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Paternal co-exposure to nicotine, ethanol, and caffeine induces neurobehavioral deficits in offspring through abnormal programming of sperm imprinting genes

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ABSTRACT: Paternal adverse environmental exposure affecting offspring health via epigenetic pathways has emerged as a forefront topic in the fields of developmental origins of health and disease and disease prevention. This study focuses on three substances widely present in daily dietary intake—nicotine, ethanol, and caffeine—and establishes a paternal mixed exposure (PME) rat model to investigate their combined effects on offspring neurodevelopment and underlying mechanisms. The results revealed that PME offspring exhibited significant anxiety- and depression-like behaviors, as well as cognitive impairment, accompanied by reduced hippocampal neurogenesis and synaptic structural damage. Transcriptomic analysis indicated significant suppression of the PI3K-Akt signaling pathway. Further investigation uncovered that this regulation stemmed from disrupted epigenetic encoding at the IG-DMR region of the sperm-derived Dlk1-Dio3 imprinted domain. Through genetic manipulation targeting this imprinted domain, the study functionally validated its central role in regulating offspring neurogenesis and behavior. This research is the first to systematically elucidate, from the perspective of paternal mixed dietary factor exposure, an intergenerational inheritance mechanism by which such exposure impairs offspring neurobehavior via interference with imprinted gene epigenetic regulation. It provides important experimental evidence and theoretical support for dietary-related paternal health risk assessment and nutritional intervention strategies.

Keywords: Paternal mixed exposure; Hippocampal neurogenesis; PI3K-Akt signaling pathway; Dlk1-Dio3 imprinted domain; IG-DMR

1. Introduction

Nicotine (tobacco products), ethanol (alcoholic beverages), and caffeine (coffee, tea, energy drinks, etc.) are widely consumed food and beverage components globally, representing a significant source of daily chemical exposure for adults, particularly men of reproductive age. In recent years, the developmental origins of health and disease (DOHaD) hypothesis has traced the etiology of chronic diseases in adults back to early

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life stages, highlighting the profound impact of parental pre-pregnancy and prenatal environmental exposures on offspring health. Although previous studies have largely focused on the role of maternal nutrition and environmental exposure during pregnancy^[1-3], growing evidence indicates that paternal exposure also serves as a critical factor influencing offspring health trajectories through epigenetic programming of germ cells (sperm). Adverse paternal dietary habits, toxin exposure, and stress can disrupt key epigenetic markers in sperm that regulate embryonic development, thereby increasing the risk of neurobehavioral abnormalities and other disorders in offspring^[4-6].

Notably, consumption of these three substances is highly prevalent among males and often occurs concurrently, implying that mixed exposure rather than single-substance exposure represents the more common real-world scenario. Epidemiological studies have shown that paternal smoking or drinking can increase the risk of attention deficit hyperactivity disorder or cognitive impairment in offspring, respectively^[7-9]. Maternal caffeine intake has been documented to be associated with neurodevelopmental abnormalities in offspring^[10-12]. However, the combined effects and underlying mechanisms of paternal pregestational exposure to nicotine, ethanol, and caffeine—referred to as paternal mixed exposure (PME)—on the neurobehavioral development of offspring have not yet been systematically elucidated. This represents a significant gap in public food safety and health research.

During early embryonic development, the process of epigenetic reprogramming is crucial for erasing the gametes' differentiation memory and establishing the totipotency of the zygote^[13]. However, a subset of parent-specific epigenetic modifications can escape this reprogramming process, with genomic imprinting being the most representative example^[14]. Genomic imprinting refers to the epigenetic regulation of gene expression in a parent-of-origin-specific manner, where only one allele—either paternal or maternal—is expressed, while the other is silenced^[15]. The differential methylation status of imprinting control regions (ICRs) is a key determinant of imprinted gene expression patterns. Studies suggest that paternal alcohol exposure can alter the methylation levels of the imprinted gene *Peg3* in sperm, leading to dysregulated gene expression in the offspring's brain and impairments in learning and memory^[16]. This indicates that imprinted genes may serve as a critical epigenetic vehicle through which paternal mixed exposure (PME) influences neurodevelopment in offspring.

Based on this premise, the present study established a PME model in rats, to systematically evaluate the effects of such exposure on neurobehavioral outcomes in offspring. The investigation further focused on elucidating the epigenetic regulatory mechanisms of imprinted genes in hippocampal tissue. Integrated with bioinformatic data mining, this study aims to reveal the potential impact of PME on offspring neurodevelopment and its epigenetic basis from the perspective of food and beverage safety. The findings are expected to provide scientific evidence for dietary and lifestyle guidance for men of reproductive age, and contribute novel theoretical and experimental insights to the field of paternal origins of health and disease (POHaD).

2. Material and methods

2.1 Animals and Treatments

Specific-pathogen-free (SPF) male Wistar rats (240-270 g, 6 weeks postnatal) were obtained from the Experimental Center of Hubei Medical Scientific Academy. Animal experiments were performed in the Animal Experimental Center of Wuhan University (Wuhan, China), which was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The Animal Experiment Ethics Committee of Wuhan University Medical College approved the program (permit number: 201709). All animal experimental procedures were conducted in compliance with the regulations of the ARRIVE guidelines for reporting experiments.

All rats were fed under standard conditions (room temperature: 18-22 °C; humidity: 40-60%; light cycle: 12 h light-dark cycle) for 2 weeks and divided into control (subcutaneously injected with normal saline daily, $n = 15$) and PME groups (subcutaneously injected with nicotine (0.1 mg/kg/d), ethanol (0.5 g/kg/d), and caffeine (7.5 mg/kg/d) for 8 weeks, $n = 15$). The selected doses correspond to human-equivalent daily intakes (for a 70 kg adult) of approximately 1.14 mg nicotine, 5.67 g ethanol, and 85 mg caffeine. These doses are all well below the established safety thresholds for each substance and are intended to establish an animal model of chronic, low-level, mixed exposure to investigate its potential intergenerational health effects at subtoxic levels^[17]. After 8 weeks of administration, the male Wistar rats were mated with normal female Wistar rats in a ratio of 1:2 at 6 p.m. (each male rat was mated successfully only once). The vaginal smear was examined the next morning and the day when the sperm was observed was regarded as the gestational day (GD) 0. The pregnant female rats were picked up and kept separately. This was continued to ensure that there were at least 12 pregnant rats in each group. On GD20, one batch of pregnant rats were anesthetized with 3% isoflurane and the fetal hippocampus were immediately frozen in liquid nitrogen and preserved at -80 °C for further analysis. Moreover, all tissues were fixed in 4% paraformaldehyde solution overnight. After dehydration, tissues were embedded within paraffin for further analysis. The other batch of pregnant rats gave birth naturally, and the pups were naturally weaned at postnatal week (PW) 4. At PW12, the control and PME groups were anesthetized with 3% isoflurane and sacrificed. Again, the brains were randomly selected and fixed with 4% paraformaldehyde, and the hippocampal tissue from each group were stored at -80 °C for further analysis.

The gene knockout mouse C57BL/6J-IG-DMR^{em1Smoc} was developed by the Shanghai Model Organisms Center.

2.2 Cell culture

NE-4C neuroectodermal stem cells were purchased from ATCC (Rockville, MD). Cells were cultured in MEM Alpha supplemented with 10% FBS, 1% antibiotics and 4mM L-glutamine. Cells were maintained under the common cell culture conditions at 37 °C in an atmosphere of 5% CO₂. During the cell differentiation stage, the culture medium was changed to DMEM/F12 medium containing 1% B27 and 1% dual antibody.

