

Betaine alleviates fluoride-induced learning and memory impairment in offspring rats through DNA methylation of GNAS

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ARTICLE INFO

Keywords:

Fluorosis

Betaine

Learning and memory

DNA methylation

GNAS

ABSTRACT

The aim of this study was to explore the DNA methylation mechanisms by which maternal betaine (BET) supplementation during pregnancy alleviates fluoride-induced learning and memory impairments in offspring rats. Female and male rats were mated 2:1 in cage. After pregnancy, they were divided into control group, sodium fluoride (NaF) group, low BET group, and high BET group. The neurobehavioral test showed that slower swimming speed, longer escape latency and fewer platform shuttles were observed in NaF groups, but these were reversed by BET intervention. Furthermore, Hematoxylin and Eosin (H&E) staining revealed disrupted structures of hippocampal neurons in the NaF group, a reduction in intracellular Nissl bodies was evidenced by Nissl staining as well. However, BET treatment could improve the above changes. Targeted Bisulfite Sequencing (TBS) analysis showed that DNA methylation levels in the promoter regions of Guanine nucleotide-binding protein Gs subunit alpha (GNAS) was lower in the NaF group compared to controls, and increased after BET intervention. Furthermore, the NaF group showed significantly increased protein and mRNA expression levels of both GNAS, while it was reduced after low-dose BET intervention. Additionally, the protein and mRNA expression of DNA methyltransferases (DNMTs) were significantly reduced in NaF group, whereas a higher expression levels of these protein and mRNA were observed in the low-dose BET group. Maternal BET supplementation attenuates fluoride-induced learning and memory impairments in rats, potentially by elevating DNA methylation levels in the promoter regions of the GNAS genes.

1. Introduction

Fluoride has been shown to effectively prevent dental caries when ingested appropriately (Walsh et al., 2019). However, the human tolerance of fluoride is low and excessive intake leads to dental fluorosis and skeletal fluorosis (James et al., 2021). China is one of the countries with the most extensive, most complex and most serious endemic fluorosis in the world. After years of efforts, the prevention and treatment of endemic fluorosis in China have achieved effective results (Shi et al., 2025; Wang et al., 2025). In addition to the damage to the fluoride-exposed individual, fluoride can also harm the nervous system of offspring through the placental barrier and the blood-brain barrier

(Basha et al., 2011). Therefore, how to prevent the neurological damage of the offspring patients caused by fluoride-induced is an important issue.

As reported by Bashash, each 0.5 mg/L increment in maternal urinary fluoride was associated with a 2.5-point reduction in offspring IQ scores (Bashash et al., 2017). Animal experiments have also shown that maternal exposure to high and low fluoride during pregnancy and lactation leads to neurological dysfunction in offspring, mainly manifested as cognitive dysfunction such as learning and memory, but the mechanism is not clear (Bartos et al., 2018). Meta-analysis showed that DNA methylation was associated with learning and memory related to the development of the nervous system and the pathogenesis of complex

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mental disorders (Ehrlich, 2019). The hippocampus is a key brain region involved in learning and memory (Kutlu and Gould, 2016), and changes in hippocampal neuronal structure and function are closely related to learning and memory function (Chen et al., 2016). Animal experiments showed that the level of DNA methylation in the hippocampus of epileptic rats increased, and the mRNA expression of genes related to learning and memory decreased (Parrish et al., 2015). In addition, due to the offspring effect of epigenetic modification, it has been reported that perinatal environmental pollutant exposure caused significant changes in the whole genome DNA methylation level in the hippocampus of offspring rats compared with the control group, and the learning and memory of offspring rats were impaired (Hong et al., 2021). However, whether fluoride also affects the neurological function of offspring through DNA methylation has rarely been reported. It has been reported that abnormal DNA methylation of *GNAS* and *ArfGAP* with GTPase-like domain, ankyrin repeat and PH domain 2 (*AGAP2*) is closely related to memory, neurodevelopment and neurodegenerative changes (Wang et al., 2017; Liu et al., 2022, 2019; Quan et al., 2020). *GNAS* encodes multiple transcripts and is an important component of many signaling pathways (Pignolo et al., 2011). At the same time, it is also an important imprinted gene (Wang et al., 2017). *AGAP2* encodes a superfamily member of GTPases, whose disturbed expression can lead to neurodevelopmental abnormalities (Yu et al., 2019). However, whether fluoride also affects the neurological function of offspring through *GNAS* and *AGAP2* DNA methylation has not been reported.

DNA methylation is regulated not only by methyltransferases and demethylases, but also by methyl donors (Łoboś and Regulski-Iłow, 2021). It has been reported that BET can also be used as methyl donor, osmotic agent and antioxidant (Zawieja and Chmurzynska, 2025). Previous studies have shown that BET is used as a methyl donor to form methionine, converted into S-adenosylmethionine to provide methyl for DNA methylation (Idriss et al., 2018). Preliminary studies have showed BET supplementation alleviated toxicant-induced cognitive deficits in rats, potentially through modulating hippocampal synaptic plasticity and neurogenesis (Knight et al., 2017). (Chen et al., 2021), found that BET regulate the expression of N-methyl-D-aspartate receptor and Postsynaptic density protein 95 in hippocampus, preventing and reversing the behavioral defects and synaptic dysfunction caused by repeated ketamine exposure in mice. However, it remains to be determined whether BET alleviate fluoride-induced learning and memory impairment in offspring by regulating DNA methylation pattern.

To provide further insights into the mechanisms through which dietary BET improves neurological damage of the offspring caused by fluoride-induced genotoxicity. The impact of BET on fluoride-induced learning and memory ability was evaluated by using rat model. This study will provide a new idea for the prevention of offspring developmental impairment of fluorosis, and provide a scientific basis for nutritional prevention of fluorosis leading to decreased intelligence of offspring.

2. Materials and methods

2.1. Reagents

NaF and 4% paraformaldehyde were purchased from Shanghai San gong Biological Engineering Co., Ltd. (Shanghai, China). BET was purchased from American Sigma (USA). Anti-*GNAS* (ab283266), Anti-DNMT1 (ab188453) and Anti-DNMT3A (ab188470) were purchased from American abcam. Anti-*AGAP2* (14690-1-AP) and Anti-DNMT3B (48739) were purchased from Wuhan SAB (China). The PCR primers was purchased from Shanghai San gong Biological Engineering Co., Ltd. (Shanghai, China).

2.2. Animal experiments

A total of 32 female and 16 male Sprague Dawley (SD) rats (age of 4 weeks; weight of 75 ± 2 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (License No.: SCXK (Liao) 2020-0001), and all experiments were approved by the Animal Care Review Committee of Zunyi Medical University (Approval number: Ethics Review [2020] No. 2-314). All experiments were performed in compliance with the requirements of the National Act on the Use of Experimental Animals (PR China). After a week of acclimation, 12 rats were randomly selected as the control group (male and female 2:1) to be given double distilled water. The remaining 36 rats were in the fluoride group to be given NaF ($29.5 \text{ mg} \cdot \text{kg} \cdot \text{d}^{-1}$). The dosage of fluoride was set at 1/5 of the LD50 of fluoride. After 90 days of gavage, a chronic fluorosis model was established according to dental fluorosis (Ding et al., 2024). The 12-hour urine of female rats was collected at night, then the Morris water maze test was performed, then, the rats were mated at 19:00 every day (male and female 2:1) and the vaginal secretions of the female rats were taken at 8:00 the next day. The vaginal smears were found to have vaginal plugs or sperm observed under a microscope, which was recorded as the 0.5 day of pregnancy. Pregnant rats in the control group continued to receive double-distilled water, and the twenty-four NaF-treated pregnant rats were numbered 1-24 and randomly assigned into three equal groups using the Excel RAND Function. According to the sorted random values, the rats were divided into three groups with 8 rats in each group. As follows: (1) the NaF group (NaF, $29.5 \text{ mg} \cdot \text{kg} \cdot \text{d}^{-1}$), (2) the low BET group (NaF + 100 BET, $100 \text{ mg} \cdot \text{kg} \cdot \text{d}^{-1}$), and (3) the high BET group (NaF + 400 BET, $400 \text{ mg} \cdot \text{kg} \cdot \text{d}^{-1}$). BET gavage dose reference literature (Hui et al., 2024; Lin et al., 2024; Sarlak et al., 2022). The three fluoride-treated groups (NaF, NaF + 100 BET, NaF + 400 BET) continued to be exposed to fluoride during pregnancy and lactation until weaning on the 21st day of birth. Then, thirty offspring rats (female : male = 1:1) were randomly selected from four groups (control, NaF, NaF + 100 BET, and NaF + 400 BET) and housed in separate cages. The BET intervention started from the first day of pregnancy until the birth of the offspring rats. During the feeding period, all rats were free to drink and feed, and the general conditions of eating and drinking water were observed every day until the end of 60 days after birth.

