sineo, China). The digested samples were redissolved with 1 mL of 65% nitric acid, 1 mL of 30% hydrogen peroxide, and 3 mL of deionized water and subjected to an Agilent 7700 inductively coupled plasma mass spectrometer (ICP−MS/MS, Agilent, USA) for total arsenic analysis.

Measurement of Arsenic Species in Rat Hippocampus. Arsenic species were measured according to previous methods.[30] Rat hippocampus tissue (15 mg) was incubated with 1 mL of 1% nitric acid at 100 °C for 90 min. After centrifugation at 14,000 rpm for 5 min at 4 °C, the supernatant was collected and filtered. Arsenic species was isolated using a PRP-X100 (4.1 × 250 mm, 10 μm) anion exchange column (Hamilton, Switzerland). The contents of arsenic species were measured using an Agilent 1200 high-performance liquid chromatograph coupled with Agilent 7700 ICP−MS/MS (Agilent, USA). Mobile phase A was 25 mmol/L $(NH_4)H_2PO_4$ (pH 8.0), and mobile phase B was deionized water. The gradient elution procedure was as follows: 0 min, 0% A; 15 min, 100% A; 20 min, 0% A; 25 min, 0% A. Mixed calibration standard solutions of iAs^{III}, iAs^{V}, MMA, DMA, AsB, and AsC were prepared in serial concentrations. Chromatographic peak areas were used for quantification according to the generated external calibration curves. Chromatographic peaks for the sample extracts were identified by a comparison of retention time with the standards. The calibration standard and blank samples were analyzed every 5 samples in the analytical series.

Quantitative Real-Time PCR. Total RNA from rat hippocampus (20 mg) was extracted by Eastep Total RNA Extraction Kit (Promega, USA) and cDNA was prepared with 1 μg of RNA using a HiScript III RT SuperMix for qPCR kit (Vazyme, China). For real-time polymerase chain reaction (PCR), a 20 μL reaction mixture containing 10 μL of SYBR Green Master Mix reagents (Roche, Switzerland), 1 μL of primers, 4 μL of distilled water, and 5 μL of cDNA was prepared and performed on a Light Cycler 480II real-time PCR system (Roche, Switzerland). The reaction procedure was as follows: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 30 s. *β-Actin* was used as a reference gene to normalize the gene expression levels. The fold changes (treatment/control) of the genes were analyzed by the $2^{-\Delta\Delta CT}$ method. The primer sequences of tested genes are listed in Table S1.

Protein Extraction and Western Blotting. Total proteins of rat hippocampal tissue were extracted using RIPA Lysis Buffer (Thermo Fisher Scientific, USA), and histone extraction was performed according to previous methods.[31] Total proteins (50 μg) and histones (10 μg) were separated by SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk and incubated overnight with the primary antibodies of anti-β-actin, anti-ARC, anti-BDNF, anti-c-FOS, anti-MGLUR1, anti-NMDAR1, anti-GRIA2, anti-AS3MT (Cell Signaling Technology, USA), anti-H3, anti-H3K4me1, anti-H3K4me2, anti-H3K4me3, anti-H3K4ac, anti-H3K9me1, anti-H3K9me2, anti-H3K9me3, and anti-H3K9ac (Epigentek, USA) with a 1:2000 dilution at 4 °C. The membranes were then washed with Tris-buffered saline and Tween 20 (TBST) and incubated with horseradish peroxidase (HRP)-conjugated goat antimouse or antirabbit IgG secondary antibody for 1 h (1:10,000 dilution). The blots were generated using enhanced chemiluminescence (ECL) and visualized on an imaging system (Tanon, China). Protein band intensity was quantified using ImageJ software.

Chromatin Immunoprecipitation (ChIP)-PCR. ChIP assays were performed using a chromatin immunoprecipitation kit (ChIP-IT Express Enzymatic, Active Motif, USA). In brief, 150 mg of hippocampus tissue was cross-linked by 37% formaldehyde at room temperature for 15 min. The reaction was then quenched by glycine. The tissue was homogenized with lysis buffer to release the nucleus, and the homogenate was sonicated to shear the DNA into fragments of 200−1000 bp. The sheared chromatin (IP) was incubated with protein G-agarose beads for 1 h and with antibodies of anti-H3K4me2, anti-H3K9me3, and anti-H3K4ac overnight. The histone/DNA complexes were reverse cross-linked, and the DNA was then purified. For PCR, 2 μL of DNA was used for the following procedure: 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s and 65 °C for 1 min. The primer sequences for amplification of gene promoters are shown in Table S2. The IP/INPUT ratio of each target sequence was calculated using the formula: $\text{IP/INPUT} = 2^{Ct(\text{INPUT DNA})-Ct(\text{IP DNA})}$.

Determination of Histone Modification-Related Metabolites in Rat Hippocampus. The levels of histone modification-related metabolites *S*-adenosine methionine (SAM), *S*-adenosine homocysteine (SAH), Methionine (Met), α-Ketoglutaric acid (α-KG), Acetyl-CoA, CoA-SH, nicotinamide adenine dinucleotide (NAD^+), and reduced form of NAD^+ (NADH) in rat hippocampus were determined by ELISA kits according to the manufacturer's instructions (MEIMIAN, China). Briefly, 20 mg of hippocampal tissue was homogenized in PBS and centrifuged. The collected supernatants and standards were then added into the microplate, followed by incubation at 37 °C for 30 min. The liquids were discarded, and the wells were washed. Then, the HRP-labeled antibody was added, and the mixture was incubated at 37 °C for 30 min. After the sample was washed, the chromogenic solution was added for reaction at 37 °C in the dark for 15 min, and then the termination solution was added. Absorbance was measured at 450 nm using a microplate reader (Tecan, Switzerland). The detection limits of the metabolites are shown in Table S3.

Statistical Analysis. Data were expressed as the mean ± standard deviation (SD). SPSS software (version 22.0) was used for statistical analysis of the obtained data. The significance of difference among multiple groups was determined by one-way ANOVA followed by Tukey's test. $p < 0.05$ was considered to be statistically significant.