2.3 Behavioral tests

Animals must be placed in the testing room for more than 3 days to adapt to the environment before testing behavior. The daily inspection time is from 8 a.m. to 6 p.m.

Elevated plus maze (EPM) test

This apparatus comprises 2 open arms and 2 closed arms, interconnected by a central platform. Prior to the commencement of the experiment, the apparatus was thoroughly cleaned with 75% ethanol to prevent the influence of odors and rat secretions on the test results. At the start of the experiment, rats were placed in the central platform area, allowing them to freely explore for 5 min. The rats' activity trajectories and behavioral parameters were recorded using the Smart v3.0 intelligent video tracking system (Panlab, Spain).

Open field test (OFT)

The apparatus consists of an open box with black interior walls. Rats were placed at the center of this arena and allowed to freely explore for a period of 5 min. The exploratory trajectory and behavioral parameters of the rats were documented using the Smart v3.0 intelligent video tracking system.

Forced swim test (FST)

The experimental rats were placed in a transparent cylindrical tank (30 cm in diameter $\times$ 60 cm in height) filled with water (22–25 °C) to a depth of 30 cm. Swimming behaviors were continuously recorded for 5 min using a video tracking system, with particular focus on measuring immobility time. Prolonged immobility time was considered as an indicator of behavioral despair.

Tail suspension test (TST)

Rats were individually suspended upside down at the center of the apparatus for a 6-min session, with the total immobility time during the last 4 min being recorded.

Morris water maze (MWM) test

The device refers to the inner blackened cylinder with a diameter of 180 cm and a height of 60 cm. The cylinder is artificially divided into four quadrants, and the platform is placed in one of the four quadrants, which is 2–4 cm below the water. The experiment was divided into two parts: training trials and probe trials. The training trails refer to putting the rats into the water from four different quadrants facing the cylinder sequentially (water temperature 23 ± 2 °C), and the video camera records the swimming of the rats in the water within 90 s. If the rat does not find the platform within 90 s, it must be guided to the platform. After 5 consecutive days of training, on the 6th day, the rats were put into the water from a certain quadrant, and the time on the platform was recorded. During the probe trials, the platform was withdrawn, and the movement of rats was observed within 90 s.

Novel object recognition (NOR)

Rats were placed in a familiar environment containing two new objects and allowed to explore these objects for 5 min. After 24 h, during the testing phase, one of the two familiar objects was replaced with a third new object, and the rats were returned to this environment to explore for 5 min. The rats' activity trajectories and behavioral parameters were recorded using the Smart v3.0 intelligent video tracking system.

Finally, a recognition index was calculated as follows: (time spent on the novel object - time spent on the familiar object) / total time spent exploring both objects.

2.4 RNA extraction, reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR) analysis

RNA was extracted from the hippocampal tissues using TRIzol reagents (Invitrogen Co., Carlsbad, CA, USA). The Reverse Transcription Kit (Takara Biotechnology, Japan) was used to prepare cDNA from total RNA. All primers were synthesized by Sangon Biotechnology Co. (China). The results were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized with *glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* gene. The corresponding primers are provided in Table S1.

2.5 Perifosine administration

In vitro experiments: Perifosine (MedChemExpress, HY-50909, USA) was dissolved in dimethyl sulfoxide (DMSO) to prepare a 10 mM stock solution (stored at -20 °C protected from light). The stock solution was subsequently diluted in culture medium to a working concentration of 20 μ M and applied to cells for 24-h treatment. Vehicle control groups were administered equivalent volumes of DMSO (final concentration $\leq 0.1\%$).

In vivo experiments: Rats received daily oral gavage of Perifosine (30 mg/kg/day) for 2 consecutive weeks. Behavioral testing commenced on day 7 of treatment, and hippocampal tissues were collected under anesthesia 6 h after the final dose.

2.6 Immunofluorescence staining

The brain tissue of rats was collected, fixed with 4% paraformaldehyde, immersed in sucrose solutions (20%, 25%, and 30%, sequentially), and preserved at 4 °C. The brain tissue was then embedded in the Tissue Tek O.C.T. compound (Sakura Finetek, USA), and continuous coronal slices (10 μ m) were prepared for immunofluorescence. After antigen retrieval and blocking using 10% sheep serum, incubate with the following primary antibodies overnight at 4 °C: Rabbit anti-Mini-Chromosome Maintenance Complex Component 2 (MCM2) antibody (diluted 1:200, ab95361, abcam, USA), Mouse anti- Doublecortin (DCX) antibody (diluted 1:200, ab254133, abcam, USA), Rabbit anti-c-Fos antibody (diluted 1:200, GB11069-100, Servicebio, China), washing with PBS and incubation in FITC-conjugated (AS001, ABclonal, China) or Cy3-conjugated secondary antibody (AS007, ABclonal, China) (diluted 1:200) for 4 h at room temperature in the next day, DAPI (B0011, Baiqiandu Inc, China) was added and incubated at room temperature in the dark for 10 min. The images were taken using a fluorescence microscope (Olympus, Japan).

The NE-4C cells were washed with cold PBS and fixed with paraformaldehyde. Then, the cells were blocked in 10% sheep serum and incubated with the following primary antibodies overnight at 4 °C: Rabbit anti-Kiel 67 (Ki67) antibody (diluted 1:200, a23722, Abclonal, China) and Mouse anti-Nestin antibody (diluted 1:200, ab254048, abcam, USA). After rinsing, the cells were incubated with Cy3-labeled goat anti-

rabbit IgG (1:200, Thermo Fisher Scientific, USA) and Alexa Fluor 488-labeled goat anti-mouse IgG (1:200, Thermo Fisher Scientific, USA), followed by subsequent DIPA staining. The images were taken using a fluorescence microscope and analyzed by the Image-Pro Plus software.

2.7 Nissl Staining

The process for Nissl staining began with the fixation of brain tissues. These tissues were treated with 4% paraformaldehyde and kept at 4 °C for an extended period of 18 h. After fixation, the tissues were embedded in an optimum cutting temperature compound and then frozen at - 80 °C. For sectioning, we employed a sliding microtome (CM1950, Leica Microsystems GmbH, Wetzlar, Germany), carefully cutting the tissues into 35 µm thick sections. These sections were then washed in PBS with a pH of 7.4 to remove any residual compounds. Following the washing step, the tissues were dried at a temperature of 55 °C for 3 h, ensuring they were adequately prepared for staining. The staining was conducted using 0.9% crystal violet, maintained at a temperature of 37 °C, for a duration of 2 h. This step was crucial for achieving the desired staining intensity. After staining, the tissues underwent a sequential dehydration process using ethanol solutions at increasing concentrations of 70%, 80%, 90%, and finally 100%, each for 5 min. This dehydration helped in preserving the stained tissues. Once dehydrated, the tissues were mounted with neutral balsam to prepare them for microscopic examination. The mounted sections were then observed under a fluorescent microscope (Nikon Corporation, Tokyo, Japan) to visualize the Nissl bodies. For quantitative analysis, densitometric analysis and the determination of the mean analysis area of Nissl body positive cells were conducted using ImageJ software, providing crucial data on the staining results.