2.3. Detection of progeny dental fluorosis

After three months of fluoride dyeing, the occurrence of dental fluorosis in the parental generation was examined. According to the Diagnostic Standard for Dental Fluorosis (WS-T208-2011) in China, dental fluorosis is classified into four grades: Normal: enamel surface was smooth, shiny, without mottling or speckling, and close to light yellow; mild: small opaque white streaks appeared on the surface of enamel; moderate: enamel lost its luster and had brown staining, and wider white streaks could be observed; severe: enamel surface was lusterless, with chalky changes, and appeared to be visibly worn, shortened, or even missing.

2.4. Determination of fluoride content in parental rats and offspring rats

Before mating, female parent rats were housed in metabolic cages for 12-hour overnight urine collection. For offspring rats, 12-hour overnight urine samples were collected at postnatal day 50 prior to the Morris water maze test. All urine specimens were transferred into 10 mL centrifuge tubes and centrifuged at 2000 rpm for 10 min for subsequent urinary fluoride detection. The content of fluorine in urine was detected by ion selective electrode method according to WS / T30-1996S standard. 1.5-2 mL of blood was collected and placed in a polyethylene tube without a fluoride stopper. The brain tissue was weighed 0.2 g and placed in a 2 mL EP tube with two 3 mm steel balls and 1 mL ultrapure water grounding at 60 Hz for 180 s. Fluoride ion selective electrode method was used to determine the contents of fluoride in the blood

fluoride and brain.

2.5. Neurobehavioral test of offspring rats

Six offspring rats were selected from each litter (male and female 1 : 1) with 30 offspring rats in each group. The offspring rats of postnatal five day were used as the research object to observe the age of neurobehavioral reflex positive reaction.

2.5.1. Plane righting reflection

The offspring rats were placed on a rough board (25 cm × 50 cm) during the test. After 5 s of hand support, the offspring rats were loosened. If the body was straightened within 2 s and the limbs were returned to the board contact, repeated 3 times, as the plane righting reflex was positive. The age of positive reaction in each litter of offspring rats was recorded until all the observed offspring rats were positive, and the age of positive reaction in each group was calculated.

2.5.2. Cliff avoidance reflection

During the test, the offspring rats were placed on the edge of a 30 cm high experimental platform to make their noses and fore toes cross the edge. At the same time, one hand was placed under the experimental platform to prevent the offspring rats from falling. Within 60 s, the offspring rats returned from the edge of the experimental platform or turned over to the body as positive. The age of positive reaction of each litter of offspring rats was recorded until all the observed offspring rats were positive, and the age of positive reaction was calculated.

2.5.3. Negative chemotaxis reflection

During the test, the pups were placed on a rough slope of 25°, with their heads facing down, and they could turn around 180° within 60 s, which was regarded as positive reaction. The age of positive reaction of offspring rats in each litter was recorded until all the offspring rats were positive, and the age of positive reaction was calculated.

2.6. Morris water maze experiment

The Morris water maze was used to evaluate the spatial learning and memory ability of rats at 3 months of fluorine dyeing and at postnatal day 60 (Each offspring group comprised 9 female and 9 male rats, and each parental group included 10 rats). In brief, the diameter of the circular heating pool is 160 cm, the height is 50 cm, and the diameter of the movable platform is 12 cm. All external visual clues surrounded by the pool were kept constant for the spatial orientation of the rats. The pool is manually divided into the first, second, third and fourth quadrants. The fourth quadrant was set as the target quadrant, and the movable platform was placed in the center of the quadrant, 30 cm from the pool wall and 1.5 cm below the water surface. The whole experiment process should be kept quiet, the water temperature should be controlled at $22 \pm 3^\circ\text{C}$, and black ink should be injected into the pool to distinguish from the rats. Behavioral data from the Morris water maze were analyzed using linear mixed-effects models, with litter ID included as a random intercept to control for litter-derived variation.

2.6.1. Localization navigation experiment

The rats were moved into the Morris water maze laboratory one day in advance to adapt to the environment, and the acquired training time was 4 days to evaluate the learning and memory ability of the rats. The rats were put into the pool in a quadrant order every day. The time of each water entry of the parental rats was set to 120 s, and the time of each water entry of the offspring rats was set to 60 s. Each animal was softly put into water at one of the four starting quadrants facing the wall of the pool. The swimming time of each rat from the start location to reach the submerged platform (escape latency) was recorded. If the rats missed the hidden platform within the specified time, it was guided to stay on the platform for 15 s and the escape latency was recorded as

120 s for the parental rats and 60 s for the offspring rats. The swimming distance represented each rat's full movement path, while average swimming speed was computed as individual total distance divided by the rat's actual swimming time. The escape latency, swimming distance and swimming speed of rats in each quadrant were recorded.

2.6.2. Space exploration experiment

After the localization navigation experiment, the platform was removed, and the rats were placed in the pool from a fixed quadrant entry point. The number of times the rats crossed the platform within the specified time, the number of times they crossed the target quadrant, the percentage of the time spent in the target quadrant to the total swimming time, and the percentage of the swimming distance in the target quadrant to the total swimming distance were recorded.

2.7. HE staining

After the Morris water maze experiment, the offspring rats were anesthetized and sacrificed under the abdominal aorta. The hippocampus was quickly removed from the ice and fixed with 4% paraformaldehyde. The morphology and nuclear changes of neurons were observed under a microscope after paraffin embedding, sectioning, staining and dehydration.

2.8. Nissl staining

After the Morris water maze experiment, the offspring rats were anesthetized and sacrificed under the abdominal aorta. The hippocampus was quickly removed from the ice and fixed with 4% paraformaldehyde. After a series of operations (such as sectioning, staining, sealing), microscopic examination was performed under a microscope, and images were collected to analyze the changes of hippocampal neurons and Nissl bodies.

2.9. Transmission electron microscopy

After the Morris water maze experiment, the offspring rats were anesthetized, and then their hearts were instilled with saline until there was no blood flow, injected with 4% paraformaldehyde. The hippocampal tissues were quickly taken out on ice, cut into $1\text{ mm} \times 1\text{ mm} \times 1\text{ mm}$ thin slices, and then placed into a special electron microscope fixative containing 2.5% glutaraldehyde, and then treated with conventional electron microscope embedding, sectioning and staining, and then observed under the Transmission electron microscopy was performed. The images were collected to analyze the changes in the internal structure of hippocampal neurons.

2.10. Sequencing by reduced representation bisulfite sequencing (RRBS)

The hippocampal tissues of 3 offspring rats were randomly selected from each group, and DNA methylation sequencing was carried out by RRBS. All the work was entrusted to Shenzhen Aisi Gene Technology Co.

2.10.1. DNA extraction and library construction and quality control

The genomic DNA of hippocampus was extracted using the universal genomic DNA extraction kit (DP705, Germany TIANGEN). After DNA quality inspection, 100–300 ng genomic DNA was digested with *MspI*, repaired at the end, added with A tail and ligated with methylation-modified illumina adaptor, followed by Bisulfite treatment with EZ DNA Methylation Gold Kit (American Zymo Research). PCR amplification was performed to enrich the library fragments, and the qualified DNA library was used for sequencing.

2.10.2. Bioinformatics analysis

After obtaining the original sequencing data (Raw reads), the clean reads were obtained by trimming. The clean reads and the reference

genome were analyzed by Bsmapp software, and the methylation sites were detected to calculate the methylation level. Then, CG sites were used to find differentially methylated regions (DMRs), and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis were performed on DMR-related genes. Finally, differentially methylated target genes were screened.

