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论文题目: From Hippocampal Epigenomic Signatures to Behavioral Phenotypes: A Cross-Model Investigation of Caffeine Effects on Neurodevelopment

From Hippocampal Epigenomic Signatures to Behavioral Phenotypes: A Cross-Model Investigation of Caffeine Effects on Neurodevelopment

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Abstract

Caffeine is widely consumed by children and adolescents, not only from coffee and energy drinks but also through foods and beverages consumed in everyday life. Although its acute stimulant effects in adults are well established, much less is known about how caffeine exposure may affect the developing nervous system or the molecular pathways involved. Here, we examined caffeine-associated changes across molecular, cellular, and behavioral levels. Gene set enrichment analysis (GSEA) of published adult mouse hippocampal H3K27ac and H3K27me3 CUT&Tag data identified enrichment of pathways related to synaptic function, neurotrophic signaling, learning, and chromatin regulation. For example, neurotrophin signaling showed significant negative enrichment in the H3K27me3-ranked dataset (NES = -2.31, FDR $q = 0.0067$).

We next examined whether changes associated with caffeine exposure could be detected in developmental models. In primary neonatal rat neurons, caffeine treatment reduced neurite complexity, as assessed by MAP2 immunofluorescence and Sholl analysis. In zebrafish larvae, caffeine exposure produced a concentration-dependent increase in mortality and reduced expression of *sox2*, *nestin*, *bdnf*, and *gdnf*, genes involved in neurodevelopment and neurotrophic signaling. Caffeine-treated larvae also showed locomotor hyperactivity and impaired cognition-related sensory behavior. Together, these findings suggest that caffeine exposure during development may alter neurodevelopment-related gene expression and neuronal morphology, with measurable behavioral changes. Because the epigenomic analysis was derived from adult mouse hippocampus whereas the functional experiments used developmental models, further studies using developmentally matched mammalian models and physiologically relevant exposure paradigms are needed to determine the mechanisms involved and the relevance of these findings to adolescent caffeine exposure.

Keywords: caffeine; neurodevelopment; CUT&Tag; GSEA; zebrafish

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1. Introduction

Caffeine is a widely consumed psychoactive stimulant, and exposure begins well before adulthood. Recent data from the U.S. Centers for Disease Control and Prevention (CDC), collected between August 2021 and August 2023, showed that 62.6% of U.S. youth aged 2–19 consumed caffeine on a given day [1]. Mean intake increased with age, 5.0 mg per day among children aged 2 to 5, 12.0 mg among those aged 6 to 11, and 30.7 mg among adolescents aged 12 to 19. The sources also varied by age. Among children aged 2–11, sweet bakery products accounted for 28.1% of total caffeine intake, followed by flavored milk (17.4%) and candy (16.3%). Among adolescents, the leading sources were soda (20.3%), sweet bakery products (19.1%), tea (14.6%), coffee (14.0%), and candy (10.7%) [1]. Thus, caffeine exposure in youth does not come only from coffee or energy drinks, but also through a variety of foods and beverages consumed in everyday life.

This widespread exposure raises a basic question about the developing nervous system. Guidelines for safe caffeine intake are largely based on adult data and, as noted by the CDC, have not been established through research including children [1]. The nervous system continues to develop throughout childhood and adolescence, but the effects of caffeine during these developmental stages are much less well defined than in adults [2,3]. In particular, how different levels of caffeine exposure affect neural development remains unclear.

The uncertainty is not limited to how much caffeine young people can safely consume. In addition, most of what is known about the influence of caffeine on the brain comes from studies in adults. One controlled human experiment found that ten days of daily caffeine intake reduced gray matter volume, with the largest reduction noted in the medial temporal lobe, including the hippocampus [4]. However, the developing nervous system is still undergoing synaptic pruning, myelination, and refinement of neural circuits [2,3]. Findings obtained from adults may not fully reflect what occurs earlier in life.

Some studies have examined caffeine exposure during development. Early-life caffeine exposure in mice altered cortical network development and hippocampal network activity [5], while adolescent caffeine exposure in rats increased basal *Crf* expression in the amygdala [6]. Changes in anxiety-related behavior and neuroendocrine signaling have also been detected later in adulthood after adolescent exposure [6]. Human studies have reported similar concerns. In children aged 9–10 from the ABCD study, caffeine intake was associated with poorer cognitive flexibility and other cognitive measures [7]. Although these studies suggest that caffeine can affect the developing nervous system, the mechanisms underlying these effects are still not fully understood.

To address this research gap, this study integrates bioinformatic analysis of public hippocampal epigenomic data with functional assays in primary neonatal rat neurons and zebrafish larvae, examining caffeine-associated alterations across molecular, cellular, and behavioral levels. We asked whether pathway-level signals associated with caffeine in the adult hippocampal dataset pointed to biological processes related to those observed in developmental models. The epigenomic analysis was used to identify broader caffeine-associated molecular and regulatory pathways. These findings were considered alongside neurodevelopment-related gene expression, neuronal morphology, and behavior to determine whether different lines of evidence pointed toward related effects on neural development. The overall study design, integrating public hippocampal epigenomic analysis with *in vitro* neuronal and *in vivo* zebrafish models, is summarized in Figure 1.