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RESULTS

Arsenic Exposure Impaired the Learning and Memory Ability of F0 Male Rats. After $NaAsO_2$ exposure for two months, the body weight (BW), hippocampus weight (HW), and hippocampus coefficient (HC, HW/BW) of F0 male rats were not significantly altered in comparison to the control ($p > 0.05$) (Table S4). The total arsenic levels in the serum and hippocampus were both increased in a dose-dependent manner after exposure. Moreover, Spearman correlation analysis found a highly positive correlation between serum and hippocampal arsenic levels ($r = 0.955$, $p < 0.01$) (Figure 1A), indicating that arsenic could cross the blood−brain barrier and accumulate in rat hippocampus. The MWM test showed that, with the elevation of the arsenic exposure dose, the distance and time of rats swimming in the target quadrant (where the platform was placed) were gradually reduced. At the same time, 10 mg/L $NaAsO_2$ exposure caused a significant decrease in the times of platform crossing for rats, while the first incubation period was increased (Figure 1B). These results suggest that arsenic exposure can cause learning and memory impairment in the rats. As shown in Figure 1C, HE staining of the hippocampal CA1 region showed that there were four or five layers of pyramidal cells with round cell structures and regular nuclei in the control group. After arsenic exposure, the pyramidal cells were deformed, and the number of dead cells, which were characterized by neuronal shrinkage and nuclear condensation, significantly increased. Moreover, Nissl staining results showed that the neurons appeared in a dense arrangement, and the Nissl bodies were abundant in the hippocampal CA1 region of the control group, whereas the hippocampal neurons loosened and the amount of Nissl bodies reduced after arsenic exposure (Figure 1D).

The effects of arsenic exposure on key learning and memory-related gene expression in the rat hippocampus were further determined. It was shown that arsenic caused significant down-regulation of *Bdnf*, *c-Fos*, *mGlur1*, *Nmdar1*, and *Gria2*. However, *Arc* was up-regulated while *Egr1*, *Slc17a7*, *Gria1*, and *Camk2a* were not significantly altered (Figure 2A). Furthermore, the protein levels of ARC, BDNF, c-FOS, MGLUR1, NMDAR1, and GRIA2 were further analyzed. Consistent with the PCR results, the expression levels of BDNF, c-FOS, MGLUR1, NMDAR1, and GRIA2 were all decreased by arsenic (Figure 2B), which may contribute to the observed cognitive dysfunction in rats. In summary, these data indicate that arsenic can accumulate in the rat hippocampus, which causes hippocampal neuronal injury, key gene inhibition, and consequent learning and memory impairment.

Arsenic Exposure Altered Histone Modifications in Hippocampus of F0 Male Rats. Considering the role of histone modifications in gene expression, we examined arsenic exposure-regulated histone methylation and acetylation in rat hippocampus. As can be seen in Figure 3A, arsenic significantly decreased H3K4me1/2/3 and H3K4ac levels, while increasing H3K9me1/2/3 and H3K9ac. Since histone modification is regulated by specific histone-modifying enzymes, we further determined the gene expression of the involved enzymes in the rat hippocampus. For H3K4 methylation, H3K4me1/2/3 methyltransferase *Mll1*, H3K4me1/2 demethylase *Kdm1a*, and H3K4me2/3 demethylase *Kdm5a* were all down-regulated by arsenic (Figure 3B); for H3K9 methylation, arsenic also reduced the expression of H3K9me1/2 methyltransferase *G9a*, H3K9me3 methyltransferases *Suv39h1* and *Suv39h2*, as well as

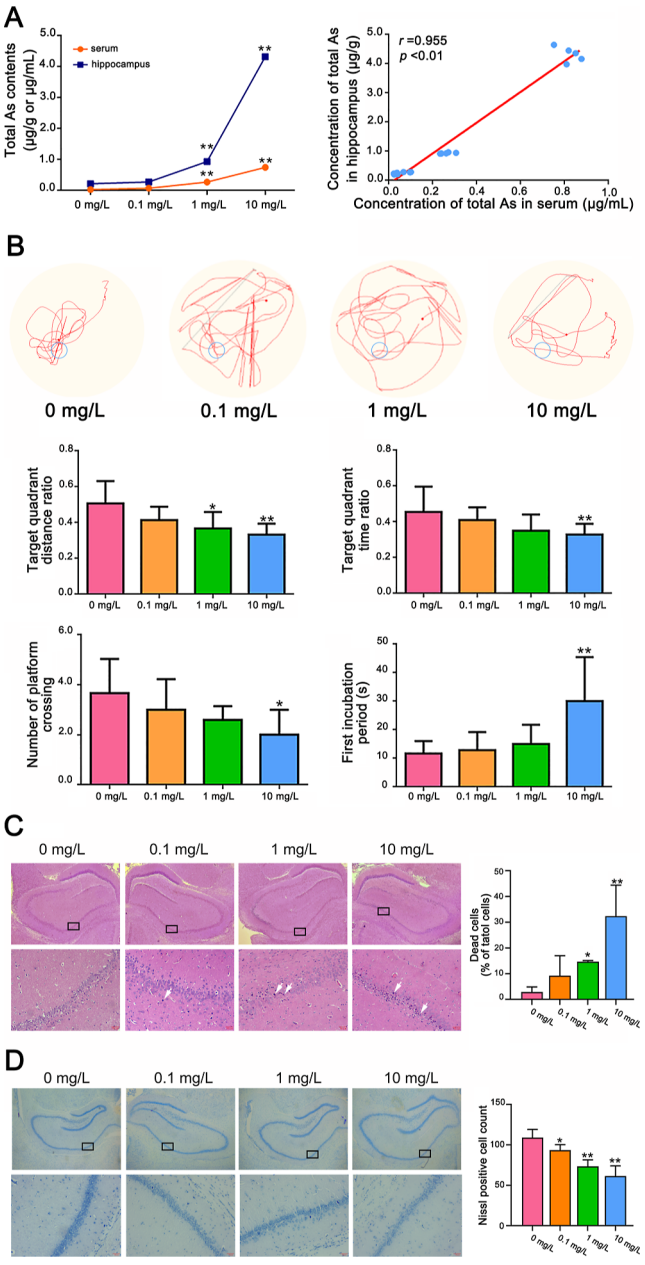

Figure 1. (A) Concentrations of total arsenic in serum and hippocampus of F0 male rats, and the Spearman correlation between serum and hippocampal arsenic levels. (B) Results of the MWM test in F0 male rats. Blue circle represents the location of the target platform in the trace diagram. (C) HE staining of the brain and the proportion of dead pyramidal cells in the hippocampal CA1 region of F0 male rats. Arrows indicate the dead cells. (D) Nissl staining of the brain and the number of normal neurons in the hippocampal CA1 region of F0 male rats. Data are expressed as mean ± SD ($n = 10$), *$p < 0.05$, **$p < 0.01$.