2.11. TBS detection of DNA methylation levels in the promoter region of *GNAS* and *AGAP2* genes

Hippocampal tissues of four offspring rats were randomly selected from each group, and the methylation level of each CpG site in the promoter region of 6 DMRs of *GNAS* gene and 2 DMRs of *AGAP2* gene was detected by TBS method in the hippocampus of rats. All the work was entrusted to Shenzhen Ace Gene Technology Co. The online Meth-Primer software (Li and Dahiya, 2002) was used to design the primer sequences for the methylation of the target genes *GNAS* and *AGAP2*. A total of 2 primer pairs were designed for the 6 DMRs targeting the *GNAS* gene and 1 primer pair for the 2 DMRs targeting the *AGAP2* gene. The primer sequences are shown in Supplement Table 1. The extracted DNA samples were subjected to sulfite conversion and recovery using ZYMO EZ DNA Methylation-Gold Kit with 50–200 ng genomic DNA. PCR amplification was performed using Acegen Targeted Methyl Panel Kit (Shenzhen Acegen). Agencourt AMPure XP was used to purify and recover the targeted amplification products for the preparation of DNA small fragment library, and Acegen DNA Library Prep Kit (Shenzhen Acegen) was used to construct a double-end 8bp-index library. The qualified library was sequenced using the Illumina platform, and the clean reads were detected using the software Trimmomatic-0.36 cji, and then the clean reads and the target sequence were compared and analyzed using Bsmapp (v2.73). The methylation level was calculated according to the following formula: the methylation level of the C site = the number of reads supporting methylation / (total number of reads), and the methylation level of each site in each sample was calculated.

2.12. Western Blot detection of protein expression in hippocampal tissues of offspring rats

Each 20 mg hippocampal tissue was homogenized with 250 μ L of the currently prepared lysis working solution (Radio-Immunoprecipitation Assay buffer: phenylmethylsulfonyl fluoride: protein phosphatase inhibitor = 100: 1: 1), centrifuging at 12000 r/min for 15 min. The protein concentration of hippocampal samples (μ g / μ L) was calculated by Bicinchoninic Acid (BCA) working solution (BCA reagent: Cu reagent = 50:1). The protein loading buffer was mixed with the sample at a ratio of 1: 4, followed by gel preparation, loading, and electrophoresis. After the membrane was transferred, the Polyvinylidene fluoride (PVDF) membrane was taken out and washed with Tris buffered saline tween (TBST) once. TBST is composed of Tris Base, NaCl, Tris-HCl and Tween. Then it was placed in 5% skim milk and sealed on a shaker at room temperature for 90 min. After the antibody was incubated, the cleaned PVDF membrane was taken out and an appropriate amount of configured luminescent developer was added to cover the entire membrane for exposure. The gray value of the protein bands obtained after exposure was analyzed by Image J software, and the internal reference protein Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for relative quantification.

2.13. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) for mRNA expression in hippocampal tissue of offspring rats

(1) Total RNA extraction

Hippocampal tissue (~50 mg) was homogenized in 1 mL Trizol. After adding chloroform (1:5, v/v), the mixture was centrifuged at 12000 r/min for 15 min at 4 °C. The aqueous phase

(400 μ L) was mixed with isopropanol, incubated for 15 min, and centrifuged. The RNA pellet was washed with 75% ethanol, centrifuged at 7500 r/min for 15 min at 4 °C, air-dried, and dissolved in Diethyl pyrocarbonate (DEPC) water. RNA concentration was measured in triplicate.

(2) Reverse transcription reaction

The RNA concentration of each group was adjusted to 500 ng / μ L with DEPC water. The RT reaction solution of each component was prepared according to Supplement Table 2 and then 200 μ L was added to the enzyme-free EP tube, and finally the RNA sample was added. After mixing, reverse transcription reaction was carried out. The conditions were set as follows: 37 °C 15 min, 85 °C 5 s, 4 °C. After the reaction, the cDNA was placed in a refrigerator at -20 °C for storage.

(3) Real Time PCR reaction

The PCR primers (Shanghai San gong Biological Engineering Co., Ltd. Table 1) were diluted to 10 μ M with DEPC water according to the label tips. The PCR reaction solution was prepared according to Supplement Table 3, and the reaction solution was added to the eight-barrel tube. qRT-PCR reaction was performed using TB Green Premix Ex Taq Kit (TaKaRa) in a PikoReal System (Thermo). The thermal cycling was set as follows: initial heating at 95 °C for 1 min (1 cycle), followed by incubating at 95 °C for 15 s and 60 °C for 30 s (40 cycles). The relative expression of the target gene was calculated by relative quantitative method ($Q=2^{-\Delta\Delta C_t}$) with GAPDH as the internal reference.

2.14. Statistical methods

IBM SPSS Statistics 29.0 (IBM Corp., 2022) was used for statistical analysis of the data, and the data are presented as mean $\pm$ standard error of the mean ($\bar{x} \pm SEM$). Normality was first assessed using the Shapiro-Wilk test, supplemented by visual inspection of Q-Q plots. Homogeneity of variances was evaluated using Levene's test. If both assumptions were met, mean consumption was compared among groups using one-way ANOVA, followed by Bonferroni test for multiple comparison correction. If either assumption was violated, the Kruskal-Wallis test was used instead, followed by Dunn's pairwise tests with Holm's adjustment for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Establishment of parental rat model

A total of 35 rats showed dental fluorosis in the NaF group, and the detection rate was 97.2%. In addition, compared with the control group, the urinary fluoride level in the NaF group was increased ($P < 0.001$, Table S4). In the Morris water maze test, the NaF group exhibited

Table 1
Real Time PCR reaction primers sequence.

Gene name		Sequences	Amplicon size (bp)
GNAS	F	CAAGTGGGCGGAGACTACA	178
	R	AGGCTGGGCTGTGAT	
AGAP2	F	GCTGGGCGAGCTAGTAATG	192
	R	GGCTGCTCGATGTAAGGAC	
DNMT1	F	AGAGTCGCAGGCACAGAGAGTC	235
	R	TTTCATCGTCATCGTCCACATCCAAG	
DNMT3A	F	CAAAGCCATCTACGAAGTCCTCCAG	206
	R	AGCCGTTCTCTTGTGCGTTTCATC	
DNMT3B	F	GATGGAGATGGTGAAGCGGATGATG	221
	R	CGATGGCGTACTGCTGCTCTTAG	
GAPDH	F	GACATGCCGCTGGAGAAAC	129
	R	AGCCAGGATGCCCTTTAGT	

Note: F (Forward) represents the upstream primer. R (Reverse) represents the downstream primer.

significantly prolonged escape latency on days 2 and 4, along with increased swimming distance compared to the control group ($P < 0.05$; Fig.S1a-b). Additionally, the number of platform shuttles and the percentage of swimming time and swimming distance were decreased in the NaF group ($P < 0.05$, Fig.S1c-d). The swimming route in the NaF group was more complicated than that in the control group (Fig.S1e). The above experimental results showed that after three months of fluoride exposure, the parental rats had different degrees of chronic fluorosis which were considered that the fluorosis model was successfully replicated.

3.2. Analysis of fluoride contents and neurobehavioral of offspring rats

The fluoride-exposed group increased the contents of fluoride in the blood and brain of the offspring rats ($P < 0.05$, Fig. 1). Furthermore, compared with the NaF group, NaF + 100BET group showed a lower level of fluoride in the blood and brain ($P < 0.05$, Fig. 1). The content of brain fluoride in the NaF + 400BET group was lower than that in the NaF group ($P < 0.05$, Fig. 1).

As shown in Supplement Table 5 (Fig. 1), the age of positive reaction in the among NaF groups was later than that in the control group ($P < 0.05$, Fig. 1c-e). In the cliff avoidance reflex and negative chemotaxis reflex experiments, the age of positive reaction in NaF + 100BET group was earlier than that in the NaF group ($P < 0.05$, Fig. 1d-e). In

addition, there was no significant difference in swimming distance across all groups in the localization navigation experiment, ($P = 0.219$, Fig. 2a). The NaF group demonstrated significantly reduced swimming speed compared to controls ($P < 0.05$), while the NaF + BET groups had significantly ($P < 0.05$) higher the swimming speed relative to the NaF group ($P < 0.05$; Fig. 2b). Similarly, the NaF group showed increased escape latency compared to the control group, and BET intervention reduced the escape latency. ($P < 0.05$; Fig. 2c). Additionally, in the space exploration experiment, the number of crossing the target quadrant and the percentage of swimming time in the target quadrant were not statistically different among the groups ($P > 0.05$, Fig. 2d-f), while BET treatment groups could significantly ($P < 0.05$, Fig. 2f) increase the platform shuttle times, indicating that BET intervention may have a good effect on the spatial learning and memory ability of offspring rats caused by fluorosis in parental rats. There was no significant difference in gender interaction among the four indicators of swimming escape latency, swimming distance, swimming speed and platform crossover number ($P > 0.05$), and no gender effect was observed in the modeling treatment.