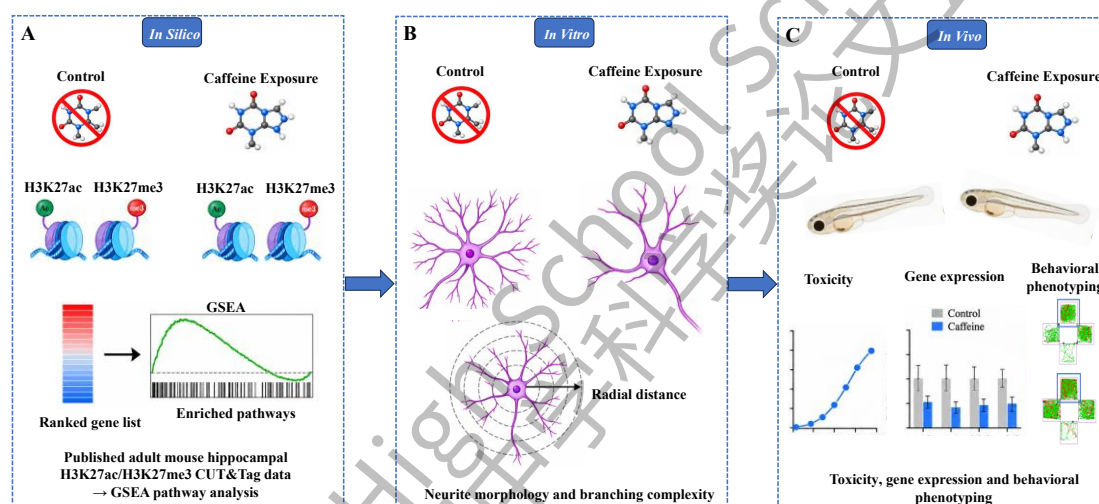


Figure 1. Schematic overview of the study design and conceptual framework. The study integrated (A) secondary analysis of public hippocampal H3K27ac/H3K27me3 CUT&Tag data, (B) primary neonatal rat neurons assessing caffeine-associated changes in neurite morphology, and (C) zebrafish experiments examining toxicity, neurodevelopment-related gene expression, and behavior.

2. Materials and methods

The experimental and analytical procedures used in the three components of this study are summarized in Figure 2.

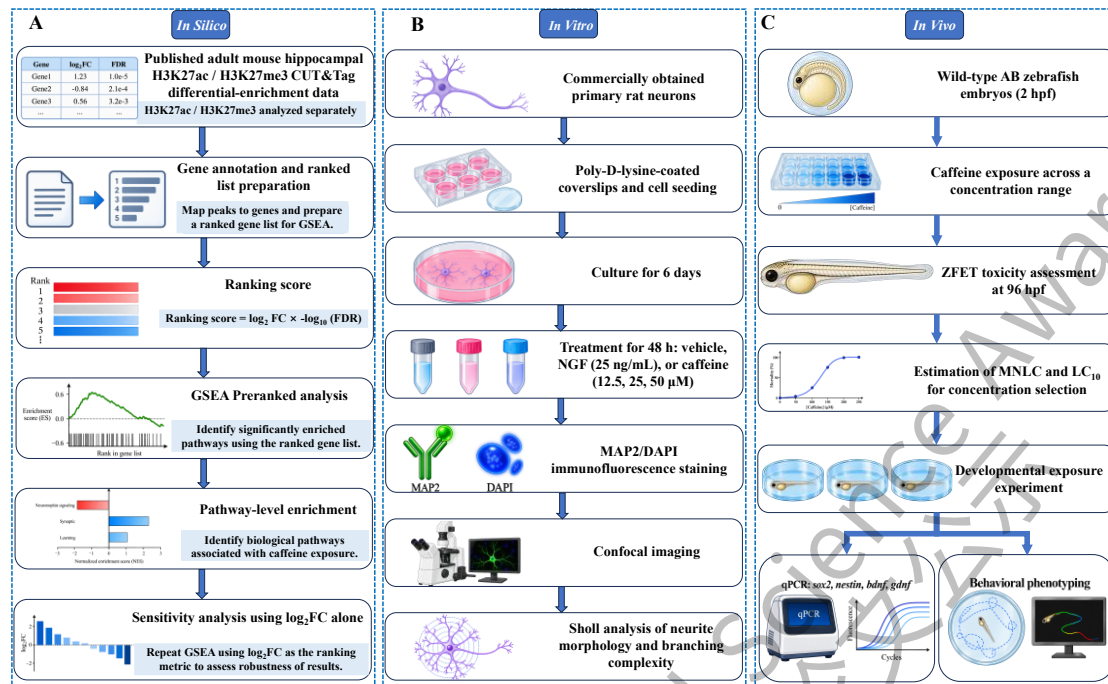


Figure 2. Experimental and analytical workflow of the study. (A) *In silico* analysis of published adult mouse hippocampal H3K27ac and H3K27me3 CUT&Tag differential-enrichment data, including gene annotation, ranked-list generation, GSEA Preranked analysis, pathway-level enrichment, and sensitivity analysis using an alternative ranking metric. (B) *In vitro* workflow for primary rat neuronal culture, caffeine treatment, MAP2/DAPI immunofluorescence staining, confocal imaging, and Sholl analysis of neurite morphology and branching complexity. (C) *In vivo* zebrafish workflow, including caffeine exposure, ZFET toxicity assessment and concentration selection, followed by developmental exposure, qPCR analysis of *sox2*, *nestin*, *bdnf*, and *gdnf*, and behavioral phenotyping.

2.1 Secondary analysis of public hippocampal epigenomic data

Bioinformatic analysis was performed using publicly available H3K27ac and H3K27me3 CUT&Tag differential-enrichment data from adult mouse hippocampus [8]. Published processed data, rather than raw sequencing reads, were used. The published processed source tables contained annotated differential-enrichment regions meeting an FDR threshold of less than 1×10^{-5} . For each region, a signed ranking score was calculated as $\log_2 FC \times [-\log_{10}(FDR)]$, with $\log_2 FC$ defined for caffeine versus control. Ranked gene lists were generated in R. When multiple regions mapped to the same gene, the region with the largest absolute ranking score was retained while preserving its sign, leaving one value per gene. The resulting H3K27ac and H3K27me3 .rnk files were analyzed separately using the GSEAPreranked module in GSEA 4.4.0 (Broad Institute) with the No_Collapse option [9]. Mouse MSigDB M5 ontology gene sets were used [10,11], including Gene Ontology Biological Process, Cellular Component, and Molecular Function gene sets [12], together with the Mammalian Phenotype Tumor Ontology

subcollection. Gene sets containing fewer than 5 or more than 500 genes were excluded. Enrichment was evaluated using enrichment score (ES), normalized enrichment score (NES), nominal P value, and FDR q value.

As a sensitivity analysis, ranked lists were regenerated using $\log_2FC$ alone. For genes associated with multiple regions, the region with the largest absolute $\log_2FC$ was retained while preserving its sign. The same gene-set database and GSEA settings were used for the repeated analysis.

