Basic Study

**AlCl₃ exposure regulates neuronal development by modulating DNA modification**

Xue-Jun Cheng, Fu-Lai Guan, Qian Li, Gong Dai, Hai-Feng Li, Xue-Kun Li

**ORCID number:** Xue-Jun Cheng 0000-0003-3573-9510; Fu-Lai Guan 0000-0002-3702-2022; Qian Li 0000-0003-3996-0888; Gong Dai 0000-0003-3691-9521; Hai-Feng Li 0000-0002-1843-0565; Xue-Kun Li 0000-0002-6985-6363.

**Author contributions:** Li XK designed the study; Cheng XJ performed the isolation and culture of adult neural stem cells, proliferation and differentiation assays, qRT-PCR, Western blot, and DNA dot-blot; Guan FL and Dai G performed the quantification analysis of immunofluorescence staining; Li Q performed neuronal culture and Sholl analysis; Li XK and Li HF wrote the manuscript; all authors reviewed and approved the final manuscript. Xue-Jun Cheng and Fu-Lai Guan contributed equally to this study.

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**Abstract**

**BACKGROUND**

As the third most abundant element, aluminum is widespread in the environment. Previous studies have shown that aluminum has a neurotoxic effect and its exposure can impair neuronal development and cognitive function.

**AIM**

To study the effects of aluminum on epigenetic modification in neural stem cells and neurons.

**METHODS**

Neural stem cells were isolated from the forebrain of adult mice. Neurons were isolated from the hippocampi tissues of embryonic day 16-18 mice. AlCl₃ at 100 and 200 μmol/L was applied to stem cells and neurons.

**RESULTS**

Aluminum altered the differentiation of adult neural stem cells and caused apoptosis of newborn neurons while having no significant effects on the proliferation of neural stem cells. Aluminum application also significantly inhibited the dendritic development of hippocampal neurons. Mechanistically, aluminum exposure significantly affected the levels of DNA 5-hydroxymethylcytosine.
INTRODUCTION

Epigenetic modifications mainly include histone posttranslational modifications, DNA and RNA methylation and demethylation, and non-coding RNAs. Previous studies have indicated that epigenetic pathways play a critical function in diverse biological processes. DNA methylation, mainly on the fifth carbon of cytosine [5-methylcytosine (5mC)] in mammalian, is established by DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, and DNMT3B. In embryonic and postnatal neuronal development, the deficiency of DNMTs affects embryonic viability, cell survival, synaptic development, and learning and memory; however, neuronal activity could influence DNA methylation, suggesting that DNA methylation is important for normal neuronal function.

Recent studies have shown that 5mC can be further converted to 5-hydroxymethylcytosine (5hmC) by ten-eleven translocation (TET) proteins including TET1, TET2, and TET3. 5hmC is significantly enriched in the brain relative to many other tissues and cell types, is acquired during postnatal neurodevelopment and aging, and displays spatial and temporal dynamics. Recent studies have shown consistently that Tet1 regulates neuronal activity, the formation and extinction of memory, and neurogenesis. Recent studies have shown that 5mC can be further converted to 5-hydroxymethylcytosine (5hmC) by ten-eleven translocation (TET) proteins including TET1, TET2, and TET3. 5hmC is significantly enriched in the brain relative to many other tissues and cell types, is acquired during postnatal neurodevelopment and aging, and displays spatial and temporal dynamics. Recent studies have shown consistently that Tet1 regulates neuronal activity, the formation and extinction of memory, and neurogenesis. Recently, DNA N6-methyladenine (6mA) modification has been uncovered, which regulates gene expression and is involved in neuronal outcomes induced by environmental stress.

Aluminum is a neurotoxin and is associated with neuronal inflammation, memory impairment, and neurological disorders through different mechanisms. Aluminum exposure (50-100 mg/kg in vivo) significantly exacerbates amyloid beta (Aβ) deposition, plaque formation, and tau phosphorylation; causes cognitive dysfunction and mitochondria oxidative; and therefore induces Alzheimer’s disease-like phenotypes in rats. Aluminum exposure (25 mg/kg in vivo or 0.5 mmol/L in vitro) also stimulates the expression of pro-inflammatory cytokines including TNF-α and IL-6, induces the production of reactive oxygen species (ROS), and then causes neuroinflammation and DNA damage. However, it remains largely unknown whether aluminum has a neurotoxic effect by altering epigenetic states.