2.8 Golgi staining

To examine neuronal morphology in the hippocampus, we employed the Golgi staining technique, utilizing a rapid Golgi staining kit (FD Neurotechnologies, Ellicott City, MD, USA) following the manufacturer's instructions. Our analysis focused on quantifying the spines along the initial segment of 15-30 µm of the primary dendrite emanating from the large apical dendrites, selecting only neurons with well-defined dendrites, secondary structures, and soma. These neurons were randomly chosen from specific fields for analysis. We normalized the total spine count to reflect the number per 10 µm of the dendrite span for relative spine density measurement. Additionally, we conducted Sholl analysis to assess the length of the primary dendrite and its secondary branching. For each section, five neurons were captured using Nikon act-1 software, resulting in three neurons per animal section for analysis. This analysis was carried out in ImageJ by an observer blinded to the experiment specifics. We calculated the total length of dendritic and branching intersections as the sum of Sholl outputs, extending up to 150 µm from the soma. Moreover, we also counted the total number of primary branches in the same hippocampal area for further comparative studies and data interpretation.

2.9 Transmission electron microscopy (TEM) analyses

The hippocampal tissue was cut into 1 mm³ and successively fixed in 2.5% glutaraldehyde and 1% osmium acid. The samples were then rinsed using 0.1 M phosphoric acid and dehydrated step by step using ethanol before being embedded and sliced. The ultrastructural changes of neurons were observed under the transmission electron microscope (Hitachi, Japan) after the sections were counterstained by 3% uranium acetate-citric acid. Image analysis was performed using Image-Pro Plus software (version 6.0, Media Cybernetics Inc, MD).

2.10 RNA-Seq and bioinformatics analysis

Hippocampal sequencing of rat, after the total RNA of the sample was extracted, the rRNA was removed by the ribosomal kit, and the RNA was fragmented (the average length of the fragment was about 200 nt). Reverse transcription was used to synthesize the single-stranded cDNA, and then double-stranded cDNA was synthesized and purified, followed by terminal repair and splice primers, PCR amplification, and purification, and then the library was qualitatively tested and finally sequenced. The Linux software used for upstream bioinformatics analysis included Trimmomatic, Bowtie2, Hisat2, Samtools, Gfold, etc. R packages used for downstream bioinformatics analysis included Clusterprofiler, Pheatmap, Limma, GEOquery, ggplot2, etc. In addition, download GSE199661, GSE155145, GSE234834, GSE53028 from GEO Datasets for supplement bioinformatics analysis.

2.11 Bisulfite sequencing PCR (BSP)

BSP analysis was performed by G&C Biotechnology (China). Genomic DNA was extracted using commercial kits, subjected to bisulfite treatment, and amplified via PCR with target-specific primers. PCR products were sequenced to determine site-specific methylation status.

2.12 Statistical analysis

Statistical analysis was performed using Graphpad Prism 10 (Graphpad Software, La Jolla, CA, USA). All measurements provided were evaluated using the unpaired t-test, one-way ANOVA or two-way ANOVA and presented as mean $\pm$ SEM. The statistical significance was set at $P < 0.05$.

3 Results

3.1 PME induces emotional and cognitive behavioral changes in offspring

We first conducted a series of behavioral tests on PW12 PME offspring to assess alterations in their cognitive and emotional behaviors. The OFT results showed that, compared with the control group, PME male offspring exhibited no significant changes in total movement distance, indicating that their motor ability remained intact. However, the number of total grid crossings, number of entries into the central zone, and time spent in the central zone were all significantly reduced (Fig. 1B), suggesting decreased exploratory behavior and increased anxiety levels. In the EPM test, compared with controls, PME male offspring showed significantly reduced total distance moved, open arm entries, and time spent in the open arms, while the time

spent in the central zone was not significantly altered (Fig. 1C), indicating increased anxiety levels, behavioral inhibition, and reduced exploration. Both the TST and FST demonstrated significantly increased immobility time in PME male offspring (Fig. 1D, E), reflecting enhanced behavioral despair, reduced escape behavior, and increased depression-like behaviors. In the MWM test, PME male offspring exhibited significantly prolonged escape latency and reduced platform crossings, with no significant changes in time spent in the target quadrant compared with controls (Fig. 1F), indicating impaired spatial learning and memory function. The NOR test further showed a significant decrease in the recognition index in PME male offspring relative to controls (Fig. 1G). Collectively, these findings indicate that PME induces anxiety- and depression-like behavioral alterations as well as cognitive impairments in PW12 male offspring.

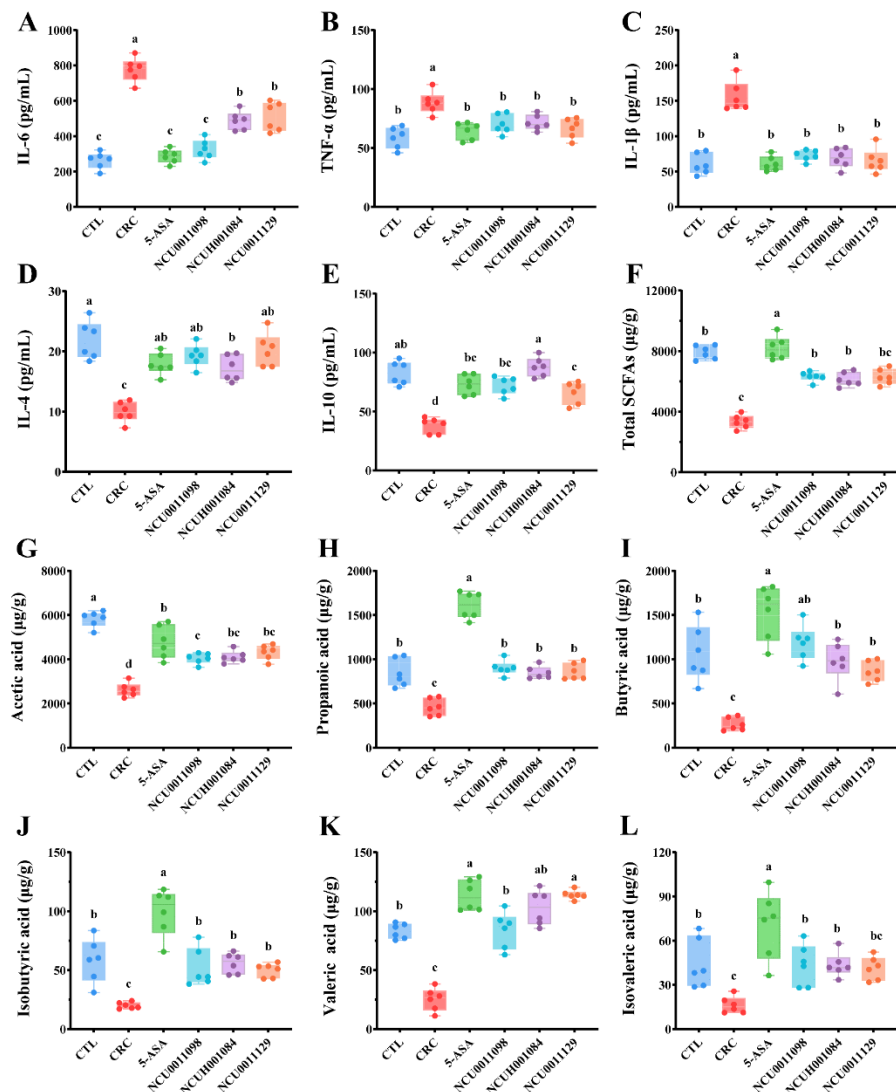


Fig. 1. PME male offspring rats exhibit anxiety- and depression-like behaviors as well as cognitive deficits. (A) Schematic diagram of animal experiments. (B) Schematic diagram of the OFT, representative trajectory plots, and statistical results (including total distance moved, number of total grid crossings, number of entries into the center zone, and time spent in the center zone) ($n = 8$ rats per group). (C) Schematic diagram of the EPM test, representative trajectory plots, and statistical results (including total distance moved, number of open arm entries, time spent in the central zone, and time spent in the open arms) ($n = 8$ rats per group). (D) Schematic diagram of the FST and statistical results of immobility time ($n = 8$ rats per group). (E) Schematic diagram of the TST and statistical results of immobility time ($n = 8$ rats per group). (F) Schematic diagram of the MWM test, representative trajectory plots, and statistical results (including escape latency, number of platform crossings, and time spent in the target quadrant) ($n = 8$ rats per group). (G) Schematic diagram of the NOR test, representative trajectory plots, and statistical results of the recognition index ($n = 8$ rats per group). Data are presented as mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$.