H3K9me1/2 demethylase *Jhdm2a* and H3K9me3 demethylase *Jmjd2a* (Figure 3C). For histone acetylation, the level of acetyltransferase *Gcn5* was significantly decreased, while the level of deacetylase *Hdac1* was unchanged after exposure (Figure 3D). Therefore, it is suggested that the decrease of H3K4me1/2/3 was mainly ascribed to the down-regulation of *Mll1*, the down-regulation of *Jhdm2a* led to the increase of H3K9me1/2, and the decreased *Jmjd2a* resulted in H3K9me3

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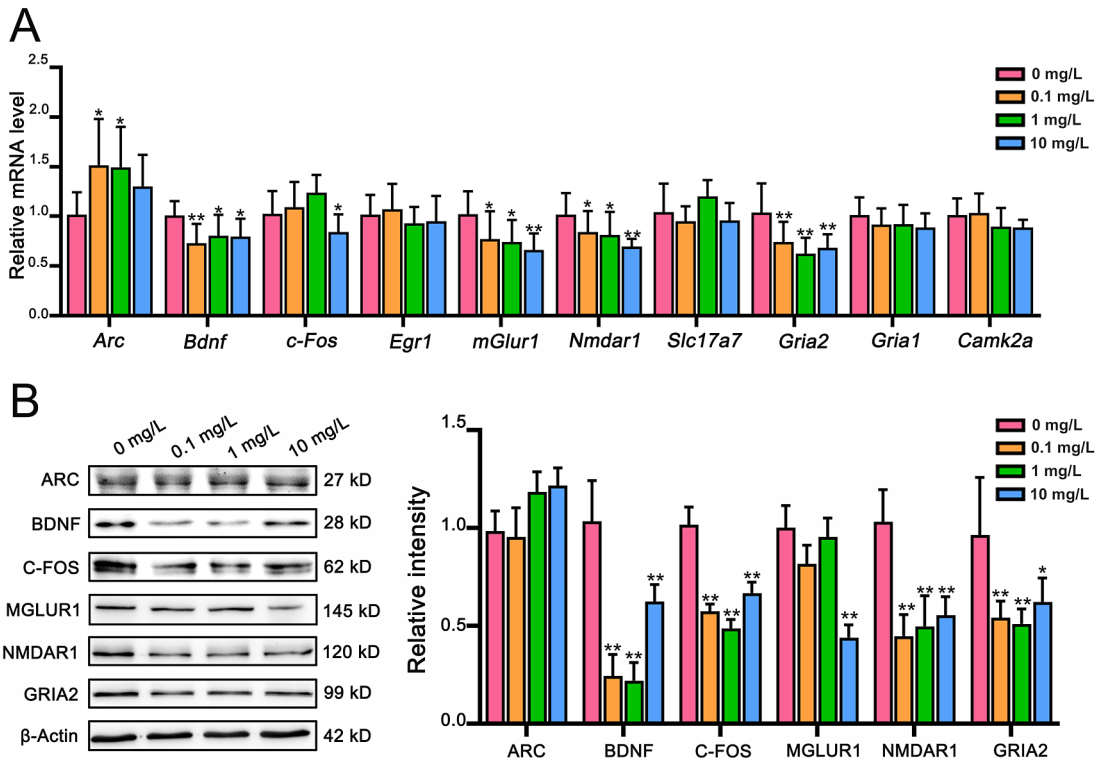

Figure 2. (A) Effects of arsenic exposure on mRNA levels of learning and memory-related genes in the hippocampus of F0 male rats. Data were expressed as mean ± SD (n = 10), *p < 0.05, **p < 0.01. (B) Western blot analysis of learning and memory-related protein expressions in the hippocampus of F0 male rats. The target protein levels were normalized to β-Actin abundance. Data were expressed as mean ± SD (n = 3), *p < 0.05, **p < 0.01.

enhancement. In addition, the depletion of *Gcn5* may be the main reason for the decreased level of H3K4ac.

Histone modifications within the gene promoter regions can directly regulate gene transcription. H3K4 methylation and histone acetylation are known to promote gene transcription, while H3K9 methylation inhibits gene expression. In view of the learning and memory-related gene repression induced by arsenic (Figure 2), the levels of H3K4me2, H3K9me3, and H3K4ac in the promoters of *Bdnf*, *c-Fos*, *mGlur1*, *Nmdar1*, and *Gria2* were representatively determined by ChIP-PCR. The results showed that H3K4me2 and H3K4ac in *Bdnf*, *c-Fos*, *mGlur1*, *Nmdar1*, and *Gria2* promoters were both significantly decreased while H3K9me3 was increased after arsenic exposure (Figure 3E), which further confirm that the reduced H3K4me2 and H3K4ac, as well as the elevated H3K9me3 are responsible for the suppression of the five genes. Taken together, the results indicate that arsenic-induced dysregulation of specific histone-modifying enzymes would contribute to the attenuated H3K4me1/2/3 and H3K4ac, as well as the enhanced H3K9me1/2/3, which synergistically inhibited the expression of *Bdnf*, *c-Fos*, *mGlur1*, *Nmdar1*, and *Gria2*, ultimately leading to learning and memory dysfunction in rats.