In the present study, we found that aluminum (AlCl₃) skewed the differentiation of adult neural stem cells (aNSCs) toward glial cells and induced apoptosis of newborn neurons. Furthermore, aluminum inhibited the morphological development of
neurons generated upon aNSC differentiation and hippocampal neurons. Finally, we found that AlCl₃ exposure differentially altered the level of DNA methylation and hydroxymethylation in aNSCs and neurons by regulating the expression of DNA methyltransferases and dioxygenases. In summary, our results suggest that AlCl₃ exerts a neurotoxic effect by modulating DNA modifications.

**MATERIALS AND METHODS**

**Animals**

Mice were housed in a standard condition of the Animal Center of Zhejiang University on a 12 h light/dark cycle with free access to food and water. All animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Zhejiang University.

**Isolation and culture of adult neural stem cells**

The isolation and culture of adult neural stem cells were performed according to an established protocol. The aNSCs were cultured in DMEM/F-12 medium containing 20 ng/mL FGF-2 (Catalog No. 100-18B-B, PeproTech), 20 ng/mL EGF (Catalog No. 100-15, PeproTech), 2% B27 supplement (Catalog No. 12587-010, Thermo Fisher Scientific), 1% antibiotic-antimycotic (Catalog No. 15140-122, Thermo Fisher Scientific), and 2 mmol/L L-glutamine (Catalog No. 25030-149, Thermo Fisher Scientific) in a humidified incubator supplied with 5% CO₂ at 37 °C.

**AlCl₃ exposure**

AlCl₃ was dissolved with nuclease free water to 50 mmol/L and applied to cells at a final concentration of 100 μmol/L or 200 μmol/L. The cells were collected at scheduled time-point for in vitro assay.

**Proliferation and differentiation assays in vitro**

For in vitro proliferation assay, aNSCs were cultured on coverslips with medium supplied with 5 mmol/L BrdU for 8 h. For in vitro differentiation assay, aNSCs were cultured on coverslips with proliferation medium, and then transferred into differentiation medium containing 1 mmol/L retinoic acid (Catalog No. R-2625, Sigma) and 5 mmol/L forskolin (Catalog No. F-6886, Sigma) for 48 h.

**Isolation and culture of embryonic hippocampal neurons**

Primary neurons were isolated from the hippocampus of E16-E18 mice and seeded in cell climbing slices (Corning, 354087) or plates that were coated with poly-D-lysine (5 μg/mL, Sigma, P0899-10). Approximately 1 × 10⁶ cells per well were seeded for a slice, while 1.5 million cells per well were seeded for a 6-well-plate. After growing in the plating medium for 4 h, which consisted of MEM (Gibco, 11095-080), 10% FBS (Gibco, 10091-148), 1% L-Glu (Gibco, 5030-149), 1% sodium pyruvate (Gibco, 11360-070), and 0.45% D-glucose (Amresco, 0188), the medium was changed to a maintaining medium that consisted of neurobasal (Gibco, 21103-049), 0.25% L-Glu (Gibco, 25030-149), 0.125% GlutaMax (Thermo, 35050061), and 2% B27 (Gibco, 17504-044). The medium was renewed half of the liquid volume every 3 d.

**Immunofluorescence staining and quantification**

To detect the function of proliferation and differentiation, cell samples were washed with PBS for 30 min followed by blocking with PBS containing 3% normal goat serum and 0.1% triton X-100 for 1 h at room temperature. Samples were incubated with primary antibodies overnight at 4 °C. For BrdU immunostaining, samples were treated with 1M HCl at 37 °C for 30 min before blocking. The following primary antibodies were used: GFAP (Catalog No. Z0334, DAKO), Tuj1 (Catalog No. G712A, Promega), Caspase 3 (Catalog No. A83623, Millipore), and BrdU (Catalog No. ab6326, Abcam). The second day, after being washed with PBS for 30 min, sections were incubated with fluorophore-conjugated secondary antibodies for 1 h at room temperature. After final washes, samples were mounted on glass slides and cover slipped with mounting medium. Images were captured using a Nikon invert microscope, and the numbers of BrdU⁺, Tuj1⁺, GFAP⁺, and Caspase3⁻Tuj1⁺ cells were quantified with image J software (NIH).
**Total RNA isolation, reverse transcription, and quantitative real-time PCR**

Total RNA was extracted with TRIzol reagent (Catalog No. 15596018, Thermo Fisher Scientific) following the manufacturer’s protocol. The concentration was determined using a NanoDrop 2000 spectrophotometer, and 500 ng of total RNA was subjected to reverse transcription. All real-time PCR reactions were performed in triplicate using power SYBR Green PCR master Mix (Catalog No. Q71502, Vazyme), and the results were analyzed using the \( \Delta\Delta^Ct \) method. The sequence of all the used primers can be found in Supplementary Table 1.