In PME female offspring, however, the behavioral profile was distinct and milder. In the OFT, total movement distance was unchanged, but the number of total grid crossings, entries into the center zone, and time spent in the center were all reduced (Fig. S1A), indicating diminished exploratory drive. In the EPM, total distance moved and time spent in the central zone were decreased, while entries into and time spent in the open arms were not significantly altered (Fig. S1B). Neither the FST nor the TST showed a significant increase in immobility time (Fig. S1C, D). No significant deficit was observed in the NOR test (Fig. S1E). In the MWM, PME female offspring exhibited prolonged escape latency, reduced platform crossings, and a significant decrease in time spent in the target quadrant (Fig. S1F), pointing to impaired spatial learning and memory. Therefore, the neurobehavioral impact of PME was less extensive in female offspring, leading us to focus subsequent mechanistic investigations on males.

3.2 PME offspring exhibit abnormal neurogenesis and synaptic structural damage in the hippocampus.

To investigate the programming mechanisms underlying neurobehavioral abnormalities in PME offspring, we first examined the fetal brain structure at GD20. PME fetuses displayed a significantly reduced brain weight compared with controls (Fig. 2A). Numerous studies have indicated that abnormal hippocampal neurogenesis leads to synaptic dysfunction, which is associated with anxiety- and depression-like behaviors as well as cognitive deficits^[18]. We therefore assessed hippocampal neuronal activity by c-Fos immunofluorescence and found that the number of c-Fos-positive neurons was markedly decreased in PME fetuses (Fig. 2B). Consistently, Nissl staining revealed a significant reduction in Nissl-positive neurons in the hippocampus of PME fetuses (Fig. 2C). Given that the intrauterine period is a high-frequency phase for neurogenesis and the dentate gyrus (DG) is the primary site of hippocampal neurogenesis^[19], we performed co-immunofluorescence staining for MCM2 (a marker of cell proliferation) and DCX (a marker of newborn neurons) to evaluate neurogenesis in PME offspring. The number of MCM2/DCX co-localized cells in the DG was significantly lower in PME fetuses than in controls (Fig. 2D), indicating impaired hippocampal neurogenesis *in utero*. Importantly, consistent alterations were observed in the hippocampus of PW12 PME offspring (Fig. 2B, E, F). Abnormal neurogenesis adversely affects subsequent neuronal function and morphology. Golgi staining demonstrated that PME offspring exhibited reduced dendritic branching, dendritic spine density, and branching points in hippocampal neurons (Fig. 2G, H), indicating dendritic structural degeneration and decreased synaptic connectivity. To further assess ultrastructural alterations, we performed TEM analysis of the DG region and found that PME offspring displayed reduced synaptic vesicle density, widened synaptic clefts, decreased postsynaptic density (PSD) thickness, and shortened active zone length (Fig. 2I, J). Together, these findings indicate that PME induces profound abnormalities in hippocampal neurogenesis during intrauterine development, which persist postnatally and are accompanied by progressive synaptic structural impairments.

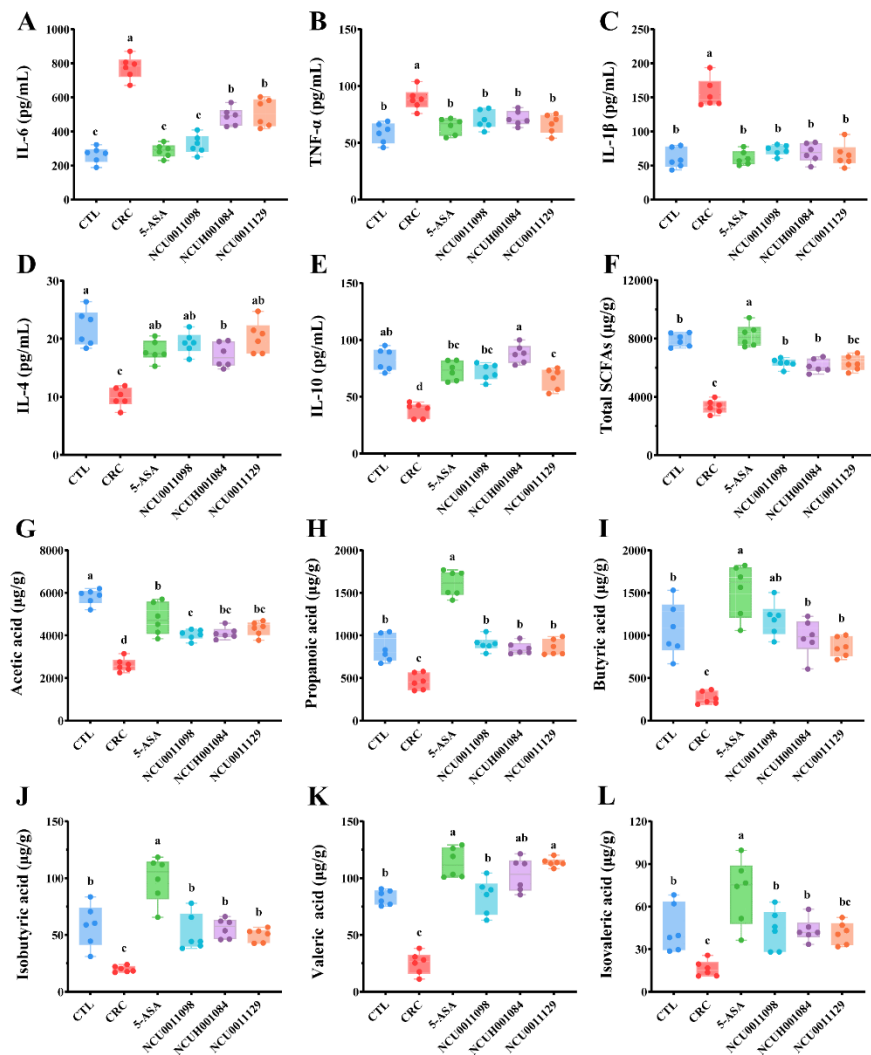


Fig. 2. Aberrant hippocampal neurogenesis and synaptic structural impairments in PME offspring. (A) Brain weight of GD20 fetuses (n = 15 rats per group). (B) c-Fos immunofluorescence staining and quantification of c-Fos-positive cells (n = 6 rats per group). (C, E) Nissl staining and quantification of Nissl-positive neurons (n = 6 rats per group). (D, F) Double immunofluorescence staining of Mcm2/DCX and quantification of colocalized cells (n = 6 rats per group). (G) Representative reconstruction of the hippocampal DG region neurons. (H) Quantification of dendritic branch number, spine density, and branch points (n = 3 rats per group). (I) Ultrastructure of neuronal synapses. (J) Quantification of synaptic vesicle numbers, synaptic cleft width, PSD thickness, and active zone length (n = 3 rats per group). Data are presented as mean ± SEM, *P < 0.05, **P < 0.01.