Arsenic Methylation and Hippocampal Metabolites Are Involved in Histone Modification Changes. Arsenic methylation in organisms may affect histone methylation by competing with the common methyl donor SAM.[21] Thus, we analyzed the arsenic species in the rat hippocampus. As a result, among the six species, only DMA and inorganic arsenate (iAs^V) were dominantly detected in the hippocampus tissue, while iAs^{III}, MMA, AsB, and AsC were not significantly detected (Figure 4A). Compared with the control, the proportion of DMA significantly increased from 85.62 to 95.51%, while the proportion of iAs^V decreased after exposure to 1 and 10 mg/L arsenite (Figure 4A). Furthermore, the expression level of AS3MT was up-regulated in rat hippocampus with arsenic exposure (Figure 4B), indicating that inorganic arsenic was mainly metabolized to organic DMA in rat hippocampus.

Histone modifications are dynamically regulated by metabolism,[22] and thus the contents of several key metabolites involved in histone methylation and acetylation were further determined in rat hippocampus. After arsenic exposure, the levels of histone methylation-related metabolites SAH and Met were increased (Figure 5B,D) while SAM was not significantly changed (Figure 5A), and SAM/SAH was decreased (Figure 5C); the demethylation-related metabolite α-KG was also increased by arsenic (Figure 5E). Moreover, the levels of histone acetylation-related metabolites A-CoA and CoA-SH were both increased in arsenic-treated rats (Figure 5F,G). For deacetylation-related metabolites, arsenic decreased NAD^+ but increased NADH, and NAD^+/NADH was decreased (Figure 5H–J). Overall, it is suggested that the arsenic-induced SAH increase resulted in H3K4me1/2/3 depletion, while the elevated CoA-SH and decreased NAD^+/NADH both reduced H3K4ac. However, the increased α-KG cannot well explain the enhancement of H3K9me1/2/3.

Transgenerational Effects of Arsenic Exposure on Learning and Memory via Histone Modification Regulation. In this study, the F1 generation was directly exposed to arsenic at the germ cell stage, while the F2 generation was never exposed to the chemical. Therefore, we further studied the intergenerational (F1) and transgenerational (F2) effects of arsenic exposure on learning and memory ability in male offspring. Like F0 rats, the BW, HW, and HC of

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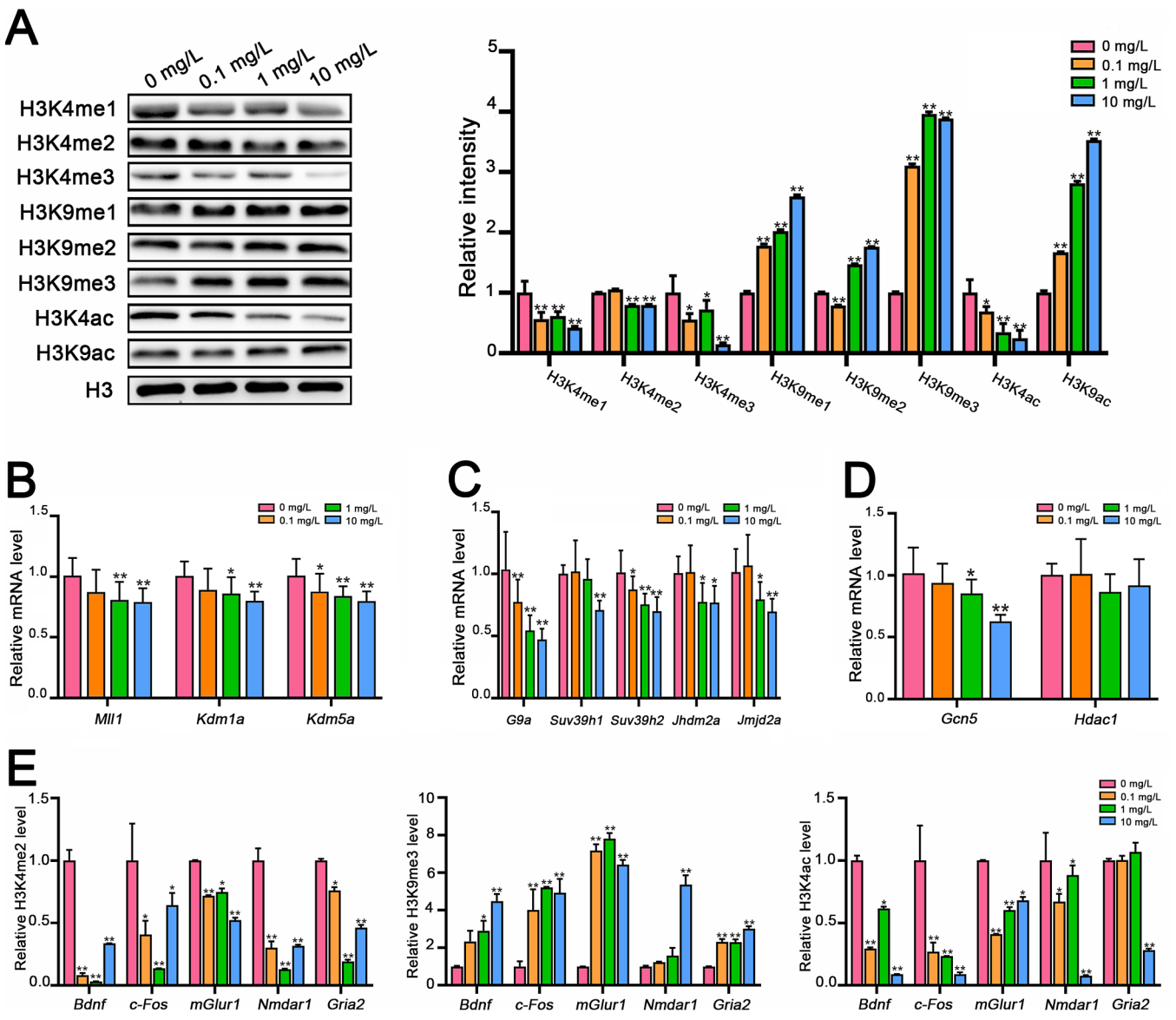

Figure 3. (A) Effects of arsenic exposure on histone modifications in F0 rat hippocampus. The relative levels of modified histones were normalized to total H3 abundance. Data were expressed as mean ± SD (n = 3), *p < 0.05, **p < 0.01. The gene expression changes of H3K4 methyltransferase and demethylases (B), H3K9 methyltransferases and demethylases (C), and HAT and deacetylase (D) in F0 rat hippocampus upon arsenic treatment. Data were expressed as mean ± SD (n = 10), *p < 0.05, **p < 0.01. (E) H3K4me2, H3K9me3, and H3K4ac levels within *Bdnf*, *c-Fos*, *mGlur1*, *Nmdar1*, and *Gria2* promoters in F0 rat hippocampus. Data were expressed as mean ± SD (n = 3), *p < 0.05, **p < 0.01.