**Western blot analysis**

Cells were washed with PBS and resuspended in RIPA (Catalog No. ab156034, Abcam) containing 1x protease inhibitor cocktail (Catalog No. 04693124001, Sigma). The samples were centrifuged at 4 °C for 20 min at 14000 rpm, and the supernatants were collected for further experiments. Protein concentrations of the samples were measured with a BioPhotometer, and 20 μg of each sample was used for electrophoresis after denaturation for 5 min at 95 °C. Samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The following primary antibodies were used: Anti-Tuj1 (Catalog No. G712A, Promega), anti-GFAP (Catalog No. 3670, Cell Signaling), and anti-GAPDH (Catalog No. AM4300, Thermo Fisher Scientific). Secondary HRP conjugated antibodies were applied for 1 h at room temperature. The signal was detected with the Tanon 5200 Detection system, and the relative level of signal intensity was normalized to that of GAPDH.

**Genomic DNA extraction and DNA dot-blot**

DNA extraction and DNA dot-blot were performed as described previously[9]. The following primary antibodies were used: 5mC (Catalog No. 61255, Active Motif), 5hmC (Catalog No. 39769, Active Motif), and 6mA (Catalog No. 61496, Active Motif).

**Statistical analysis**

All data are expressed as the mean ± SE. GraphPad Prism (GraphPad Software Inc.) was used for statistical analyses. Unpaired student’s t-test was used to determine the differences between two groups with at least three replicates. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**AlCl₃ affects the differentiation of aNSCs and survival of newborn neurons**

To determine the effects of AlCl₃ on the proliferation of aNSCs, aNSCs were exposed to AlCl₃ for 48 h, and BrdU was administered at 5 μmol/L for 8 h followed by immunofluorescence staining (Supplementary Figure 1A). The quantification results showed that the number of BrdU positive (BrdU⁺) cells did not show a significant difference between control and AlCl₃ exposure aNSCs (Supplementary Figure 1B), suggesting that AlCl₃ does not affect the proliferation of aNSCs.

To examine the effects of AlCl₃ on the differentiation of aNSCs, aNSCs were exposed to AlCl₃ for 2 d and then underwent differentiation induction. Immunostaining for neuronal cell marker Tuj1 and astrocyte marker GFAP was performed (Figure 1A). The quantification results of immunofluorescence staining showed that the number of neuronal marker Tuj1 positive cells was significantly decreased (Figure 1B), but the number of glial cell marker GFAP positive cells increased after AlCl₃ application (200 μmol/L) (Figure 1C). We also detected the expression levels of Tuj1 and GFAP by qRT-PCR and Western blot, and we found that the level of Tuj1 decreased, while the level of GFAP increased (Figure 1D-F). Taken together, these results suggest that AlCl₃ regulates the differentiation of aNSCs.

To determine whether AlCl₃ affects the survival of newborn neurons, we performed immunofluorescence staining for Tuj1 and caspase 3. Representative images (Figure 1G) and quantification results (Figure 1H) show that AlCl₃ at a dosage of 200 μmol/L significantly increased the number of Caspase3 positive cells, suggesting that AlCl₃ exposure induces apoptosis of newborn neurons.

**AlCl₃ inhibits the maturation of newborn neurons derived from aNSCs and hippocampal neurons**

Next, we aimed to investigate whether AlCl₃ regulates neuronal development. We first analyzed the effects of AlCl₃ on the development of newborn neurons generated upon