3.3 PI3K-Akt pathway suppression mediates hippocampal neurogenesis deficits in PME offspring

To elucidate the molecular mechanisms underlying impaired neurogenesis in PME offspring, we performed bioinformatic analyses of publicly available transcriptomic datasets (GEO database) derived from offspring brains exposed to exogenous substances such as alcohol and nicotine. In parallel, our laboratory had previously established a paternal preconception caffeine exposure (PPCE) model and conducted RNA sequencing of hippocampal tissue from PPCE offspring. Across alcohol, nicotine, and caffeine exposure models, the phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt) signaling pathway was consistently dysregulated (Fig. 3A-C). Given that this pathway is critically involved in neurogenesis^[20, 21], we next validated PI3K-Akt signaling in the hippocampus of PME offspring by qPCR, which confirmed a significant downregulation (Fig. 3D). To assess its functional role, we treated NE-4C neural stem cells with the Akt inhibitor Perifosine and evaluated proliferation *via* Ki67/Nestin double immunostaining. Perifosine markedly

reduced neural stem cell proliferation (Fig. 3E), suggesting that PI3K-Akt downregulation may contribute to impaired hippocampal neurogenesis in PME offspring.

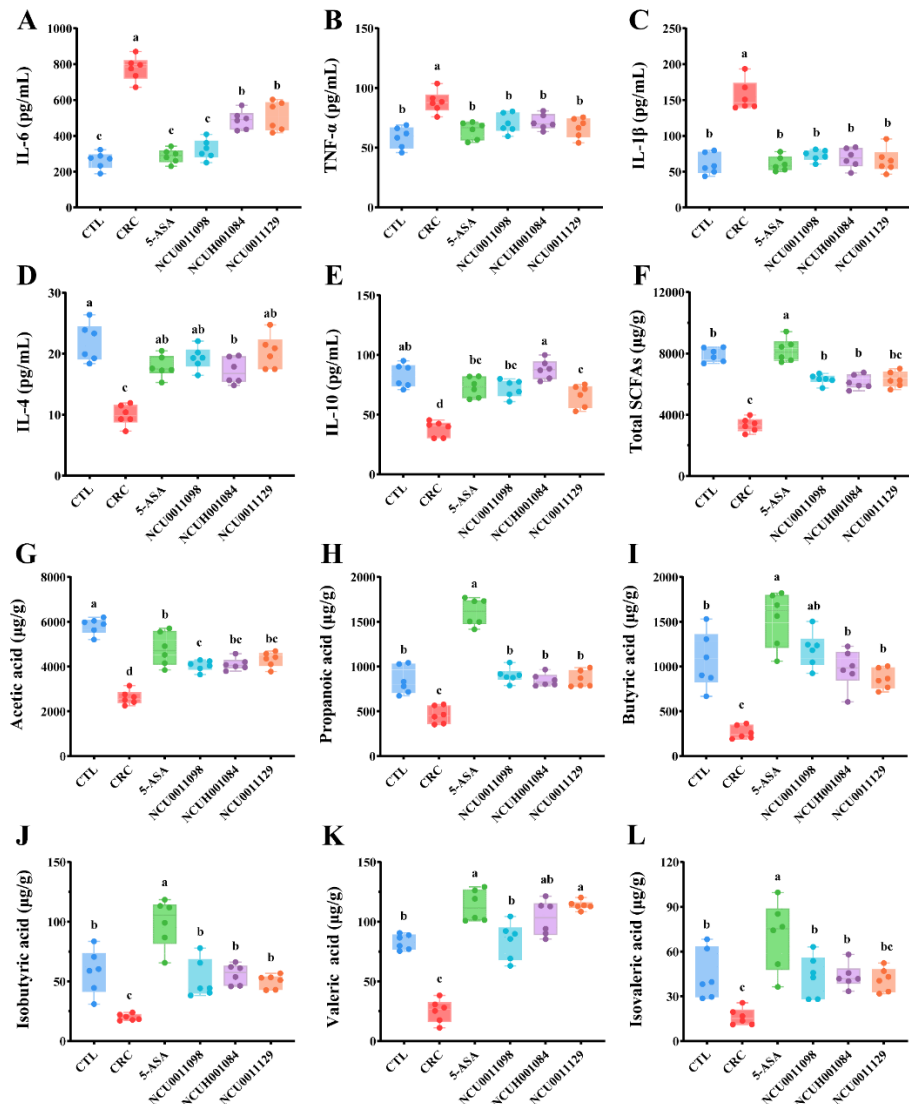


Fig. 3. Inhibition of the PI3K-Akt signaling pathway contributes to impaired hippocampal neurogenesis in PME offspring. (A) Bioinformatic analysis of transcriptomic data from offspring brains following paternal nicotine exposure, including principal component analysis (PCA), Gene ontology (GO) term enrichment, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. (B) Bioinformatic analysis of transcriptomic data from offspring brains following paternal alcohol exposure, including PCA, GO term enrichment, and KEGG pathway enrichment. (C) Bioinformatic analysis of hippocampal transcriptomic data from offspring following PPCE, including PCA, GO term enrichment, and KEGG pathway enrichment. (D) mRNA expression levels of key genes in the PI3K-Akt signaling pathway, including *Akt1*, *Akt2*, *Akt3*, *Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (Pik3ca)*, *Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta (Pik3cb)*, *Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma (Pik3cg)*, *Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (Pik3cd)* ($n = 6$ rats per group). (E) Ki67/Nestin double immunofluorescence staining and quantification of colocalized cells ($n = 10$). Data are presented as mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$.

We then administered Perifosine to normal Wistar rats (hereafter referred to as the Per group) and conducted a series of behavioral tests. In the OFT, compared with controls, Per rats showed no significant changes in total movement distance or number of entries into the central zone, but exhibited significantly fewer total grid crossings and reduced time spent in the central area (Fig. 4A). In the EPM test, Per rats displayed significantly decreased total distance traveled, open arm entries, and central time, while time spent in the open arms was unchanged (Fig. 4B). In the MWM test, escape latency and platform crossings were

unaffected, but time spent in the target quadrant was significantly reduced (Fig. 4C). Both the TST and FST revealed significantly increased immobility time in Per rats (Fig. 4D, E). The NOR test showed a significantly reduced recognition index in the Per group compared with controls (Fig. 4F). These results indicate that inhibition of PI3K-Akt signaling reproduces most of the neurobehavioral abnormalities observed in PME offspring. STRING network analysis revealed that Akt interacts with multiple downstream signaling cascades (Fig. S2A). qPCR validation in hippocampal tissue of PME offspring demonstrated dysregulation of several Akt downstream pathways to varying degrees (Fig. S2B-H). Together, these findings provide strong evidence that PI3K-Akt signaling mediates hippocampal neurogenesis deficits and consequent neurobehavioral abnormalities in PME offspring.