3.3. Observation of brain tissue pathology and ultrastructure of offspring rats

H&E staining, Nissl staining and transmission electron microscopy

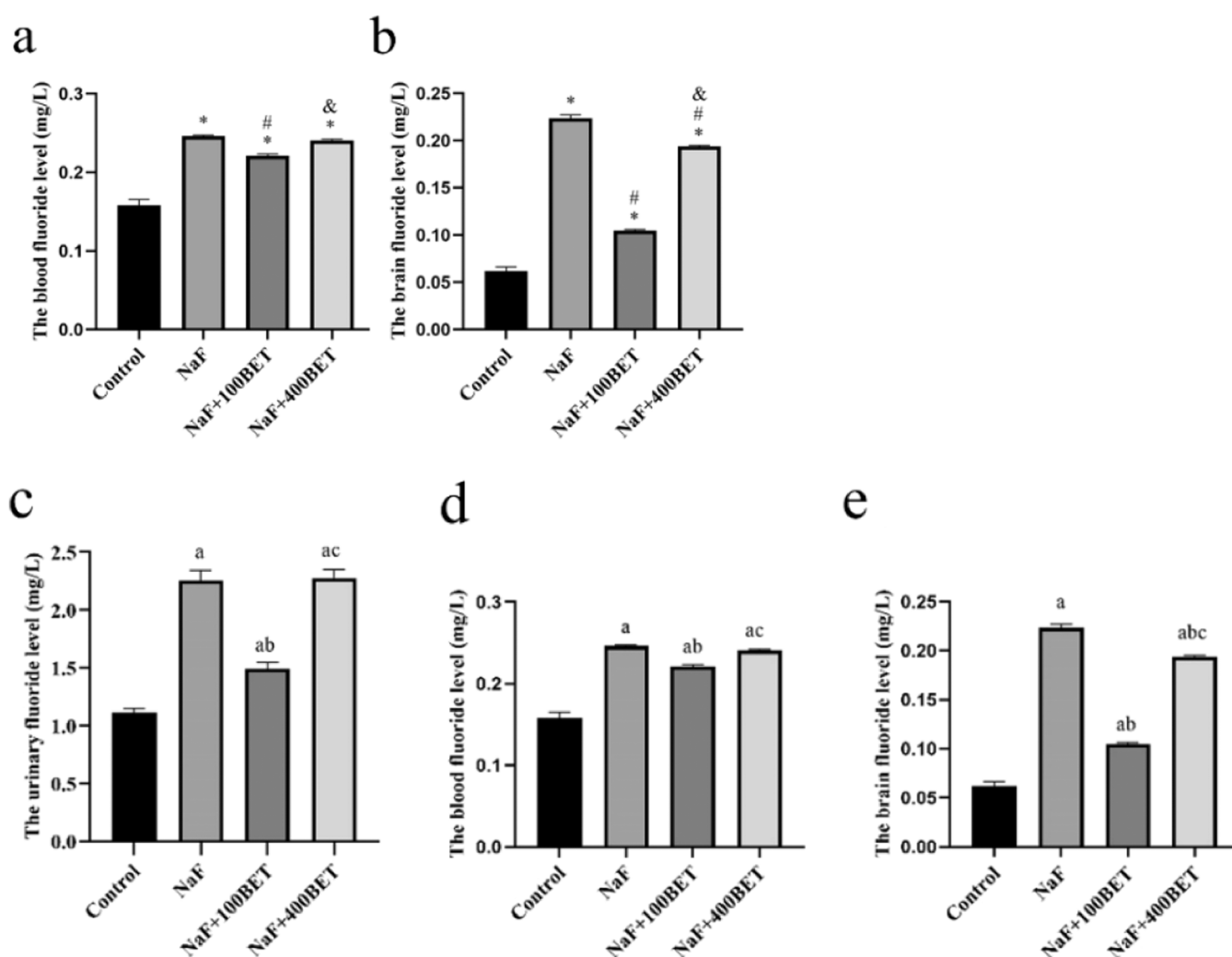


Fig. 1. Distribution of fluoride content and positive time of neurobehavioral reflex in offspring rats. Note: a Blood fluoride; b Brain fluoride; c Positive time of plane righting reflection; d Positive time of cliff avoidance reflection; e Positive time of negative chemotaxis reflection, * Means compared with the control group, $P < 0.05$; # Means compared with NaF group, $P < 0.05$; & Means compared with NaF + 100BET group, $P < 0.05$.

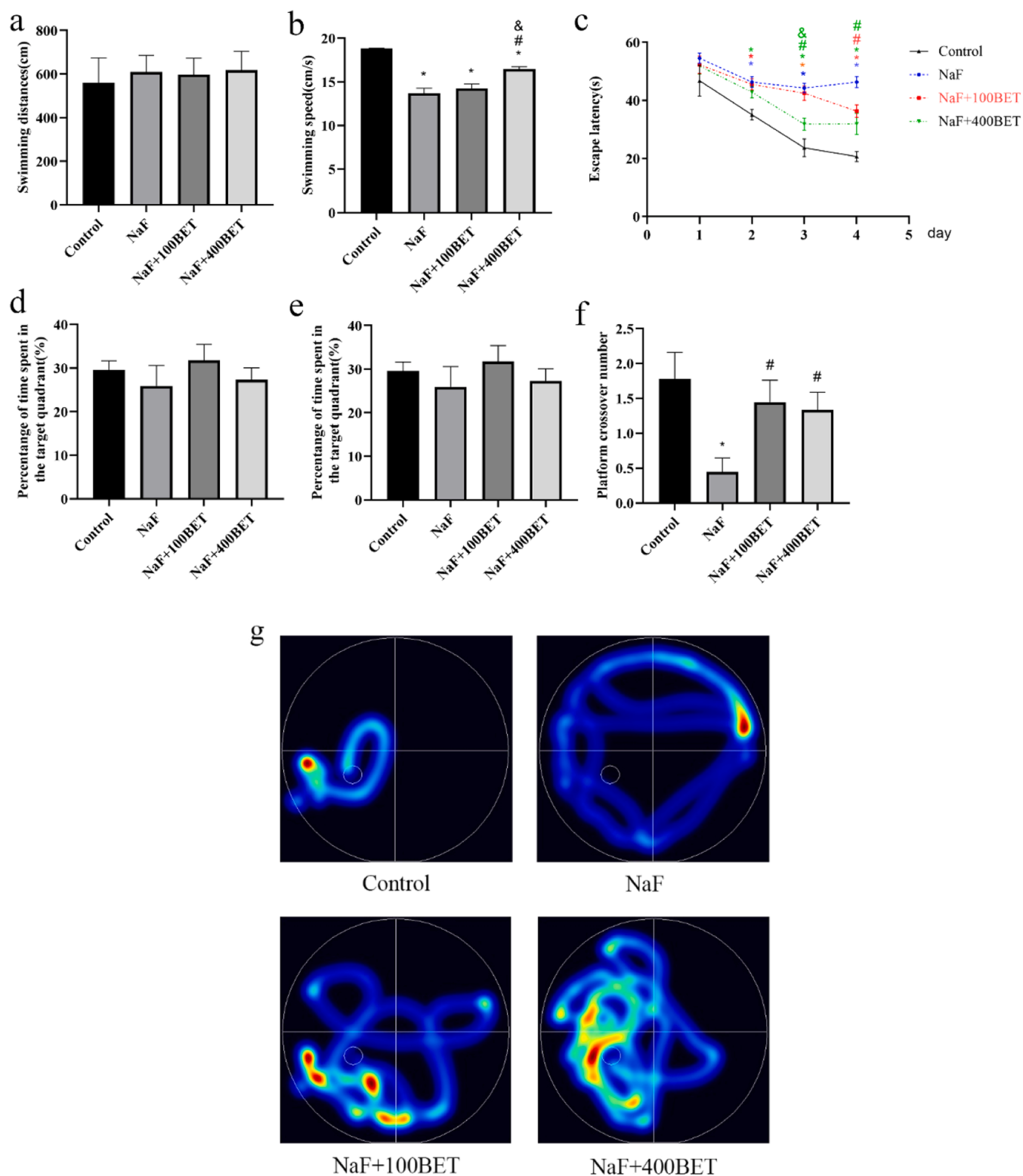


Fig. 2. Morris water maze experiment results of offspring rats. Note: **a** Swimming distance; **b** Swimming speed; **c** Escape latency; **d** The number of crossing the target quadrant; **e** The percentage of swimming time in the target quadrant; **f** Platform shuttle times; **g** swimming trajectory diagram. The offspring $n = 18$, * Means compared with the control group, $P < 0.05$; # Means compared with NaF group, $P < 0.05$; & Means compared with NaF + 100BET group, $P < 0.05$. Data were pooled from male and female rats, no significant group-by-sex interaction was detected for all Morris water maze indexes.