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**Supplementary Table 1**

[Details of Table 1 can be found in the original text or added as necessary.]
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Figure 1 AlCl3 exposure affects adult neural stem cell differentiation and neuron survival in vitro. A: Immunostaining of adult neural stem cell differentiated in the presence of AlCl3 for β-III tubulin (Tuj1) and glial fibrillary acidic protein (GFAP) expression (Scale bar: 100 μm); B and C: The number of Tuj1+ cells decreased while that of GFAP+ cells increased (n = 8); D and E: The relative mRNA level of Tuj1 significantly decreased, and that of GFAP increased (n = 3); F: Western blot assay showing that Tuj1 protein expression was significantly decreased by AlCl3 at 200 μmol/L, while GFAP protein expression was increased. GAPDH was used as a loading control; G: Representative images of apoptosis in newborn neurons (scale bar: 100 μm); H: The number of newborn neurons decreased after AlCl3 exposure (n = 8). Data are represented as the mean ± SE (n = 3). Statistically significant differences are indicated: *P < 0.05, **P < 0.01, ***P < 0.001. Tuj1: β-III tubulin; GFAP: Glial fibrillary acidic protein.
the differentiation of aNSCs. Immunostaining (Figure 2A) and Sholl analysis showed that AlCl₃ at a dosage of 200 μmol/L significantly decreased dendritic length and the number of intersections (Figure 2B-D).

Next, we isolated neurons from the hippocampal tissues of embryonic mice and examined the effects of AlCl₃ on the development of primary neurons. Immunofluorescence (Figure 2E) and Sholl analysis showed that AlCl₃ exposure at both 100 μmol/L and 200 μmol/L dosages significantly decreased the intersection number and dendritic length of hippocampal neurons (Figure 2F-H). Collectively, these results indicate that AlCl₃ exposure inhibits neuronal development.

**AlCl₃ regulates DNA methylation and demethylation of aNSCs and neurons**

Previous studies have shown the important function of DNA modifications in neurogenesis and neuronal development[22,23]. To dissect the molecular mechanisms by which AlCl₃ regulates neuronal development, we first analyzed the effects of AlCl₃ on the DNA modifications of aNSCs and neurons. DNA dot-blot and quantification results showed that AlCl₃ exposure increased the global level of 5-hmC in proliferating and differentiated aNSCs, but AlCl₃ exposure decreased the global levels of 5-mC and 6mA (Figure 3A-I).

Next, we analyzed the effects of AlCl₃ on the DNA modifications in neurons. DNA dot-blot and quantification results showed that AlCl₃ exposure increased the global levels of 5-mC and 6mA but decreased the global level of 5-hmC in hippocampal neurons (Figure 3G-L). Taken together, these results indicate that AlCl₃ alters the epigenetic state in aNSCs and neurons.

**AlCl₃ differentially regulates the expression of DNA modification related genes in aNSCs and neurons**

Next, we examined the expression of genes related to DNA modifications. We found that AlCl₃ exposure significantly increased the mRNA level of Tet2 but did not affect the mRNA levels of Tet1 and Tet3 in aNSCs (Figure 4A-C). Meanwhile, AlCl₃ exposure significantly decreased the mRNA level of DNA methyltransferases Dnmt1 (Figure 4D) but did not affect the levels of Dnmt3a and Dnmt3b (Figure 4E and F).

We then aimed to determine the effects of AlCl₃ on the expression of Tet5 and DNMTs in neurons. We found that AlCl₃ exposure decreased the mRNA levels of Tet1 and Tet3 while not affecting the level of Tet2 (Figure 4G-I) in neurons. Furthermore, AlCl₃ exposure led to a decrease in the mRNA levels of Dnmt1 and Dnmt3a but induced an increase in Dnmt3b (Figure 4J-L). Taken together, these results suggest that AlCl₃ differentially regulates the expression of genes relating to DNA methylation and demethylation in aNSCs and neurons.

**DISCUSSION**

Previous studies have shown that DNA modifications play an important role in neuronal development and function and that dysregulation of DNA modifications is involved in neurological disorders[26-29]. Machineries of regulating DNA modifications have been identified. In the present study, we found that aluminum inhibits the differentiation of aNSCs and the development of neurons. Furthermore, aluminum can induce apoptosis of newborn neurons derived upon the differentiation of aNSCs. Mechanistically, aluminum affects the global level of 5mC, 5hmC, and 6mA in aNSCs and neurons by regulating the expression of DNA modification associated genes including Tet5s and DNMTs. Taken together, our results reveal a novel mechanism for regulating adult neurogenesis.

In adult mammalian brain, two regions, the subventricular zone in the lateral ventricle and subgranular zone in the dentate gyrus of hippocampus, maintain the neurogenic capacity[27]. Adult neurogenesis is driven by aNSCs and regulated by multiple mechanisms including environmental stimuli, genetics, and epigenetics including DNA modifications[26,27,29]. Our results show that aluminum can affect the differentiation of aNSCs and induce apoptosis of newborn neurons. Therefore, our study reveals the roles of aluminum in regulating neuronal development and associated mechanisms.