2.2 Primary neonatal rat neuronal culture and caffeine treatment

A 50 $\mu\text{g/mL}$ poly-D-lysine solution was used to coat circular coverslips in 12-well plates. The plates were incubated at 37 °C for 6 h, then washed and air-dried. Commercially obtained primary neonatal rat neurons were thawed and seeded onto the coated coverslips. After seeding, the plates were left undisturbed for 5 min before being transferred to a humidified incubator at 37 °C with 5% CO_2 . Half of the culture medium was replaced every two days. After six days in culture, neurons were treated for 48 hours with control, NGF (25 ng/mL ; positive control), or caffeine at 12.5, 25, or 50 μM .

2.3 Immunofluorescence staining and neurite morphology analysis

After treatment, the coverslips were removed from the culture wells and stained for MAP2 (microtubule-associated protein 2), with DAPI used to label cell nuclei. Images were collected by confocal microscopy. Neurite morphology was analyzed in ImageJ using Sholl analysis [13]. Before analysis, images were processed by background subtraction, thresholding, and binarization. The center of the soma was set as the origin, and concentric circles were drawn at 10 μm intervals from the soma to the outermost neurites. Intersections between neurites and each circle were counted to evaluate neurite complexity.

2.4 Zebrafish husbandry and caffeine exposure

Wild-type AB zebrafish were maintained under standard aquaculture conditions [14] (28 °C, pH 6.5–8.5, conductivity 450–550 $\mu\text{S/cm}$, and hardness 50–100 mg/L as CaCO_3) at the Hunter Biotech aquaculture center (License No. SYXK (Zhe) 2022-0004). The facility was accredited by AAALAC (No. 001458), and all experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC; Review No. IACUC-2025-202511170013-01). Wild-type AB embryos at 2 hours post-fertilization (hpf) were randomly selected for caffeine exposure experiments.

2.5 Zebrafish embryo toxicity and concentration selection

For the zebrafish embryo acute toxicity assessment, embryos at 2 hpf were exposed to a range of caffeine concentrations in 6-well plates, with 30 embryos per well in 3 mL of test solution, together with a control group. Embryos were incubated at 28 °C and assessed for lethal endpoints at 96 hpf, following the general framework of the OECD Fish Embryo Acute Toxicity Test [15]. Mortality data were fitted to a concentration-response curve to estimate the maximum non-lethal concentration (MNLC) and the lethal concentration for 10% of the population (LC₁₀).

2.6 Quantitative real-time PCR (qPCR)

Total RNA was extracted from zebrafish larvae using the Universal RNA Extraction TL Kit C. RNA concentration and purity were measured with a UV–Vis spectrophotometer. For each sample, 2.00 µg of total RNA was reverse-transcribed in a 20.0 µL reaction. Expression of *sox2*, *nestin*, *bdnf*, and *gdnf*, genes involved in neurodevelopment and neurotrophic signaling, was quantified by qPCR, with β -actin used as the internal control. Three biological replicates were analyzed for each treatment group.

2.7 Zebrafish behavioral phenotyping

At 96 hpf, larval behavior was recorded using the ZebraLab tracking system (ViewPoint, France). Locomotor activity was measured as the total distance traveled during a 1 h recording period. In the color-zone preference assay, time spent in the blue zone was recorded over 10 min to assess color-zone preference. Movement tracks and heatmaps were also generated to show spatial activity patterns across treatment groups.

2.8 Statistical analysis

Statistical analysis was performed to rigorously evaluate experimental data and determine the significance of observed effects. Data are presented as mean $\pm$ standard error of the mean (SEM). Comparisons among multiple groups were performed using one-way or two-way ANOVA, followed by Tukey's or Dunnett's post hoc tests as appropriate. A *p* value < 0.05 was considered statistically significant.

3. Results

3.1 Public adult mouse hippocampal data revealed caffeine-associated epigenomic signatures

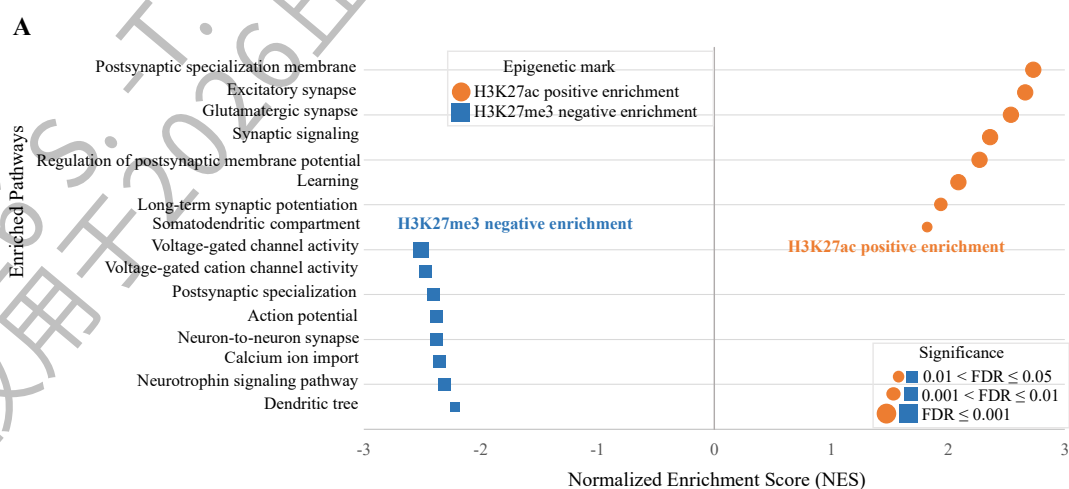
Published H3K27ac and H3K27me3 CUT&Tag differential-enrichment data from adult mouse

hippocampus [8] were analyzed using GSEAPreranked to look for biological pathways associated with caffeine exposure. Several neuronal and synaptic pathways emerged from the analysis (Figure 3A). In the H3K27ac-ranked dataset, positive enrichment was observed in pathways related to synaptic signaling, postsynaptic specialization, dendritic organization, and learning. In the H3K27me3-ranked dataset, several neuronal pathways showed negative enrichment. Neurotrophin signaling was one of the strongest examples, with an NES of -2.31 and an FDR q value of 0.0067 (Figure 3B).

A number of neuronal pathways were significant in both histone-mark analyses. These pathways had positive NES values in the H3K27ac-ranked dataset and negative NES values in the H3K27me3-ranked dataset. Five representative examples are shown in Figure 3C. The overlap included pathways related to synaptic function, neuronal structure, and neurotrophic signaling.