F1 and F2 male rats were not significantly altered in comparison to the control (Tables S5 and S6). The results of the MWM test showed that for the F1 pups descended from arsenic-exposed rats, the swimming distance and swimming time in the target quadrant, the times of platform crossing, and the first incubation period were all not significantly changed (Figure S2). However, for F2 pups, the distance and time ratio in the target quadrant and the platform crossing times were significantly decreased while the first incubation period was increased (Figure 6A). These results suggest that arsenic exposure had transgenerational but not intergenerational effects on learning and memory impairment in male offspring. Thus, we further explored the mechanism of the transgenerational effects in F2 rats. Similar to F0 rats, hippocampal pyramidal cells also underwent deformation and death (Figure 6B), and the number of normal neurons decreased (Figure 6C) in F2 pups descending from arsenic-exposed ancestors compared with those descending from nonexposed ones. In addition, the expressions of c-FOS, MGLUR1, and NMDAR1 were significantly down-regulated, in line with the changes in F0 rats (Figure 7A). The reduced H3K4me2 and the elevated H3K9me1/2/3 and H3K9ac were also consistent with the alterations found in the F0 generation (Figure 7B). Furthermore, H3K4me2 in the promoter regions of *mGlur1* and *Nmdar1* were down-regulated while H3K9me1/2/3 were up-regulated in *c-Fos*, *mGlur1*, and *Nmdar1* promoters (Figure 7C), which contributed to the repression of *c-Fos*, *mGlur1*, and *Nmdar1*. However, the inhibition of *c-Fos* cannot be reasonably explained by the change of H3K4me2 (Figure S3). Collectively, by inhibiting *c-Fos*, *mGlur1*, and *Nmdar1* expression through the repression of H3K4me2 and stimulation of H3K9me1/2/3 in the hippocampus, paternal arsenic exposure induced transgenerational effects of learning and memory defect in F2 male rats.

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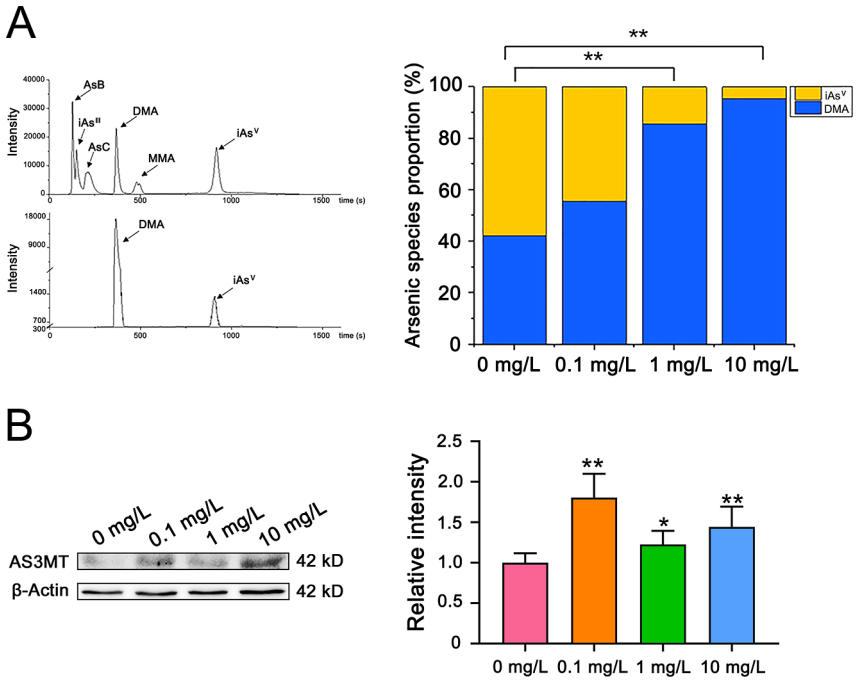

Figure 4. (A) Determination of arsenic species in the hippocampus of F0 male rats. Left panel, representative chromatograms of standards (upper) and samples (lower); right panel, the proportion changes of iAs^V and DMA after arsenite exposure. Data were expressed as mean ± SD (n = 10), $^{**}p < 0.01$. iAs^{III}, inorganic arsenite; iAs^V, inorganic arsenate; MMA, monomethylarsine acid; DMA, dimethylarsine acid; AsB, arsenobetaine; AsC, arsenocholine. (B) Effects of arsenic exposure on AS3MT expression in rat hippocampus. Data were expressed as mean ± SD (n = 3), $^{*}p < 0.05$, $^{**}p < 0.01$.