experiments were carried out to observe the brain tissue and ultra-structure of offspring rats. The H&E staining results showed that the NaF group obviously altered and loosely distributed the structures of hippocampal neurons, as well as caused a pyknosis and deep staining of

nuclear, and degeneration or death of a large number of neurons, while BET treatment could alleviate these symptoms (Fig. 3a). Likewise, Nissl staining showed that the number of intracellular Nissl bodies was less in the NaF group, whereas BET intervention could mitigate the neuronal

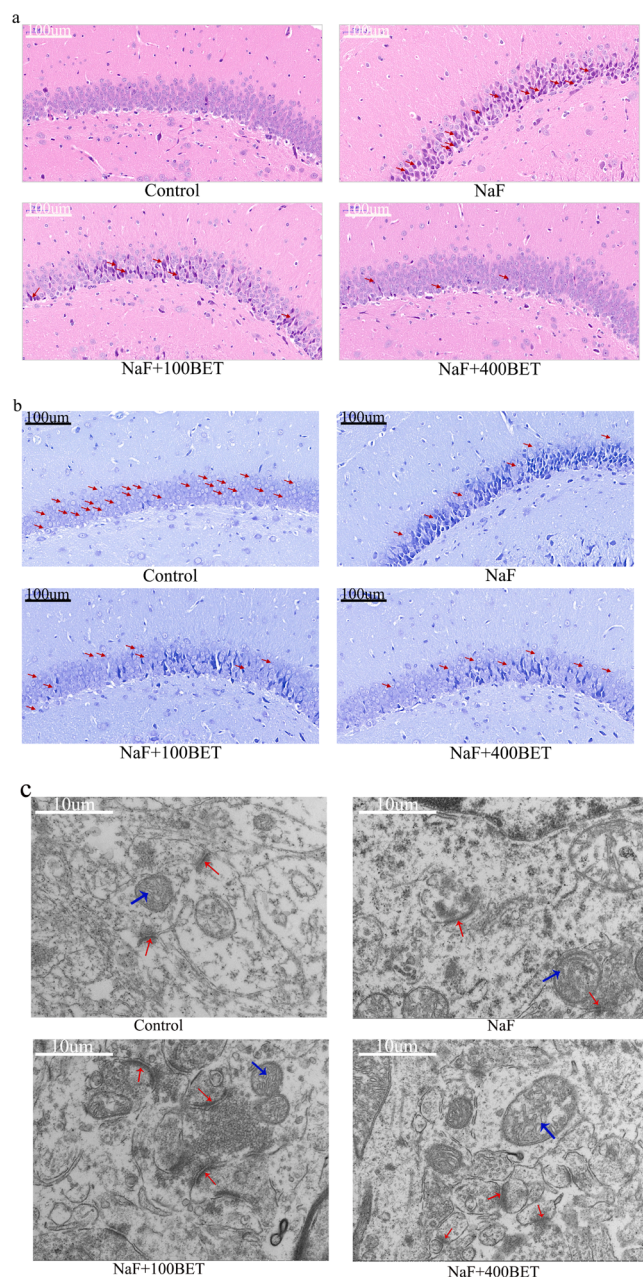


Fig. 3. Observation of brain tissue pathology and ultrastructure of offspring rats. Note: **a** HE staining of hippocampus, red arrows indicate wrinkled, deeply stained neurons. **b** Nissl staining of hippocampus, red arrows point to Nissl bodies. **c** Ultrastructure of hippocampal neurons, red arrows indicate synaptic vesicles, blue arrows indicate mitochondria. The scale bar of each image in **a** and **b** is 100 μm. The scale bar of each image in **c** is 10 μm.

damage and increase the number of intracellular Nissl bodies (Fig. 3b). In addition, compared with the control group, the NaF group showed obvious the incomplete synaptic structure, unclear the synaptic gap structure, partly damaged and fused mitochondrial cristae using the transmission electron microscope analysis, while BET treatment could reduce the disorder of synaptic structure and the damage of mitochondrial cristae (Fig. 3c).

3.4. Analysis of RRBS sequencing results

3.4.1. DMRs anchored region distribution and methylation level

The data analysis showed that the methylation level of the Cytosine (C) sites of each sample was reliable (Table S6). The methylation C sites

under different sequence environments (CG, CHG, CHH, where H represents A, C, T) with coverage depth above 5X were statistically analyzed (Table S7). The data showed that the same type of methylation sites accounted for similar proportions in different samples, while different types of methylation sites accounted for different proportions in the same sample, among which CG type accounted for the highest proportion, up to more than 97%, which was consistent with the fact that methylated C in eukaryotic genomes was mainly distributed in CG sites. CG sites were used to find differentially methylated regions (Fig. 4a).

The distribution of DMRs in the functional regions of genes was similar in all groups, and a higher number of DMRs was observed in the gene body. Further analysis showed that in NaF vs Control, a total of 932 hypermethylated regions and 945 hypomethylated regions were detected, including 1346 differentially methylated genes, and 159 DMRs were located in the promoter region. In NaF + 100BET vs NaF, a total of 776 hypermethylated regions and 1102 hypomethylated regions were detected, including 1337 differentially methylated genes, and 169 DMRs were located in the promoter region. In NaF + 400BET vs NaF, 472 hypermethylated regions and 2781 hypomethylated regions were detected, including 2186 differentially methylated genes, and 318 DMRs were located in the promoter region. ($P > 0.05$, Fig. 4b).

3.4.2. DMR-related, KEGG enrichment analysis and target gene screening

Based on the DMR annotation results, KEGG enrichment analysis was performed on genomes and their upstream and downstream regions overlapping with DMRs. In the scatter plot, the degree of KEGG enrichment was measured by fold change, q-value and the number of genes enriched in this pathway. The pathway entries co-enriched in each comparison group included: Ras signaling pathway, Olfactory transduction, Glutamatergic synapse, and Circadian entrainment (Fig. 5a). Since in mammals, abnormal methylation of gene promoter regions can cause changes in gene expression levels. We screened 21 genes overlapping in the promoter region in NaF vs Control, NaF + 100BET vs NaF, and NaF + 400BET vs NaF, of which only 7 genes had functional annotations in the KEGG enrichment. By consulting the relevant literature, it was found that *GNAS* and *AGAP2* were related to brain function in these 7 genes, which were verified as candidate genes for later experiments (Fig. 5b).

3.5. TBS detection of DNA methylation levels in the promoter region of *GNAS* and *AGAP2* genes

The methylation levels of CpG sites on the CpG islands of DMR promoter regions of *GNAS* and *AGAP2* genes were analyzed in offspring rat hippocampal tissues (Fig. 6a-b, Table S8). Compared to the NaF group, BET-treated groups showed higher methylation levels at 34 CpG sites within the *GNAS* promoter-associated CpG island ($P < 0.05$, Fig. 6a). Although no statistically significant differences were detected at 9 CpG sites in the *AGAP2* promoter CpG island across experimental groups ($P > 0.05$, Fig. 6b), BET intervention consistently increased methylation levels at these CpG sites relative to NaF exposure. Furthermore, comparative analysis of 14 hypomethylated and 20 hypermethylated CpG sites in the *GNAS* promoter DMR across all groups (Fig. 6c-d, Table S9). The results showed that the hypomethylation levels were decreased in the NaF group that in the control group, while BET intervention partially reversed the hypomethylation levels in the hippocampal tissues of offspring rats ($P < 0.05$, Fig. 6c).