The initial pathway analysis also informed the developmental experiments. Synaptic and plasticity-related signals were one reason we examined neurite morphology in primary neonatal rat neurons. Neurotrophin-related findings also contributed to the decision to measure neurodevelopmental and neurotrophic gene expression in zebrafish larvae. After the wet-lab experiments were completed, the GSEA analysis was expanded to include a broader set of pathways.

We also tested whether the pathway results depended strongly on the ranking method. The primary ranking score, $\log_2FC \times [-\log_{10}(FDR)]$, incorporates both the direction and magnitude of the differential-enrichment signal and its FDR. The analysis was repeated using $\log_2FC$ alone. The main neuronal and synaptic pathways retained the same overall direction of enrichment, although NES values changed for some pathways. The results of this sensitivity analysis are shown in Supplementary Figure S1.



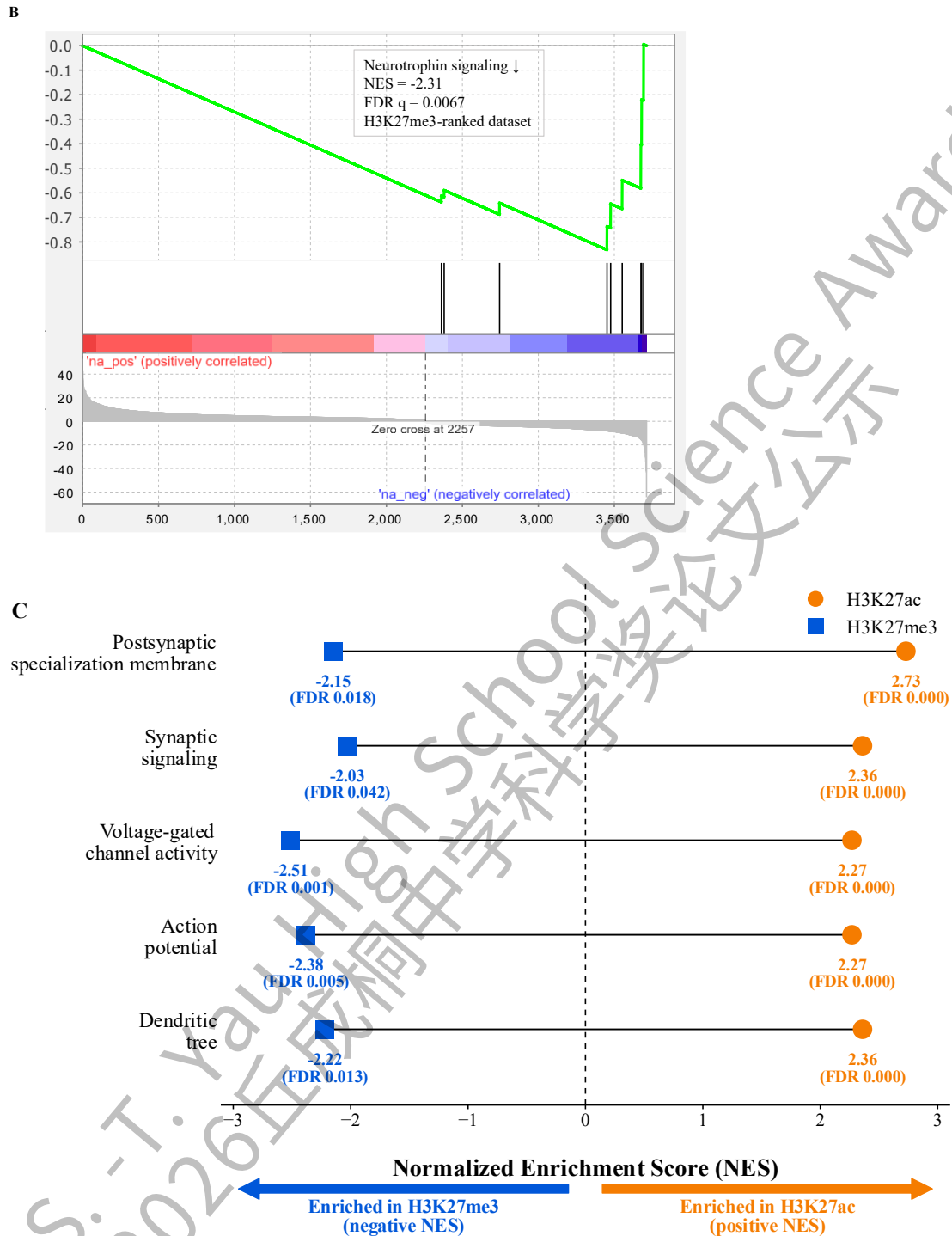


Figure 3. Caffeine-associated hippocampal H3K27ac and H3K27me3 signatures converge on neuronal and synaptic pathways. (A) GSEAPreranked analysis of published adult mouse hippocampal H3K27ac and H3K27me3 CUT&Tag differential-enrichment data. Representative neuronal pathways showing positive enrichment in the H3K27ac-ranked dataset and negative enrichment in the H3K27me3-ranked dataset are displayed by normalized enrichment score (NES). Orange circles indicate H3K27ac-associated enrichment and blue

squares indicate H3K27me3-associated enrichment; symbol size denotes FDR q-value category. (B) GSEA enrichment plot for the GO Biological Process *neurotrophin signaling pathway* in the H3K27me3-ranked dataset, showing significant negative enrichment (NES = -2.31, FDR q = 0.0067). Vertical tick marks indicate the positions of pathway genes within the ranked gene list, and the green curve represents the running enrichment score. (C) Comparison of five representative neuronal pathways significantly enriched in both mark-specific analyses, with positive NES values in the H3K27ac-ranked dataset and negative NES values in the H3K27me3-ranked dataset. Values shown beside each pathway indicate the corresponding NES and FDR q value for each histone mark.

3.2 Caffeine impaired neurite outgrowth in primary rat neurons

Primary neonatal rat neurons were cultured for six days and then exposed for 48 h to control, NGF (25 ng/mL; positive control), or caffeine at 12.5, 25, or 50 μ M. MAP2/DAPI imaging showed less extensive neuritic arborization in caffeine-treated cultures, particularly at the higher caffeine concentrations (Figure 4A).