DNA modifications are regulated by diverse factors, such as environmental stimuli and food nutrients. Nutrient Vitamin C can serve as a cofactor for Tet and improves the reprogramming and neuronal differentiation by enhancing the expression level of Tet5 and therefore increasing the global level of 5hmC[29,30]. As one type of environmental pollution, the excessive intake of aluminum could induce inflammatory
Figure 2 AlCl₃ exposure inhibits neuronal development. A: Representative images of adult neural stem cell differentiation (scale bar: 100 μm); B-D: Sholl analysis in newborn neuron indicated that the intersection number and length of dendrites both decreased after AlCl₃ exposure (n = 21); E: Representative images of hippocampal neurons (scale bar: 100 μm); F-H: Sholl analysis indicated that the intersection number and length of dendrites both significantly decreased in hippocampal neurons after AlCl₃ exposure (n = 21). Data are represented as the mean ± SE (n = 3). Statistically significant differences are indicated: aP < 0.05, bP < 0.01, cP < 0.001. TuJ1: β-III tubulin; GFAP: Glial fibrillary acidic protein.
responses and oxidative stress, and then cause toxic effects on the neural, immune, and reproductive systems. Aluminum exposure also increases apoptosis and impairs learning and memory in adult rats$^{[31]}$. These findings indicate the crosstalk between environmental signal and epigenetic modifications.

**CONCLUSION**

In summary, our findings show the neurotoxic effect of aluminum on neuronal development. One limitation of the present study is that the data were collected in vitro. A further study should be performed to examine the effects of aluminum on neuronal development and DNA modifications in vivo.
Figure 3 AlCl₃ exposure alters the levels of DNA 5-hydroxymethylcytosine, 5-methylcytosine, and N⁶-methyladenine in adult neural stem cells and neurons. A-C: Representative images of 5-hydroxymethylcytosine (5hmC), 5-methylcytosine (5mC), and N⁶-methyladenine (6mA) dot-blot assays in adult neural stem cell (aNSC) differentiation and proliferation; D-F: Quantification revealing that the relative levels of 5-mC and 6mA both decreased in aNSC proliferation and differentiation, but the level of 5hmC increased (n = 3); G-I: Representative images of 5hmC, 5mC, and 6mA dot-blot assays in neurons; J-L: Quantification revealing that the relative levels of 5hmC decreased but those of 5mC and 6mA increased in neurons (n = 3). Data are represented as the mean ± SE (n = 3). Statistically significant differences are indicated: *P < 0.05, **P < 0.01, ***P < 0.001. 5hmC: 5-hydroxymethylcytosine; 5mC: 5-methylcytosine; 6mA: N⁶-methyladenine.
Figure 4 AlCl₃ exposure regulates the expression of Tets and Dnmts at the transcriptional level in adult neural stem cells and neurons. A-F: The relative mRNA levels of Tet1, Tet2, Tet3, Dnmt1, Dnmt3a, and Dnmt3b in aNSCs; G-L: The relative mRNA levels of Tet1, Tet2, Tet3, Dnmt1, Dnmt3a, and Dnmt3b in neurons. Data are represented as the mean ± SE (n = 3). Statistically significant differences are indicated: aP < 0.05, bP < 0.01, cP < 0.001.

ARTICLE HIGHLIGHTS

Research background
With the industrial development of society, environmental pollution is becoming a serious challenge for humans. Previous studies have revealed the crosstalk between environment and epigenetics and consequent phenotypes.
Research motivation
Aluminum pollution is a common issue and its exposure induces neurotoxic effects and impairs neuronal development and cognitive function.

Research objectives
To study the effects of aluminum on epigenetics in the context of neuronal development.

Research methods
Neural stem cells were isolated from the brain of adult mice. Hippocampal neurons were isolated from the brain of embryonic mouse pups. The levels of DNA modifications were detected by dot-blot. The levels of DNA modification related genes were examined by qRT-PCR.

Research results
Our present findings uncovered the roles of aluminum in inhibiting neuronal development and promoting cell death. Our results also showed that aluminum exposure can display significant effects on DNA modifications.

Research conclusions
Our study indicated that aluminum exposure regulates neuronal development by modulating DNA modifications.

Research perspectives
Future studies should be performed to examine whether DNA modification could be a target for the treatment of neurological disorders induced by aberrant neuronal development.

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