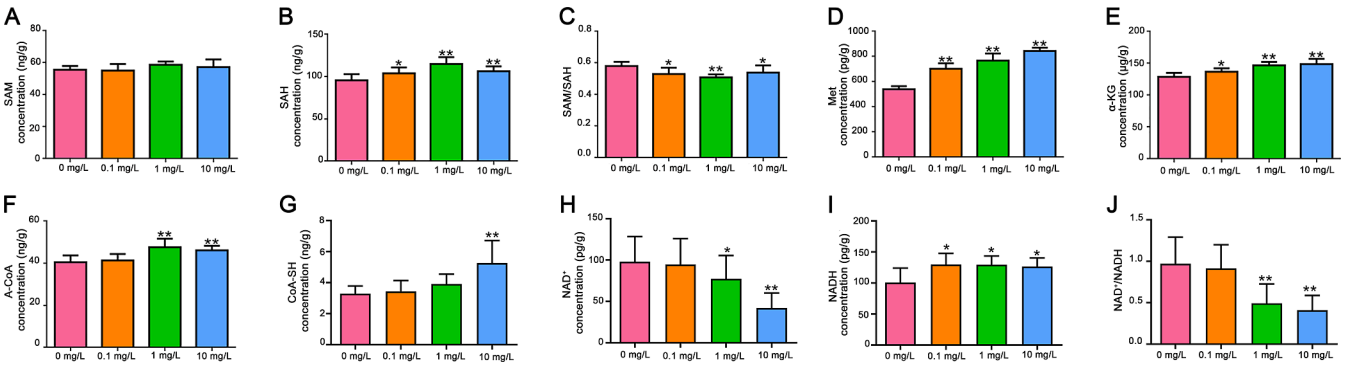

Figure 5. Effects of arsenic exposure on the concentrations of histone modification-related metabolites in F0 rat hippocampus. (A) *S*-adenosine methionine (SAM); (B) *S*-adenosine homocysteine (SAH); (C) SAM/SAH; (D) methionine (Met); (E) α-ketoglutaric acid (α-KG); (F) acetyl coenzyme A (A-CoA); (G) coenzyme A (CoA-SH); (H) nicotinamide adenine dinucleotide (NAD^+); (I) reduced form of NAD^+ (NADH); (J) NAD^+/NADH. Data are expressed as mean ± SD (n = 10), $^{*}p < 0.05$, $^{**}p < 0.01$.

DISCUSSION

Accumulating evidence has suggested that arsenic exposure impairs learning and memory ability, while the involved epigenetic mechanisms (especially histone modification) have rarely been explored. Moreover, whether arsenic-induced cognitive damage could be transmitted across generations via histone modification reprogramming remains to be elucidated. Our results showed that through self-methylation and altering relevant metabolites in the hippocampus, arsenic regulated H3K4me1/2/3, H3K9me1/2/3, and H3K4ac, which thus inhibited *Bdnf, c-Fos, mGlur1, Nmdar1*, and *Gria2* expression, leading to learning and memory impairment in rats. Furthermore, paternal arsenic exposure induced transgenerational effects of cognitive disorder in rat offspring by regulating specific histone modifications.

A variety of genes are known to be involved in memory acquisition, storage, and consolidation.[32] Brain-derived neurotrophic factor (BDNF), FBJ osteosarcoma oncogene (c-FOS), metabotropic glutamate receptor 1 (MGLUR1), *N*-methyl-D-aspartate receptor subunit 1 (NMDAR1), and glutamate receptor 2 (GRIA2) are closely related to synaptic plasticity and neuronal excitability, which play important roles in learning and memory function. It has been demonstrated that arsenic exposure inhibits the expression of these genes/proteins in rodent brain tissues.[33−36] Consistently, the present study suggested that together with the altered hippocampal structure, the down-regulation of BDNF, c-FOS, MGLUR1, NMDAR1, and GRIA2 contributed to the cognitive dysfunction in rats exposed to arsenic.

Histone modification has been reported to play important roles in modulating learning and memory processes by

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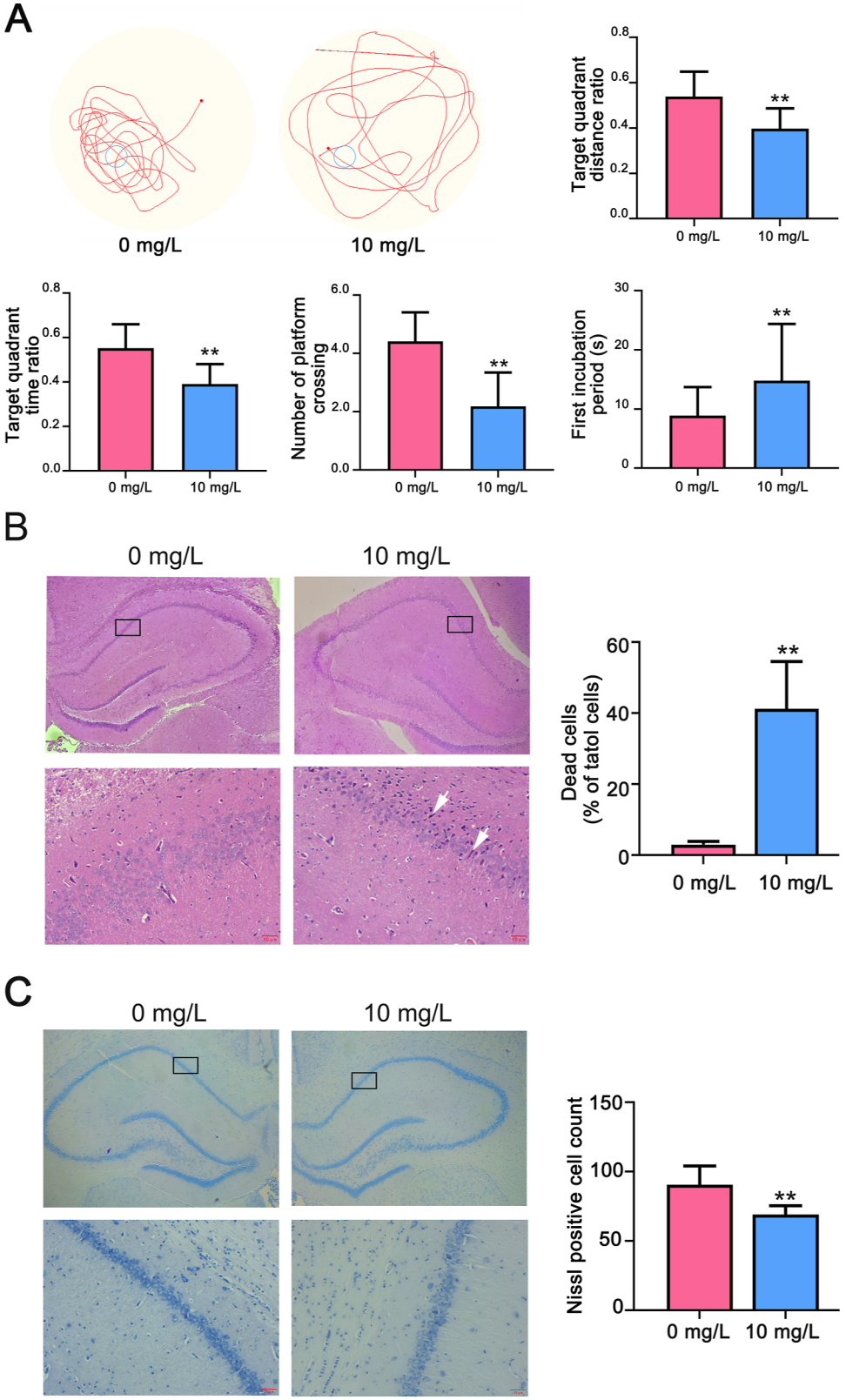