Sholl analysis confirmed a reduction in neurite complexity following caffeine exposure (Figure 4B). Compared with control and NGF-treated neurons, caffeine-treated cells generally showed fewer neurite intersections over a broad range of distances from the soma. Significant differences were detected at multiple radial distances, with the clearest reductions occurring in the 25 and 50 μ M groups. The 12.5 μ M group showed a smaller effect. Group differences across radial distance were analyzed by two-way ANOVA followed by Dunnett's post hoc test (n = 3 independent experimental replicates per condition).

Together, the imaging and Sholl analysis showed that caffeine exposure was associated with reduced neurite complexity in primary neonatal rat neurons, with larger changes at the higher concentrations tested.

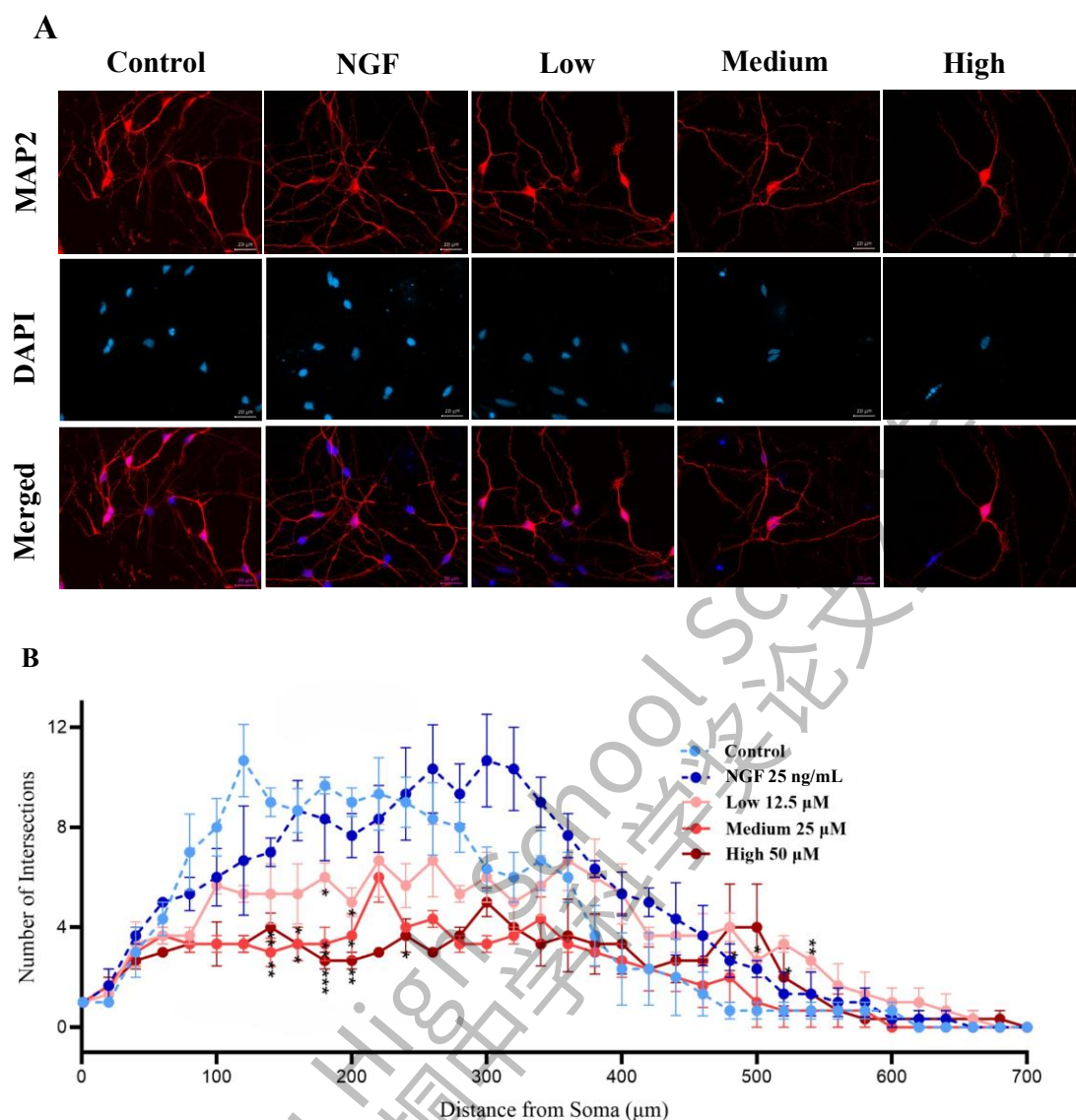


Figure 4. (A) Representative MAP2 immunofluorescence images of primary neonatal rat neurons from control, NGF, and caffeine-treated groups, illustrating reduced neurite outgrowth following caffeine exposure. (B) Sholl analysis of MAP2-stained primary neonatal neurons demonstrating dose-dependent reductions in neurite complexity following caffeine treatment. Two-way ANOVA followed by Dunnett's post hoc Test, $n = 3$ independent experimental replicates per condition; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control group.

3.3 A sublethal caffeine exposure range was established in zebrafish larvae

Before the zebrafish gene-expression and behavioral experiments, we first established an exposure range that avoided overt lethality. Wild-type AB embryos were exposed to caffeine from 2 to 96 hpf across a concentration series of 62.5–1000 μM , with 30 embryos in each condition. Mortality increased with caffeine concentration. No mortality was observed at 62.5 or 125 μM , while mortality reached 10% at 250 μM , 70% at 500 μM , and 100% at 1000 μM .

No gross phenotypic abnormalities were observed in the control group, and embryos exposed to 62.5 or 125 μM appeared similar to controls (Table 1).

The concentration–mortality data showed a close fit to the fitted curve ($R^2 = 0.999$). The maximum non-lethal concentration (MNLC) was estimated at 128 μM , and the LC_{10} was 252 μM (Figure 5B). Based on the MNLC, three sublethal concentrations were selected for the downstream experiments: 14.2 μM (1/9 MNLC), 42.7 μM (1/3 MNLC), and 128 μM (MNLC), designated as Low, Medium, and High, respectively. These concentrations were then used for the gene-expression and behavioral assays.

Table 1. Results of the exploratory zebrafish embryo toxicity experiment used to estimate MNLC and LC_{10} ($n = 30$ per condition).