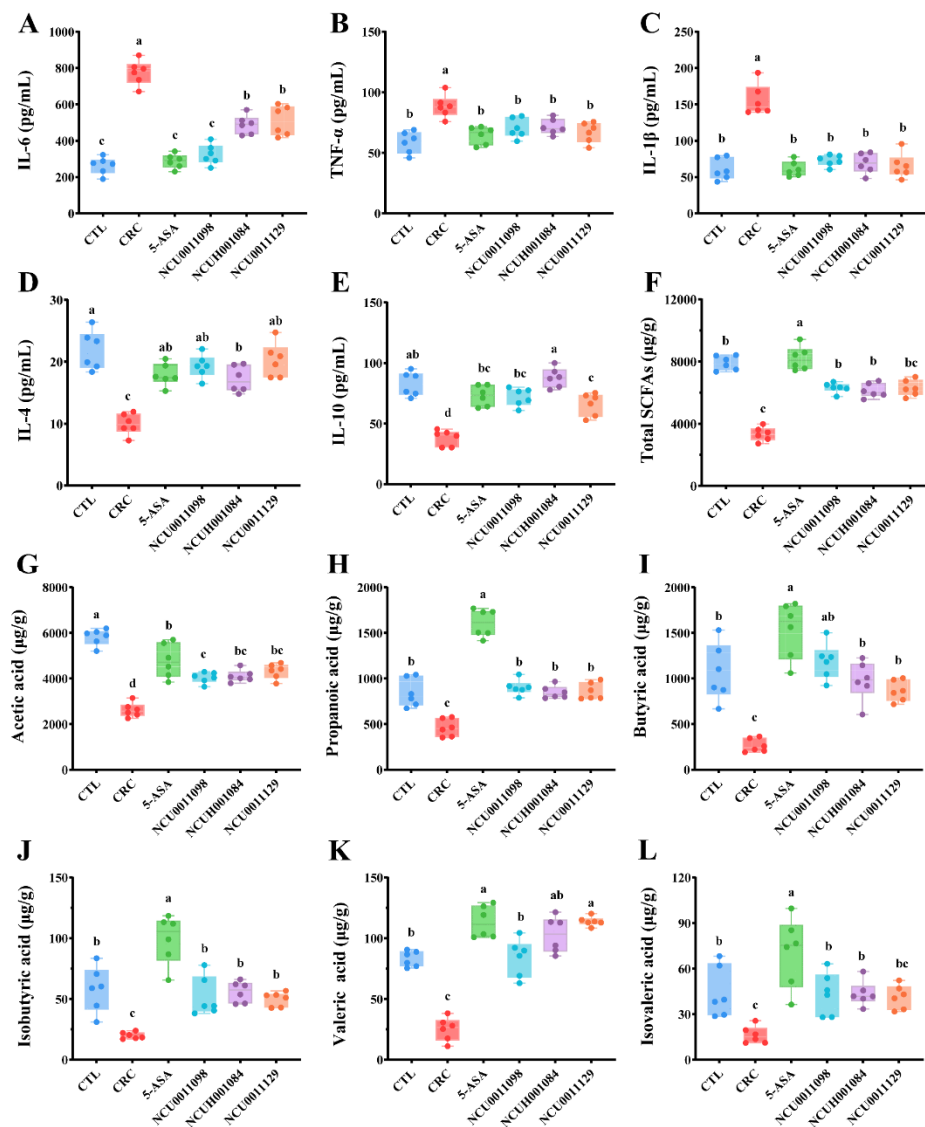


Fig. 4. Inhibition of the PI3K-Akt signaling pathway mediates neurobehavioral deficits in PME offspring. (A) Representative trajectory plots and statistical results of the OFT (including total distance moved, number of total grid crossings, number of entries into the center zone, and time spent in the center zone) ($n = 8$ rats per group). (B) Representative trajectory plots and statistical results of the EPM test (including total distance moved, number of open arm entries, time spent in the central zone, and time spent in the open arms) ($n = 8$ rats per group). (C) Representative trajectory plots and statistical results of the MWM test (including escape latency, number of platform crossings, and time spent in the target quadrant) ($n = 8$ rats per group). (D, E) Quantification of immobility time in the FST and TST ($n = 8$ rats per group). (F) Representative trajectory plots and recognition index in the NOR test ($n = 8$ rats per group). (G) STRING analysis showing downstream effectors of PI3K-Akt signaling. (H) mRNA expression levels of key genes in the mechanistic target of rapamycin (mTOR) signaling pathway, including *late endosomal/lysosomal adaptor MAPK* and *mTOR activator 1*

(*Lamtor1*), *Ras homolog enriched in brain (Rheb)*, *regulatory-associated protein of mTOR (Raptor)*, *Eukaryotic translation initiation factor 4E binding protein 1 (Eif4ebp1)*, and *Mtor* (n = 6 rats per group). (I) mRNA expression levels of key genes in the forkhead box O (FoxO) signaling pathway, including *Foxo1*, *Foxo4*, *Protein arginine methyltransferase 1 (Prmt1)*, and *cyclin-dependent kinase 2 (Cdk2)* (n = 6 rats per group). (J) mRNA expression levels of key genes in the glycogen synthase kinase-3 beta (*Gsk3b*) signaling pathway, including *Gsk3b* and *P21* (n = 6 rats per group). (K) mRNA expression levels of key genes in the BCL2-associated agonist of cell death (*Bad*)/caspase 9 (*Casp9*) signaling pathway, including *Bad* and *Casp9* (n = 6 rats per group). (L) mRNA expression levels of key genes in the extracellular signal-regulated kinase (ERK) signaling pathway, including *RAF proto-oncogene serine/threonine-protein kinase (Raf1)*, *mitogen-activated protein kinase 2 kinase 1 (Map2k1)*, *Erk* and *ETS like transcription factor-1 (Elk1)* (n = 6 rats per group). (M) mRNA expression levels of key genes in the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling pathway, including *inhibitor of nuclear factor kappa B kinase complex (Chuk)* and *Nfkb1* (n = 6 rats per group). (N) mRNA expression levels of key genes in the P53 signaling pathway, including *p53* and *mouse double-minute homolog 2 (Mdm2)* (n = 6 rats per group). Data are presented as mean $\pm$ SEM, * P < 0.05, ** P < 0.01.

3.4 Paternal sperm *Dlk1-Dio3* imprinted domain IG-DMR hypomethylation mediates offspring hippocampal PI3K-Akt signaling suppression.

Since paternal environmental exposures can only influence offspring phenotypes through sperm^[22], the alterations in PI3K-Akt signaling observed in PME offspring must originate from upstream factors at the sperm level. To address this, we accessed sperm sequencing data from paternal alcohol and nicotine exposure models via the GEO database. Notably, paternal alcohol exposure induced widespread transcriptional alterations within the *Dlk1-Dio3* imprinting domain (Fig. 5A). The *Dlk1-Dio3* domain represents the largest imprinted cluster in mammals, encompassing three paternally expressed protein-coding genes (*Dlk1*, *RTL1*, and *Dio3*) and several maternally expressed non-coding transcripts, including *Meg3*, *Anti-RTL1*, *Meg8*, and multiple miRNAs^[23]. Similarly, sperm methylation profiling following paternal nicotine exposure revealed increased methylation within the *Dlk1-Dio3* imprinted domain (Fig. 5B). We further analyzed RNA-seq data from hippocampal tissue of PPCE offspring using Linux-based bioinformatic pipelines to obtain genomic locus information. Genome browser inspection revealed clear expression changes of genes within the *Dlk1-Dio3* domain, such as *Dlk1*, *Anti-RTL*, and *Meg3* (Fig. 5C). Given that imprinted genes escape global DNA methylation reprogramming^[24], they represent strong candidates mediating PME-induced neurodevelopmental abnormalities. We next investigated whether the *Dlk1-Dio3* imprinting domain could target PI3K-Akt signaling pathway. STRING interaction analysis and TargetScan predictions revealed that the *Dlk1-Dio3* locus is associated with multiple signaling pathways, most prominently PI3K-Akt and Notch signaling cascades (Fig. 5D), both of which play critical roles in neural stem cell proliferation and differentiation. Consistently, in hippocampal tissue of PME offspring, expression of *Dlk1*, *Rtl1*, and *Dio3* was significantly decreased, while *Anti-RTL1*, *Meg3*, and *Meg8* were markedly upregulated compared with controls (Fig. 5E). Together, these findings suggest that altered expression of *Dlk1-Dio3* imprinted genes mediates suppression of PI3K-Akt signaling, thereby leading to reduced hippocampal neurogenesis and neurobehavioral abnormalities in PME offspring.