Figure 6. (A) Results of the MWM test in F2 male rats. Blue circle represents the location of the target platform in the trace diagram. (B) HE staining of the brain and the proportion of dead pyramidal cells in the hippocampal CA1 region of F2 male rats. Arrows indicate the dead cells. (C) Nissl staining of the brain and the number of normal neurons in the hippocampal CA1 region of F2 male rats. Data are expressed as mean ± SD (n = 10), **p < 0.01.

regulating relevant gene expression.[37] Additionally, arsenic exposure can disturb the states of cellular histone modifications.[19] However, there are still few studies of arsenic-induced cognitive impairment due to histone modification changes hitherto.[38] It is known that histone H3K4 methylation and acetylation are transcriptional activators, while H3K9 methyl-

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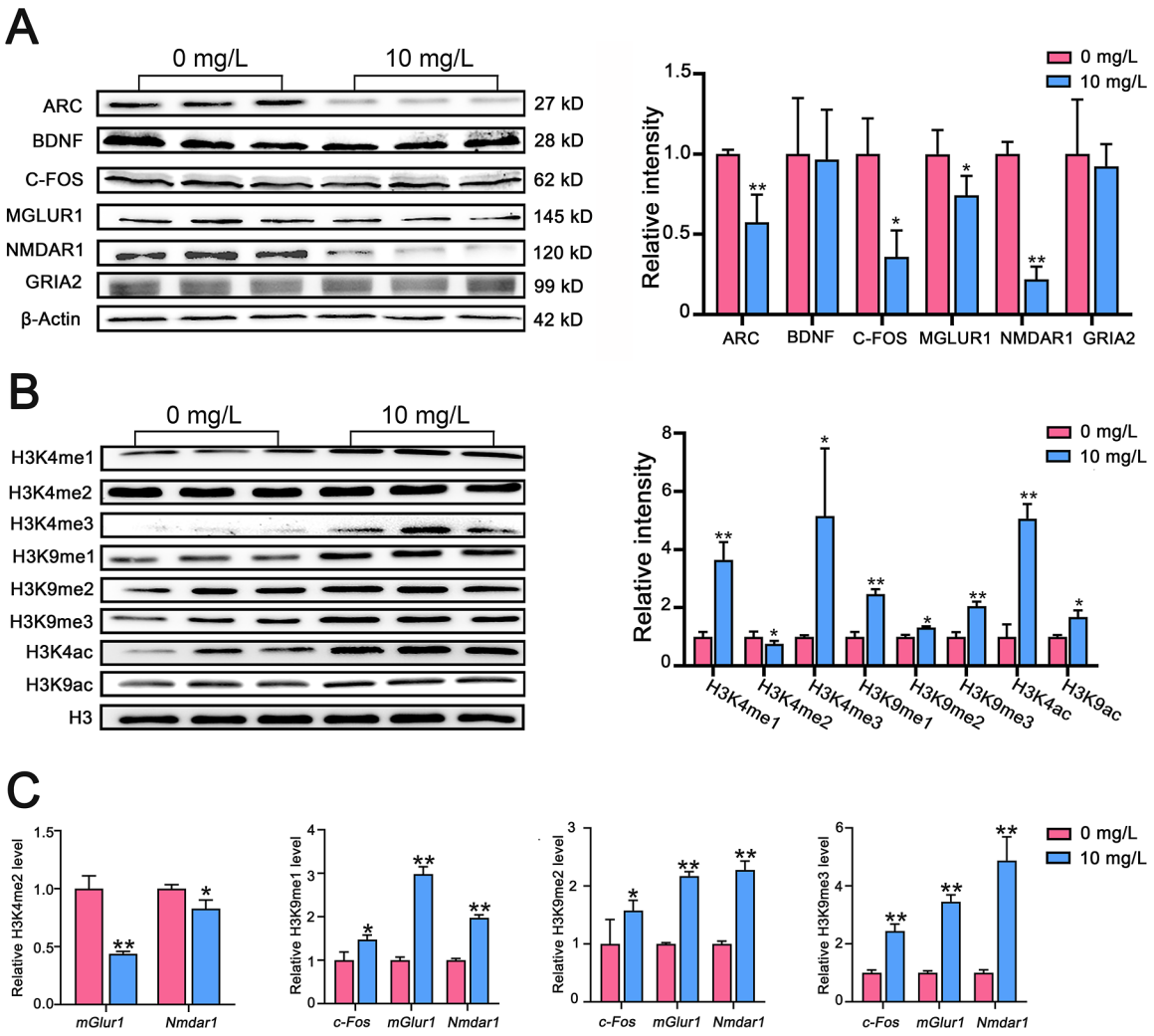

Figure 7. Western blot analysis of learning and memory-related proteins (A) and histone modifications (B) in the hippocampus of F2 male rats. (C) H3K4me2 levels within *mGlur1* and *Nmdar1*, and H3K9me1/2/3 levels within *c-Fos*, *mGlur1*, and *Nmdar1* promoters in F2 rat hippocampus. Data were expressed as mean ± SD (n = 3), *p < 0.05, **p < 0.01.