Group	Concentration (μM)	Number of deaths	Mortality Rate (%)	Phenotype
Normal	-	0	0	No obvious abnormalities observed
Control	-	0	0	No obvious abnormalities observed
Caffeine	62.5	0	0	No obvious abnormalities observed
	125	0	0	No obvious abnormalities observed
	250	3	10	-
	500	21	70	-
	1000	30	100	-

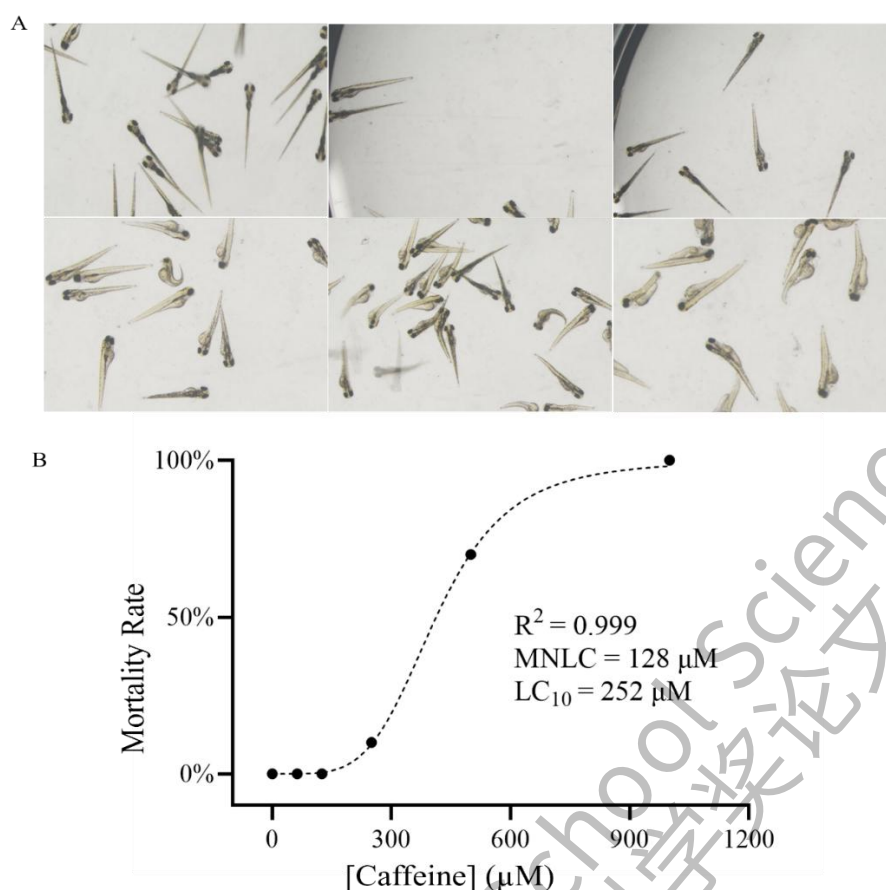


Figure 5. (A) Zebrafish embryo mortality across caffeine concentrations, shown from left to right: (i) control, (ii) 62.5 μM , (iii) 125 μM , (iv) 250 μM , (v) 500 μM , and (vi) 1000 μM . (B) Concentration–mortality curve from the zebrafish embryo acute toxicity test (ZFET) showing caffeine-induced lethality and regression-based estimation of MNLC (maximum non-lethal concentration) and LC_{10} (10% lethal concentration).

3.4 Caffeine exposure altered the expression of genes involved in neurodevelopment and neurotrophic signaling in zebrafish larvae

qPCR was used to examine the expression of *sox2*, *nestin*, *bdnf*, and *gdnf* in zebrafish larvae after caffeine exposure. Expression of all four genes was significantly lower than in controls at each caffeine concentration tested ($p < 0.0001$; Figure 6). Mean expression also decreased progressively from the Low to Medium and High groups, with the lowest levels observed at the highest caffeine concentration.

The four genes represent related but distinct aspects of neural development. *sox2* and *nestin* are associated with neural progenitor and developmental processes, whereas *bdnf* and *gdnf* are involved in neurotrophic signaling. The reduction in *bdnf* and *gdnf* was consistent with the neurotrophin-related pathway signal identified in the adult mouse hippocampal GSEA analysis.

Taken together, the qPCR results showed a clear concentration-related reduction in genes associated with neurodevelopment and neurotrophic support in caffeine-exposed zebrafish larvae.