3.6. The proteins and mRNA expression levels of *GNAS*, *AGAP2* and *DNMT*

NaF exposure upregulated both protein and mRNA levels of *GNAS* and *AGAP2* in the hippocampal tissue of offspring rats, whereas BET intervention significantly downregulated the expression of these genes and proteins. ($P < 0.05$, Fig. 7a-d). In addition, the protein expression

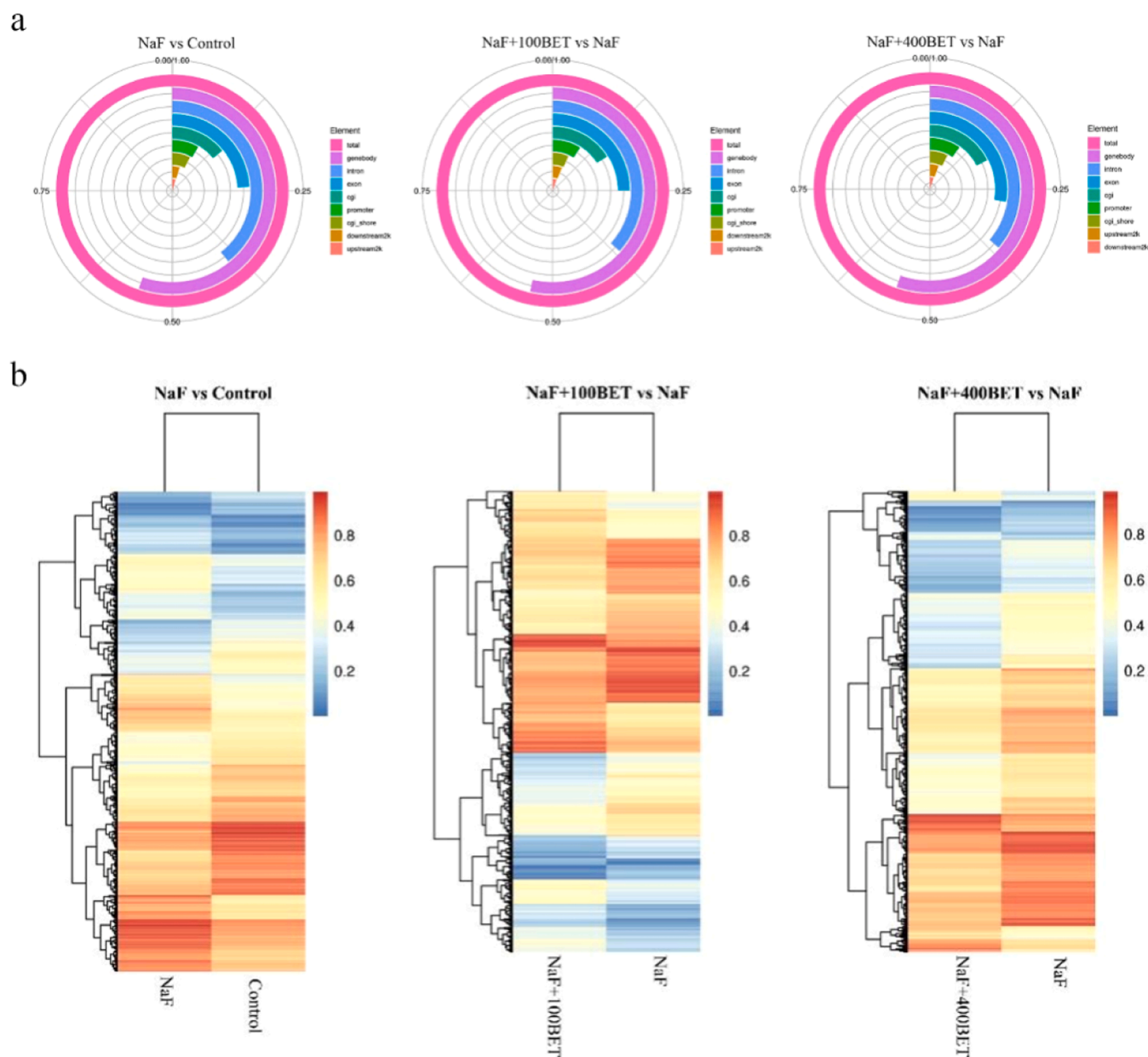


Fig. 4. DMR anchored region distribution and methylation level. Note: **a** Cluster heat map of DMR methylation level. **b** Scale map of quantity distribution of DMR anchoring areas. Horizontal coordinates represent contrasting groups, vertical coordinates represent the clustering effect of methylation level, and blue to red indicates that the methylation level is from low to high.

levels of DNMT1, DNMT3A, and DNMT3B were significantly reduced in the NaF group compared to the control group. In contrast, BET intervention markedly upregulated these proteins in offspring rats ($P < 0.05$, Fig. 7e-g). Similarly, the mRNA expression levels of DNMT1, DNMT3A and DNMT3B were significantly downregulated in the NaF group, whereas BET intervention effectively upregulated these genes in the hippocampal tissue of offspring rats ($P < 0.05$, Fig. 7h-m). These results indicated that BET intervention increased DNA methylation levels by regulating the proteins and mRNA expression levels of *GNAS*, *AGAP2* and DNMTs in the hippocampal tissue of offspring rats, which play an important role improving cognitive deficits.

4. Discussion

In this study, the parental rats successfully established a chronic fluorosis model after three months of fluoride exposure, and showed a significant decrease in learning and memory ability. More importantly,

this adverse effect of fluoride exposure in parental generation can be transmitted to offspring rats. The offspring rats showed delayed reflex development and impaired spatial learning and memory ability in neurobehavioral tests, which is consistent with previous studies on fluoride neurotoxicity (Xiang et al., 2023; Zheng et al., 2016). These behavioral deficits have their structural basis: disordered hippocampal neurons, reduced Nissl bodies, incomplete synaptic structure, and impaired mitochondrial cristae were found in the offspring rats. The hippocampus is a key brain region for learning and memory (Kutlu and Gould, 2016). Alterations in hippocampal synaptic plasticity can lead to changes in learning and memory (Liu et al., 2020; Tiwari et al., 2015). The above pathological changes induced by fluoride may be the direct cause of cognitive impairment in offspring rats.

In present study, BET intervention can significantly alleviate the negative effects of fluoride exposure, which not only reduced fluoride levels in the blood and brain of offspring rats, but also improved their neurobehavioral performance and alleviated pathological damage in the

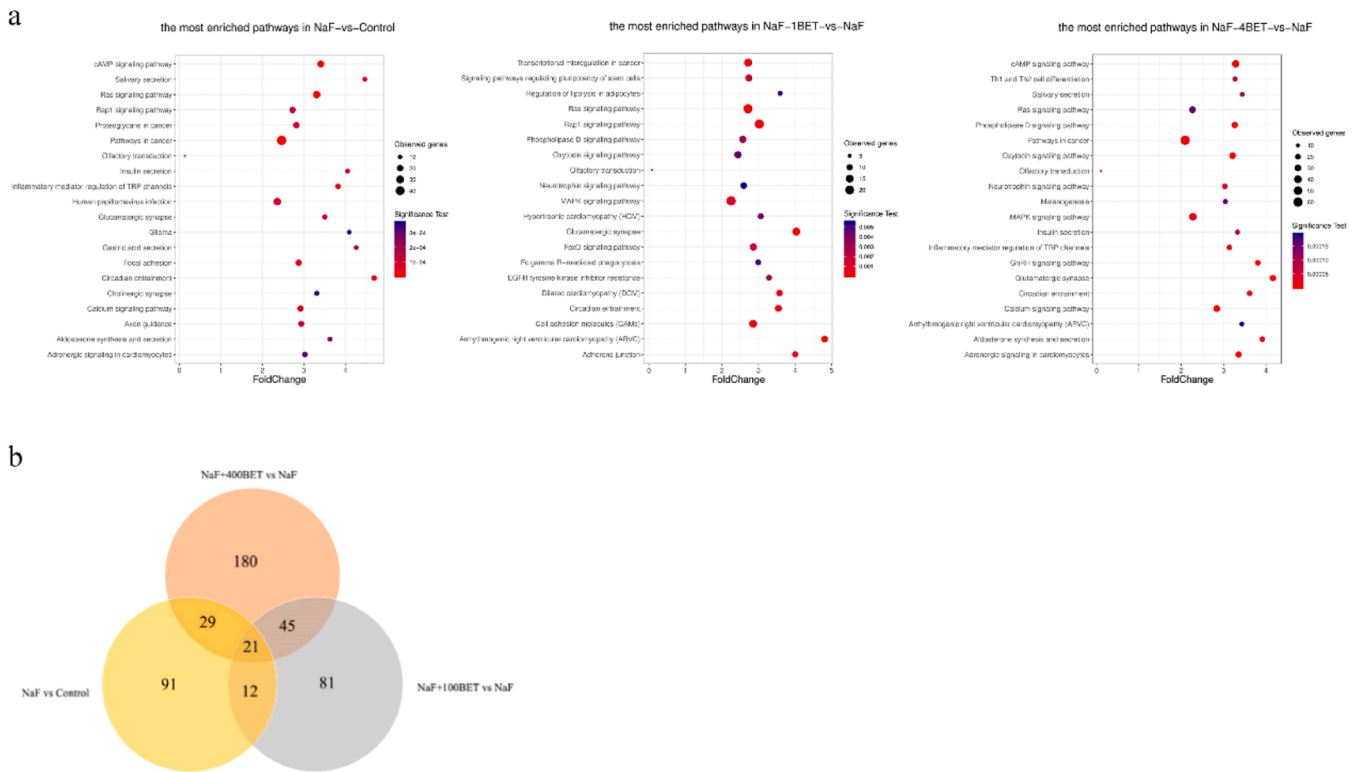


Fig. 5. DMR-related genes KEGG enrichment analysis and target gene screening. Note: **a** KEGG enrichment scatter diagram. The vertical axis indicates the pathway name, the horizontal axis indicates the fold change, the size of the dot indicates the number of DMR-related genes in the pathway, and the color of the dots corresponds to the different q-value ranges, with redder colors indicating more significant. **b** Target gene screening venn diagram.

hippocampus. Betaine, as a methyl donor nutrient, provides three methyl groups for methylation reaction, which provides higher methyl utilization efficiency compared with other methyl donors (Steluti et al., 2020) and can regulate gene methylation levels (Zhang et al., 2020). Several studies have also reported that hippocampal synaptic plasticity is associated with DNA methylation, and the relationship between fluorosis and DNA methylation has been previously reported (Ma et al., 2020; Wu et al., 2018; Wang et al., 2022; Daiwile et al., 2019). Our results suggest that BET may repair damaged neuronal function by providing methyl groups to regulate epigenetic modifications related to neural development and function.