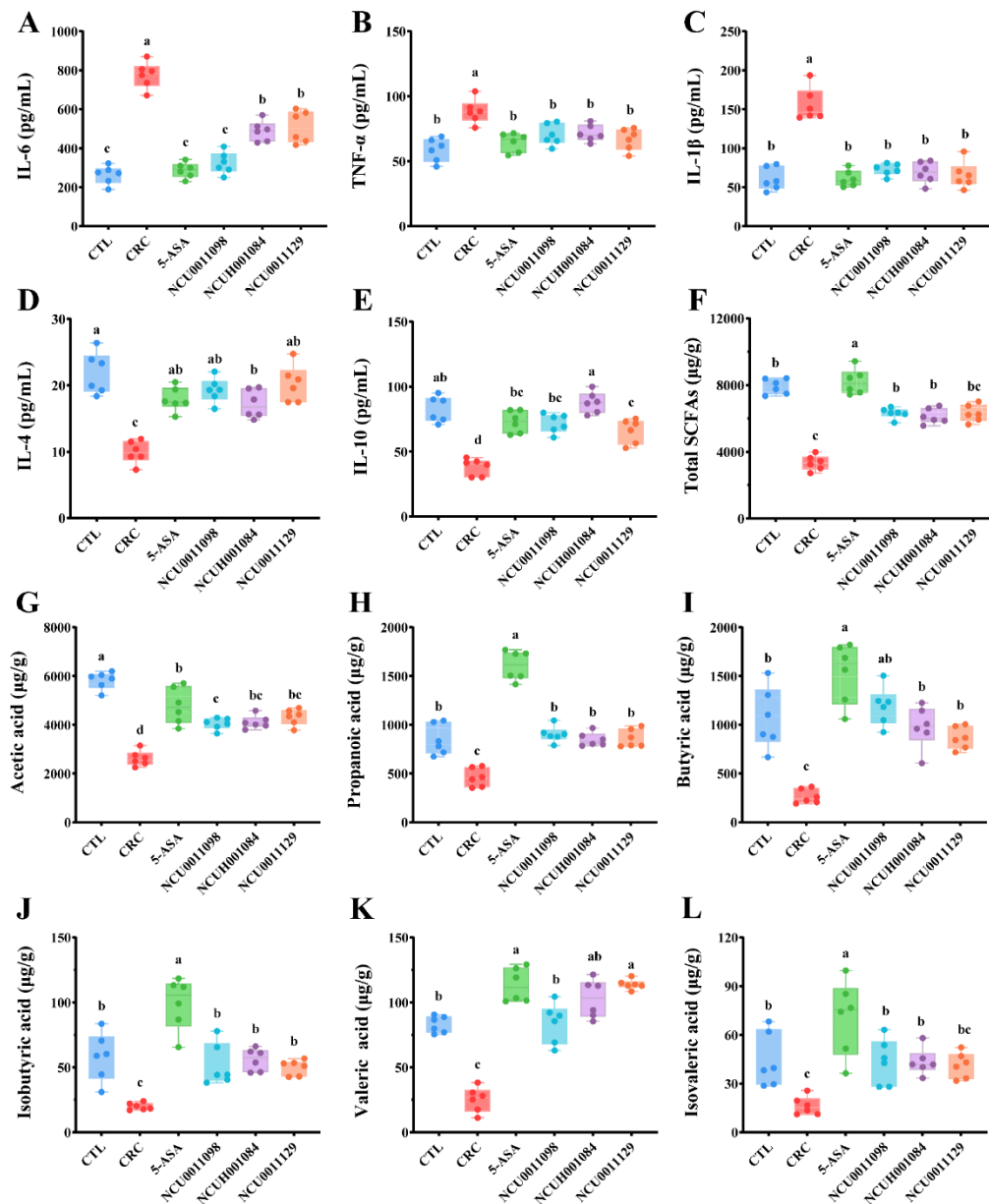


Fig. 5. PME induces hypomethylation of the paternal sperm Dlk1-Dio3 IG-DMR, thereby suppressing hippocampal PI3K-Akt signaling in offspring. (A) Transcriptomic analysis of the Dlk1-Dio3 imprinting domain in sperm from alcohol-exposed fathers. (B) DNA methylation profiling of the Dlk1-Dio3 imprinting domain in sperm from nicotine-exposed fathers. (C) Transcriptomic analysis of hippocampal tissue from PPCE offspring showing altered expression of genes within the Dlk1-Dio3 imprinting domain. (D) KEGG pathway enrichment analysis of genes within the Dlk1-Dio3 imprinting domain. (E) mRNA expression levels of *Dlk1*, *Rtl1*, *Dio3*, *Antitrl1*, *Meg3*, and *Meg8* in hippocampal tissue of PW12 PME offspring (n = 6 rats per group). (F) mRNA expression of ICR methylation maintenance-related transcriptional regulators (*Zfp57*, *Trim28*, *Ctcf*, *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Dnmt3l*) in hippocampal tissue of PW12 PME offspring (n = 6 rats per group). (G) mRNA expression of ICR methylation maintenance-related transcriptional regulators (*Zfp57*, *Trim28*, *Ctcf*, *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Dnmt3l*) in sperm from PME fathers (n = 8 rats per group). (H) BSP analysis of IG-DMR methylation in sperm from PME fathers (n = 3 rats per group). (I) BSP analysis of IG-DMR methylation in hippocampus of PME offspring rats (n = 3 rats per group). (J) Schematic representation of the knockout mouse generation strategy. (K) mRNA expression levels of key PI3K-Akt pathway genes, including *Akt1*, *Akt2*, *Akt3*, *Pik3ca*, *Pik3cb*, *Pik3cg*, and *Pik3cd* (n = 6 rats per group). Data are presented as mean ± SEM, **P* < 0.05, ***P* < 0.01.

Imprinted genes are typically clustered in the genome and regulated by specific ICRs. Given the broad alterations observed across the Dlk1-Dio3 imprinted domain, we hypothesized that dysregulation of its ICRs might underlie these changes. This domain contains three known ICRs: the IG-DMR, Meg3-DMR, and Meg8-DMR^[25, 26] (Fig. 5B), whose differential methylation states are primarily maintained by regulatory factors such as ZFP57, TRIM28, CTCF, and DNMTs. We therefore examined the expression of key epigenetic

regulators in the hippocampus of PME offspring and sperm from PME fathers. Our results showed significant downregulation of *Zfp57*, *Trim28*, and *Dnmt3l* in both PME offspring hippocampus and PME father sperm, while the expression of *Ctcf*, *Dnmt1*, *Dnmt3a*, and *Dnmt3b* remained unchanged in both tissues (Fig. 5F, G). This demonstrates that PME disrupts the integrity of the ZFP57-TRIM28-DNMT3L imprinting maintenance module not only in the offspring brain but, crucially, at its germline origin. Given that ZFP57-TRIM28-DNMTs complex is essential for maintaining differential methylation at the IG-DMR^[27], these findings implicate altered IG-DMR methylation may underlie the neurogenesis deficits in PME offspring. To directly test whether PME altered the methylation status of the IG-DMR, we performed BSP on sperm from exposed fathers and hippocampal tissue from their offspring. Consistent with our hypothesis, BSP analysis revealed a significant decrease in methylation levels at the IG-DMR in both PME father sperm and PME offspring hippocampus compared to their respective controls (Fig. 5H, I). To further establish the functional causality, we generated a mouse model with targeted deletion of the paternal IG-DMR high-CpG island region (Pat Δ IG) (Fig. 5J). Compared with wild-type littermates, Pat Δ IG fetuses exhibited markedly reduced hippocampal PI3K-Akt signaling (Fig. 5K). Together, these findings indicate that PME induces loss of imprinting at the IG-DMR of the paternal sperm *Dlk1-Dio3* domain, leading to dysregulated gene expression within this cluster, suppression of hippocampal PI3K-Akt signaling, and consequent impairments in neurogenesis and neurobehavioral outcomes in offspring.