ation is a repressor. In our study, arsenic exposure caused the down-regulation of H3K4me1/2/3 and H3K4ac, as well as the up-regulation of H3K9me1/2/3 in rat hippocampus, which would repress the expression of learning and memory-related genes (*Bdnf*, *c-Fos*, *mGlur1*, *Nmdar1*, and *Gria2*). To support our findings, it was previously reported that the enhanced H3K4me2 in the *Bdnf* promoter of the lateral-medial hypothalamic forebrain bundle leads to *Bdnf* overexpression.[39] Ionescu-Tucker et al. also found that the reduced H3K9me3 in the brain is one of the reasons for BDNF activation.[40] Therefore, we propose that arsenic exposure impaired learning and memory ability by inhibiting key gene expressions, which were attributable to the repressed H3K4 methylation and acetylation and the enhanced H3K9 methylation in rat hippocampus.

Histone modifications are known to be regulated by body metabolism, as many metabolites are substrates or activators/inhibitors of histone-modifying enzymes.[22] SAM is a major methyl donor for arsenic methylation and other methylation processes, including histone methylation. However, whether arsenic would alter histone modifications by self-methylation and affecting hippocampal metabolism is still unclear. In this work, we found that iAs^{III} was majorly methylated to DMA while SAM level was not changed in rat hippocampus after arsenic exposure. Consistently, it was previously pointed out that DMA was increased,[41] but SAM remained unchanged in brain tissues after exposure to inorganic arsenic.[29,42] It is suggested that the consumption of SAM by arsenic and histone methylation may be compensated for by the enhanced synthesis from Met (a precursor of SAM). Nonetheless, the increased conversion of SAM to SAH may in turn inhibit histone methyltransferase *Mll1*,[43] which reduces the H3K4 methylation level. The JmjC domain-containing histone demethylase relies on iron and α-KG to catalyze the lysine demethylation of histones.[44] Here, we propose that although the increase of α-KG induced by arsenic could promote the demethylation process, the down-regulated demethylases (*Jhdm2a* and *Jmjd2a*) mainly resulted in the increase of H3K9me1/2/3. In addition, SAM is also the methyl donor for DNA methylation, and α-KG is a substrate of DNA demethylase TETs.[45] It was found that arsenic exposure impairs cognition by reducing DNA methylation.[29,46] In the present study, although the SAM was not changed, the increased hippocampal α-KG could accelerate DNA demethylation. Thus, we propose that the altered histone methylation (H3K4/9me1/2/3) and DNA hypomethylation induced by

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arsenic may jointly lead to cognitive disorder in rats. However, the role of crosstalk between DNA methylation and histone modifications in arsenic-induced learning and memory impairment still needs further study.

Lipid-derived A-CoA is a major source of carbon for histone acetylation,[47] and its metabolite CoA-SH inhibits HAT.[45] Amal et al. found that arsenic exposure caused a decrease of A-CoA level in mouse neuronal cells.[48] However, in our study, the levels of both A-CoA and CoA-SH in the rat hippocampus were increased by arsenic. It is suggested that the up-regulation of A-CoA caused the accumulation of CoA-SH, which reduced H3K4ac by inhibiting the HAT *Gcn5*. The NAD^+-dependent sirtuin family of deacetylases is a subgroup of HDACs that mediate the histone deacetylation process.[45] Therefore, the decrease in NAD^+ and increase in NADH also indicated a promoted H3K4 deacetylation in rat hippocampus after arsenic exposure. Collectively, these results indicate that arsenic methylation and altered hippocampal metabolites synergistically regulated histone modification levels, thereby causing cognitive impairment in rats exposed to inorganic arsenic.

The toxic effects of arsenic can be transmitted across generations through paternal, maternal, or both parents via epigenetic mechanisms.[49] It has been reported that arsenic can cause transgenerational neurotoxicity in the F2 generation of zebrafish by increasing H3K4me3.[25] In the present study, we found that the male rats of the F2 (not F1) generation showed learning and memory impairment, indicating that paternal arsenic exposure induced transgenerational but not intergenerational effects of cognitive damage. Maternal inheritance may involve cytoplasmic factors, whereas paternal inheritance is mainly attributed to alterations in sperm epigenetic inheritance.[50] In line with the findings in F0 generation, histone H3K4me2 and H3K9me1/2/3 showed similar changes in F2 offspring derived from arsenic-exposed F0 male rats, which resulted in the down-regulation of c-FOS, NMDAR1, and MGLUR1. Taken together, we propose that paternal exposure to arsenic caused transgenerational alteration in the learning and memory ability of male offspring through histone modification regulations in rats.

Despite the important results found in our study, there are still some limitations. Firstly, besides Western blotting, the expression changes of learning and memory-related proteins deserve immunohistochemical analysis to further confirm the hippocampal alterations induced by arsenic. Secondly, since the intergenerational and transgenerational effects of paternal arsenic exposure were only studied in male offspring, the sex differences are unknown. Finally, the mechanisms by which arsenic-induced epigenetic alterations of germ cells result in somatic (hippocampus) changes need further studies.

In conclusion, this study found that arsenic repressed H3K4me1/2/3 and H3K4ac while it activated H3K9me1/2/3 through its methylation and altering hippocampal metabolism, which down-regulated the expression of cognition-related genes, leading to learning and memory defects in rats. Furthermore, this adverse effect of arsenic exposure on neural function was epigenetically transmitted to F2 male rats by altering specific histone modifications. Our work reveals the role of arsenic methylation-hippocampal metabolism-histone modification coregulation in arsenite-induced neurotoxicity and has important implications for the neurological health risk of environmental arsenic exposure in offspring.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c07989.

Primer sequences for real-time PCR and ChIP-PCR; detection limits of the metabolites measured by ELISA; rat BW, HW, and hippocampal coefficient; breeding scheme for rat offspring; MWM test for F1 male rats; and ChIP results (PDF)

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Notes

The authors declare no competing financial interest.

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