DNA methylation is a common epigenetic modification in eukaryotes, which can be inherited to the next generation through DNA replication and can stably alter animal behavior, such as learning, memory and synaptic plasticity (Daiwile et al., 2019; Meng et al., 2021; Younesian et al., 2022; Poon et al., 2020; Greeson et al., 2023). RRBS sequencing results revealed that fluoride exposure in parental rats led to a wide range of methylation differences in the hippocampus genome of offspring rats, and these differences were enriched in Ras signaling pathway, glutamatergic synapses and other pathways closely related to neurological function and synaptic plasticity (Zhu et al., 2002; Kaupp, 2010; Malenka and Bear, 2004; Colwell, 2011). This suggests that the neurotoxicity of fluoride may be achieved by interfering with the epigenetic regulation of these key pathways. We further focus on the promoter region of the gene, where the methylation status has a direct regulatory effect on gene transcription (Jones and Takai, 2001; Bird, 2002). Combined with previous studies demonstrating that DNA methylation in the promoter region is related to the transcriptional expression of genes (Yang et al., 2019), we screened the *GNAS* and *AGAP2* as candidate genes. *GNAS* encodes multiple transcripts and is an important component of many signaling pathways (Pignolo et al., 2011). *AGAP2* encodes a superfamily member of GTPases, whose disturbed expression can lead to neurodevelopmental abnormalities (Yu et al.,

2019).

Based on TBS detection, qPCR and Western Blot results, we found that parental fluoride exposure significantly down-regulated the expression of DNMTs in the hippocampus of offspring. The three main DNA methyltransferases in the central nervous system of eukaryotes including DNMT1, DNMT3A, and DNMT3B, have been shown to be involved in biological processes such as early germ cell genesis, embryonic development, learning memory, and disease genesis, which are key enzymes that catalyze and maintain DNA methylation (Uysal and Ozturk, 2017; Nguyen et al., 2020; Tajima et al., 2022). The decreased expression of DNMTs directly leads to the trend of genome-wide hypomethylation and hypomethylation in the promoter region of *GNAS* gene (Plagge, 2012; Liu et al., 2005). This hypomethylation status relieves the inhibition of *GNAS* transcription and abnormally increases its mRNA and protein levels (Liu et al., 2005). Overexpression of *GNAS* may disrupt normal intracellular signal transduction, and ultimately damage synaptic function and plasticity, manifested as cognitive deficits. The addition of BET effectively up-regulated the expression of DNMTs. The recovery of DNMTs levels in turn promotes the re-methylation of the promoter regions of key genes such as *GNAS* and restores their expression levels to normal, and the BET intervention showed a trend of increasing methylation level of *GNAS*. These findings indicate that BET alleviates fluoride induced learning and memory impairment in offspring rats primarily by restoring DNMTs expression and subsequent *GNAS* promoter methylation. In addition, no significant differences were observed in the methylation level of the *AGAP2* promoter region. Therefore, BET alleviated fluoride-induced neuronal injury mainly by elevating DNA methylation of *GNAS*, rather than *AGAP2*.

Studies have confirmed multiple biological roles of BET, such as a critical methyl donor participating in homocysteine metabolism, an antioxidant suppressing oxidative damage, and a suppressor of neuro-inflammation (Zawieja and Chmurzynska, 2025). Nevertheless, the present study specifically focused on BET's methyl-donating capacity to

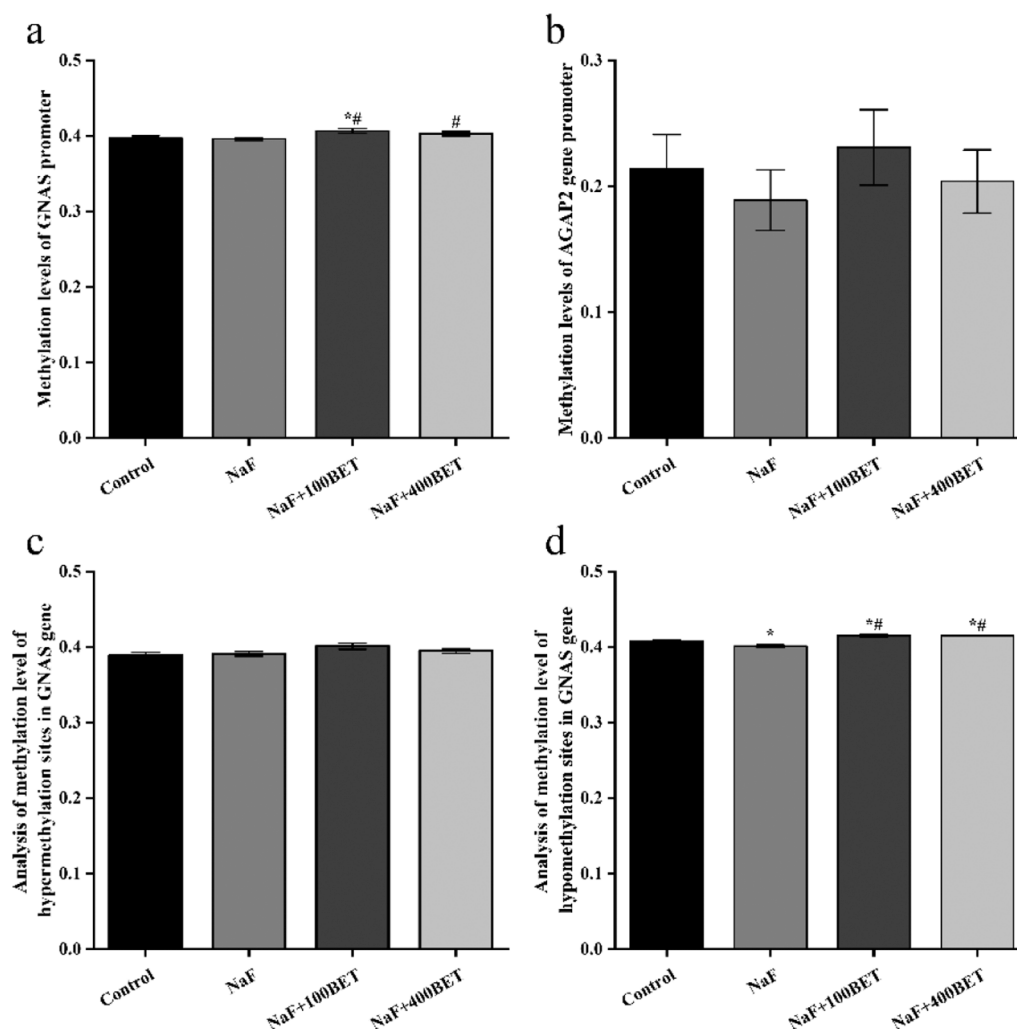


Fig. 6. Methylation levels of GNAS and AGAP2 gene promoters. Note: **a** Methylation levels of GNAS gene promoters; **b** Methylation levels of AGAP2 gene promoters; **c** Methylation level of hypermethylation site in GNAS gene; **d** Methylation level of hypomethylation site in GNAS gene; * Means compared with the control group, $P < 0.05$; # Means compared with NaF group, $P < 0.05$.

alleviate fluoride-induced learning and memory impairment in rat offspring via DNA methylation alterations. This targeted research focus does not negate or rule out any other potential pathways underlying BET's protective effects. Other biological functions of BET related to fluoride neurotoxicity is worthy of further investigation in our follow-up research.