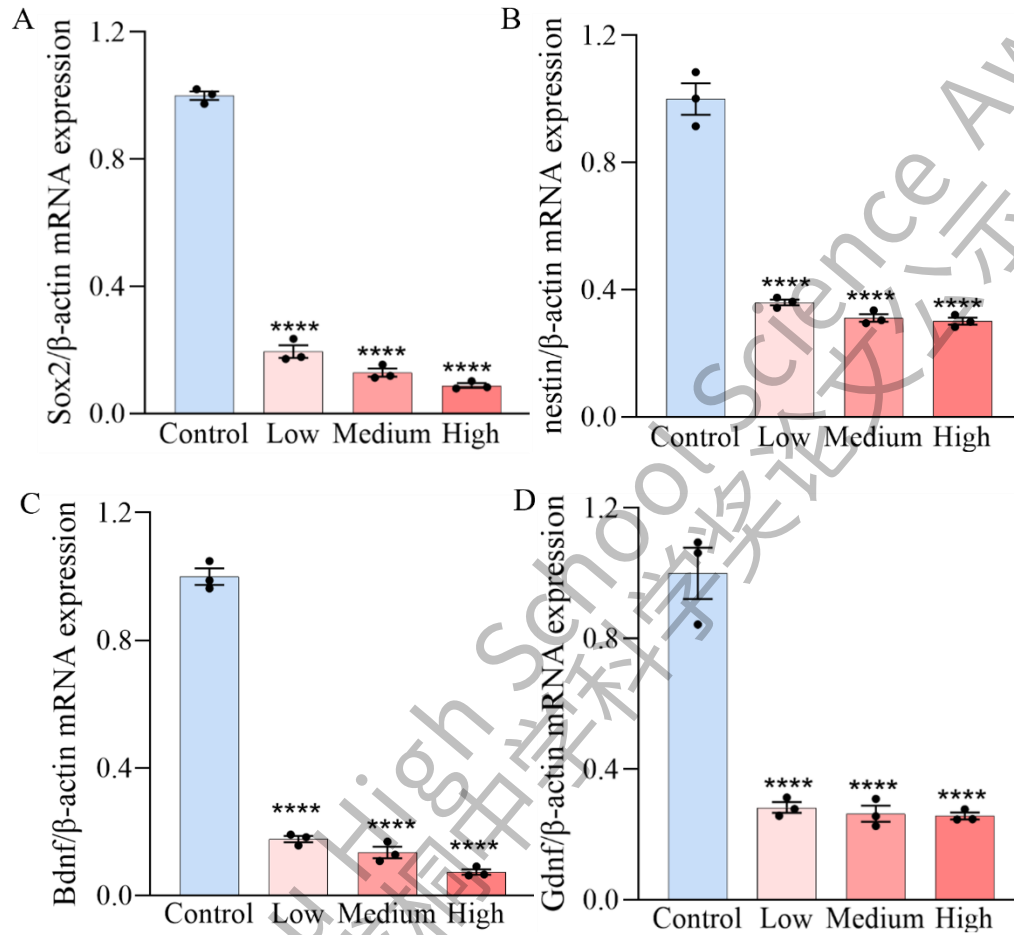


Figure 6. qPCR analysis showing reduced expression of neurodevelopmental and neurotrophic genes (*sox2*, *nestin*, *bdnf*, and *gdnf*) in zebrafish larvae following caffeine exposure. One-way ANOVA followed by Tukey's post hoc test, $n = 3$ biological replicates per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus control group.

3.5 Caffeine altered locomotor activity and color-zone preference in zebrafish larvae

Larval behavior was examined at 96 hpf after exposure to the three sublethal caffeine concentrations. Caffeine-treated larvae traveled a greater total distance than controls during the 1 h recording period, indicating increased locomotor activity (Figure 7A). The increase was significant in the Low, Medium, and High groups and became larger with increasing caffeine concentration, with the greatest total distance observed in the High group.

A separate color-zone preference assay was used to examine spatial behavior. The mean

proportion of time spent in the blue zone decreased across the caffeine-treated groups (Figure 7B). The change was modest in the Low and Medium groups and did not reach statistical significance, whereas larvae in the High group spent significantly less time in the blue zone than controls. Movement traces and spatial heatmaps showed the corresponding differences in activity and zone occupancy across treatment groups (Figure 7C).

These results show that developmental caffeine exposure was associated with increased locomotor activity and altered color-zone preference in larval zebrafish, with the clearest changes occurring at the highest sublethal concentration.

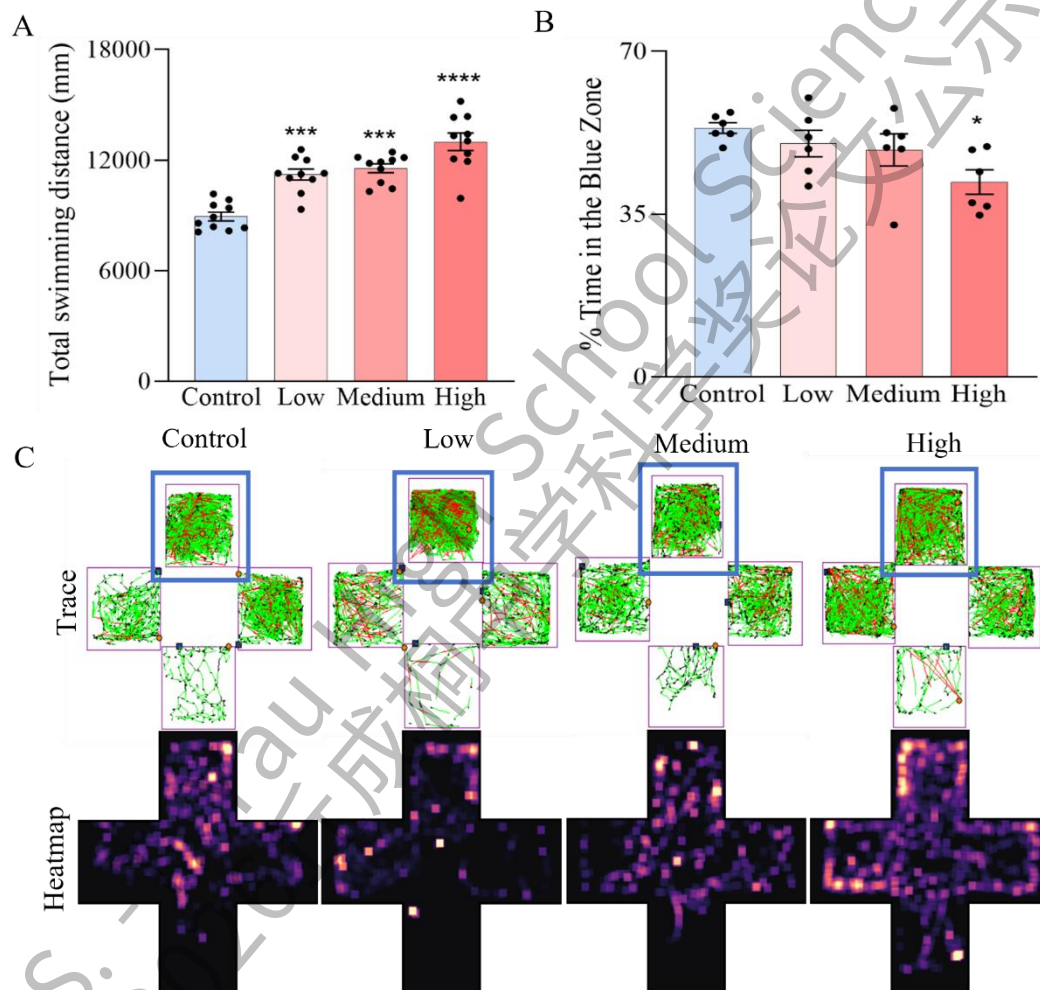


Figure 7. Behavioral phenotypes in zebrafish larvae following developmental caffeine exposure. (A) Total swimming distance during the 1-h recording period, showing locomotor hyperactivity in caffeine-exposed larvae. (B) Percentage of time spent in the blue zone during the color-zone preference assay. Reduced blue-zone preference, particularly in the High group, was consistent with impaired cognition-related sensory behavior. (C) Representative movement traces and spatial heatmaps showing activity patterns and zone occupancy across treatment groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus control group.