4. Discussion

This study aimed to investigate the impact of PME on offspring neurodevelopment and its underlying epigenetic mechanisms. We demonstrate that PME induces anxiety- and depression-like behaviors as well as cognitive deficits in offspring rats. Mechanistically, PME disrupts the stability of the *Dlk1-Dio3* imprinting domain in paternal sperm, an alteration that escapes post-fertilization epigenetic reprogramming and is transmitted to the offspring hippocampus. This disruption leads to an imbalance in parent-of-origin-specific gene expression within the domain (downregulation of paternally expressed genes and upregulation of maternally expressed non-coding RNAs), which in turn suppresses the PI3K-Akt signaling pathway, thereby impairing hippocampal neurogenesis and synaptic function. To our knowledge, this work provides the first systematic evidence that PME induces intergenerational neurobehavioral abnormalities through dysregulation of imprinting gene networks.

The most important finding of this study is the first direct linkage of paternal mixed exposure to three highly coexisting lifestyle substances—nicotine, ethanol, and caffeine—with a defined epigenetic hub, the *Dlk1-Dio3* imprinting domain. Unlike previous studies focusing on single exposures, our mixed-exposure model more faithfully mimics real-world human scenarios, and the observed synergistic effects bear greater relevance to public health. Mechanistically, this study achieves notable depth on two fronts: First, we systematically identified a critical downstream signaling node, demonstrating that suppression of the PI3K-Akt pathway is central to impaired hippocampal neurogenesis and behavioral abnormalities in offspring, and we further established its causal role through inhibitor experiments at both cellular and animal levels. Second,

and more innovatively, we traced the origin of PME effects to a disruption of ZFP57-mediated imprint maintenance. Second, and more innovatively, we traced the origin of PME effects to a germline disruption of the imprinting maintenance module. We demonstrate that PME induces coordinate downregulation of *Zfp57*, *Trim28*, and *Dnmt3l* in paternal sperm, which constitutes the initial lesion. As a key “epigenetic gatekeeper”, ZFP57 binds methylated IG-DMR and recruits the TRIM28-DNMTs complex to safeguard parental imprinting memory during early embryonic reprogramming^[28]. Our findings indicate that PME compromises this protective complex at its source. This germline deficiency, transmitted to the offspring, manifests as persistent downregulation of the same module in the hippocampus, resulting in structural disorganization of the *Dlk1-Dio3* domain. Interestingly, while previous studies reported that nicotine alone induces hypermethylation at the IG-DMR, our PME model produced hypomethylation. This apparent divergence likely reflects the complex interactive effects inherent to mixture exposures: ethanol is known to interfere with one-carbon metabolism and promote hypomethylation^[29], which may counteract or override nicotine’s hypermethylation tendency when combined. This observation underscores that the epigenetic outcomes of mixed exposures cannot be simply extrapolated from single-substance studies, highlighting the importance of directly investigating real-world mixture scenarios. Together, these results provide a novel molecular perspective and compelling experimental evidence addressing the fundamental question of how paternal environmental exposures can breach the barrier of epigenetic reprogramming.

Nevertheless, several limitations should be acknowledged. First, although our exposure paradigm was calibrated based on human-relevant doses, inherent species differences in metabolic pathways and exposure duration between rats and humans warrant caution in extrapolating these findings. Second, and of particular relevance to the interpretation of our model, our experimental design employed a mixed-exposure paradigm to mirror common real-life scenarios but did not include single-substance exposure groups. Therefore, we cannot distinguish whether the observed intergenerational neurobehavioral and epigenetic effects are driven predominantly by one component (nicotine, ethanol, or caffeine) or result from synergistic interactions among them. Third, although we observed coordinated downregulation of the ZFP57-TRIM28-DNMT3L module in paternal sperm and linked it to epigenetic and behavioral deficits in offspring, the precise molecular trigger by which PME induces this downregulation remains unclear. Fourth, while we report distinct behavioral phenotypes in female offspring, the current study lacks mechanistic exploration (e.g., imprinting regulation, PI3K-Akt signaling) in females, which limits our understanding of the sex-specific neurodevelopmental impacts of paternal exposure. In addition, the use of mouse NE-4C cells for *in vitro* validation, while supportive, involves species differences and immortalized cell line limitations that should be considered when interpreting the findings. Furthermore, the mechanistic validation using a mouse IG-DMR knockout model, while providing causal evidence for the role of this imprinting domain, involves cross-species considerations that should be taken into account when extrapolating these findings to the rat model. Future studies using rat-specific genetic models would further strengthen the direct applicability of these mechanistic conclusions.

Finally, although the behavioral phenotypes were robust, we cannot fully exclude contributions from other brain regions (such as the prefrontal cortex) or neural circuits.

To address these outstanding questions, we propose the following future directions. First, upstream mechanistic interrogation: utilizing *in vitro* spermatogonial stem cell culture systems, we will expose cells to individual and combined active metabolites of nicotine, ethanol, and caffeine throughout the spermatogenesis process. This will allow us to dynamically monitor the expression of the ZFP57-TRIM28-DNMT3L module and the establishment of IG-DMR methylation, thereby identifying the critical window and molecular mechanisms by which PME disrupts the imprinting maintenance machinery at its germline origin. Second, downstream causal validation: by generating hippocampus-specific ZFP57 conditional knockout mice, we aim to establish the causal chain of “ZFP57-imprinting domain-PI3K-Akt-neurogenesis” both at the molecular and behavioral levels. Third, investigation of sex-specific mechanisms: we will perform parallel molecular and epigenetic analyses (e.g., Dlk1-Dio3 imprinting status, PI3K-Akt pathway activity) in female offspring to elucidate whether the observed sexual dimorphism arises from shared or distinct regulatory pathways. Fourth, translational extension: a prospective paternal cohort study will be undertaken to examine the methylation status of the Dlk1-Dio3 IG-DMR in human sperm and correlate it with standardized offspring neurodevelopmental assessments, thereby advancing our findings toward early clinical prediction and prevention.

This study provides compelling support for the POHaD theory, underscoring that the health of men of reproductive age is not merely an individual concern but a determinant of the neuropsychiatric well-being of the next generation. From a public health perspective, our findings highlight the urgent need for comprehensive interventions discouraging the use of tobacco, alcohol, and high-caffeine beverages among men of reproductive age. Building on these results, we propose a novel hypothesis: paternal environmental insults may exert their intergenerational impact by “hijacking” imprinting maintenance machinery (e.g., the ZFP57-TRIM28 complex), thereby coordinately regulating multiple imprinting domains and producing multi-organ consequences in offspring. Future studies should move beyond the Dlk1–Dio3 locus to explore the role of additional imprinting domains in mediating PME effects, ultimately aiming to construct a global map of paternal epigenetic inheritance.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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