Meanwhile, based on the results of this study, we found that the effect of high-dosage BET intervention was not as obvious as that of low-dosage BET intervention, indicating that appropriate supplementation of BET acts as an efficient methyl donor to alleviate fluoride-induced aberrant DNA methylation. However, excessive methyl supply derived from high-dose BET (400 mg/kg) exerts adverse developmental impacts. Studies have confirmed that overexposure to methyl donors during early life can trigger harmful biological consequences (Yadon et al., 2019). Consistently, excessive maternal folate supplementation during gestation impairs methylenetetrahydrofolate reductase activity, resulting in fetal growth retardation and cognitive dysfunction in offspring (Bahous et al., 2017). In short, moderate-dose BET achieves an optimal methyl-donating balance to counteract fluoride neurotoxicity, while the excess methyl supply from high-dose BET weakens its protective efficacy. Therefore, the most suitable amount for BET to improve learning and memory impairment in offspring rats remains to be studied.

In the present study, we first explored that BET alleviates fluoride-induced learning and memory impairments in rat offspring from the

perspective of DNA methylation, with functional alterations primarily linked to methylation modifications of the GNAS gene. Nevertheless, several limitations exist in our work. First, we only observed the effects of fluoride exposure and BET intervention on offspring neurobehavior at the animal level, and did not verify the epigenetic changes directly induced by fluoride or the protective mechanism of BET in vitro. Second, only two doses of BET (100 and 400 mg/kg) were used in this study, and the dose-response relationship remains unclear. Future research will adopt a broader range of BET doses to clarify potential dose-response effects. Additionally, we did not directly administer a DNMT inhibitor to examine whether it mimics the effects of fluoride. Future experiments will utilize DNMT inhibitors to verify the above conjecture.

5. Conclusion

BET intervention during pregnancy reduces fluoride-induced learning and memory impairment in offspring rats. The mechanism may be that BET increases the DNA methylation level of the GNAS gene promoter region, thereby down-regulating its expression and exerting a protective effect.

CRedit authorship contribution statement

Jun Liu: Writing – review & editing, Funding acquisition,

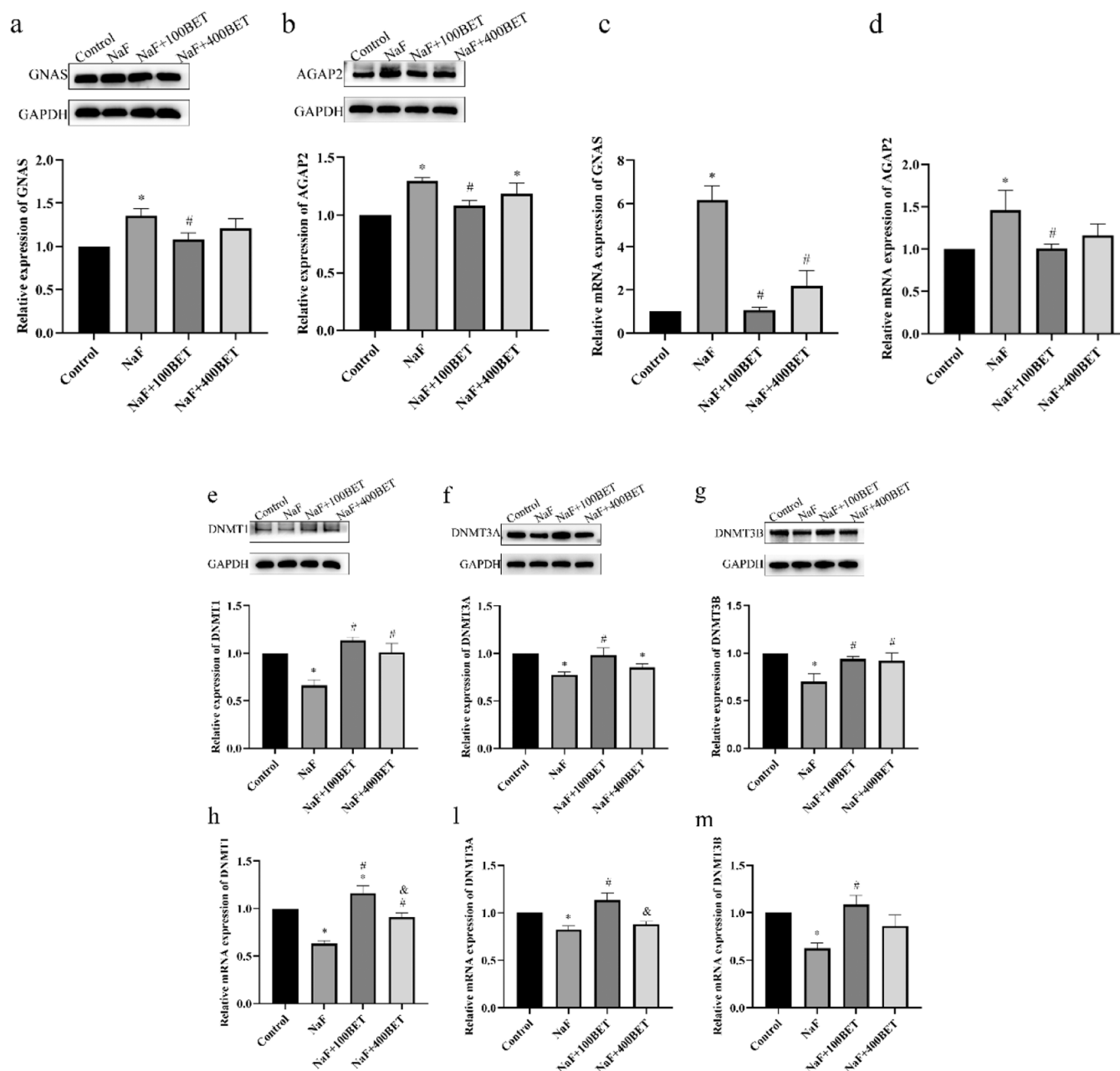


Fig. 7. The expression of GNAS, and AGAP2 and DNMTs protein and mRNA of GNAS, and AGAP2 and DNMTs gene. Note: **a** Expression of GNAS protein in hippocampus. **b** Expression of AGAP2 protein in hippocampus. **c** Expression of GNAS gene mRNA in hippocampus. **d** Expression of AGAP2 gene mRNA in hippocampus. **e** Expression of DNMT1 protein in hippocampus. **f** Expression of DNMT3A protein in hippocampus. **g** Expression of DNMT3B protein in hippocampus. **h** Expression of DNMT1 gene mRNA in hippocampus. **i** Expression of DNMT3A gene mRNA in hippocampus. **m** Expression of DNMT3B gene mRNA in hippocampus. $n = 4$, * Means compared with the control group, $P < 0.05$; # Means compared with the NaF group, $P < 0.05$; & Means compared with the NaF + 100BET group, $P < 0.05$.

Conceptualization. Min Liu: Writing – review & editing, Supervision, Project administration. Lianhong Li: Writing – original draft, Data curation. Wei Wu: Methodology, Formal analysis, Data curation. Yin Chen: Writing – original draft, Methodology. Yi Luo: Writing – review & editing, Visualization. Ling Tang: Writing – review & editing, Supervision. Xiaogang Zhang: Writing – review & editing, Supervision. Man Wang: Writing – review & editing, Supervision.

Funding

This work was supported by the National Natural Science Foundation of China [82360642]; Science & Technology Program of Guizhou

Province [QKHHBZ(2020)3002, QKHPTRC-GCC(2022)039–1, QKHPTRC-CXTD(2022)014, QKHJC-ZK(2023)-YB503]; Guizhou Province Foundation for postgraduate Scientific Research Fund Project [2024YJSKYJJ330] and College Students' Innovation and Entrepreneurship Program of Guizhou Province [S202510661493]; and Zunyi Science and Technology Innovation Team for Nutrition and Health [Zun KCTD (2025)89].

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.

Acknowledgement

The successful completion of this paper is inseparable from the careful guidance of Jun Liu and Min Liu. From the topic selection and design of the experiment to the specific operation, and then to the writing and modification of the paper, both teachers have devoted a lot of effort. Teacher Jun Liu's rigorous academic attitude and control of details have greatly benefited me on the road of scientific research; the key advice and encouragement given by Min Liu when the experiment encountered bottlenecks pointed out the direction for me. Here, I would like to express my sincere thanks to the teachers.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2026.120722](https://doi.org/10.1016/j.ecoenv.2026.120722).

Data availability

Data will be made available on request.

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