4. Discussion

Caffeine exposure was associated with changes in neuronal pathways, neurite morphology, gene expression, and larval behavior across the different systems examined here. The experiments were not performed in a single species or developmental stage, and they were not designed to trace one continuous molecular pathway. What emerged instead was a recurring set of effects involving neuronal growth, neurotrophic signaling, and neural function.

The adult mouse hippocampal analysis first pointed to this pattern. GSEA of H3K27ac- and H3K27me3-associated differential-enrichment data identified several neuronal and synaptic pathways that changed with caffeine exposure. The two histone marks showed different enrichment patterns, with H3K27ac-associated pathways generally shifting in a positive direction and several neuronal pathways in the H3K27me3 analysis showing negative enrichment. Neurotrophin signaling was one of the strongest signals in the H3K27me3-ranked dataset. These results place neuronal regulation and plasticity among the processes associated with caffeine exposure in the adult hippocampus. Since the analysis was performed on chromatin-associated differential-enrichment data, it does not by itself show how individual genes were transcriptionally regulated.

The primary neonatal rat neuron experiments showed a clear structural effect. Caffeine-treated neurons had fewer Sholl intersections across much of the neuritic field, and the reduction was strongest at 25 and 50 μ M. The lower concentration produced a smaller change. Neurite branching is a major part of neuronal maturation and network formation, so a reduction in arbor complexity points to an effect on neuronal development at the cellular level. The experiment does not identify the upstream molecular cause, but the phenotype itself was consistent across a broad range of radial distances rather than being restricted to a single region around the soma.

In zebrafish larvae, caffeine exposure also altered genes involved in neural development. Expression of *sox2*, *nestin*, *bdnf*, and *gdnf* decreased across the exposure groups, with the lowest mean expression seen at the highest concentration. *sox2* and *nestin* are more closely associated with neural progenitor state and developmental processes [16], whereas *bdnf* and *gdnf* are central to neurotrophic support and neuronal growth [17-19]. The fact that all four genes moved in the same direction suggests that the response was not limited to one narrow aspect of neural development.

The decrease in *bdnf* and *gdnf* is especially relevant to the pathway analysis. Neurotrophin-related signaling appeared in the adult hippocampal GSEA, and two genes from that same general biological system were reduced in developing zebrafish. These findings came from different models, so they cannot be treated as direct validation of one another. Still, the overlap

is biologically meaningful. It points to neurotrophic signaling as one of the more consistent themes in the study.

The neuron morphology data fit that interpretation reasonably well. *bdnf* and *gdnf* support neuronal growth and neurite development, while caffeine-treated primary neonatal rat neurons showed reduced arbor complexity [17-19]. That correspondence does not prove that lower *bdnf* or *gdnf* expression caused the structural phenotype, particularly because the qPCR and morphology experiments were performed in different species. It does, however, make the relationship worth testing directly. A reduction in trophic support and a reduction in neurite complexity would be expected to occur within the same general developmental framework.

Behavior changed as well. Caffeine-exposed zebrafish larvae traveled a greater total distance than controls, with the largest increase in the High group. This indicates increased locomotor activity after developmental caffeine exposure. Greater activity in larval zebrafish can accompany altered arousal or stress-related responses, although total distance traveled is not specific enough to distinguish among those possibilities. The color-zone assay produced a more selective effect: time spent in the blue zone decreased across the caffeine groups, but only the High group differed significantly from control. Because larval zebrafish normally show reproducible color-related preferences [20], the reduced blue-zone preference at the highest sublethal concentration is consistent with impaired cognition-related sensory behavior, reflecting an altered ability to respond to or express an innate sensory preference. However, because the assay did not involve training, memory acquisition, or retention, this result should not be interpreted as evidence of generalized cognitive or learning impairment.

Taken as a whole, the developmental experiments show a consistent direction of change. Caffeine exposure was associated with lower expression of developmental and neurotrophic genes, reduced neurite complexity, and altered behavior. The links among those findings remain unresolved. We did not test whether the transcriptional changes caused the structural phenotype, or whether the structural changes were responsible for the behavioral response.

The contrast with the adult hippocampal data raises a broader question about developmental context. In the adult dataset, caffeine exposure was associated mainly with pathway-level changes involving neuronal and synaptic regulation. In the developing systems, the effects appeared as changes in developmental gene expression, neuronal structure, and behavior. One possibility is that perturbing the same broad biological processes has different consequences depending on whether the nervous system is mature or still being assembled. During development, progenitor maintenance, neurite growth, trophic support, and circuit formation are all active at the same time. Changes in these processes may therefore carry different

consequences than similar changes in an adult brain. Recent work in a prenatal mammalian model has also linked caffeine exposure to a glucocorticoid-driven epigenetic cascade affecting hippocampal neurodevelopment [21].

This idea is summarized in Figure 8. The figure is a proposed mechanistic model rather than a demonstrated sequence of events. We did not measure H3K27ac or H3K27me3 directly in the developing zebrafish or primary neurons, and the adult mouse, rat neuron, and zebrafish experiments differ in species, developmental stage, and exposure conditions. The model therefore reflects the pattern across the study rather than a proven epigenetic cascade. It proposes that caffeine-associated neuronal pathway changes seen in the adult hippocampus may have different consequences when related biological systems are perturbed during development.

Several limitations follow from the design. The developmental and adult datasets were not matched for species, age, tissue, or exposure paradigm. Zebrafish embryos were exposed directly to caffeine in water, while primary neurons were treated in culture; neither reproduces the pharmacokinetics of repeated dietary intake in adolescents. The concentrations used here should therefore not be translated directly into human consumption levels. In addition, the lack of direct epigenomic measurements in the developmental models leaves an important gap between the adult pathway analysis and the developmental phenotypes.

The next experiments are fairly clear. Measuring H3K27ac, H3K27me3, and transcription in the same developing nervous system would show whether the epigenomic signals identified in the adult hippocampus are also present during development. Manipulating *bdnf*-, *gdnf*-, or related signaling pathways could test whether these changes contribute to the reduction in neurite complexity or to the behavioral phenotype. A mammalian adolescent model with repeated oral caffeine exposure would also allow developmental and adult responses to be compared under more similar conditions.

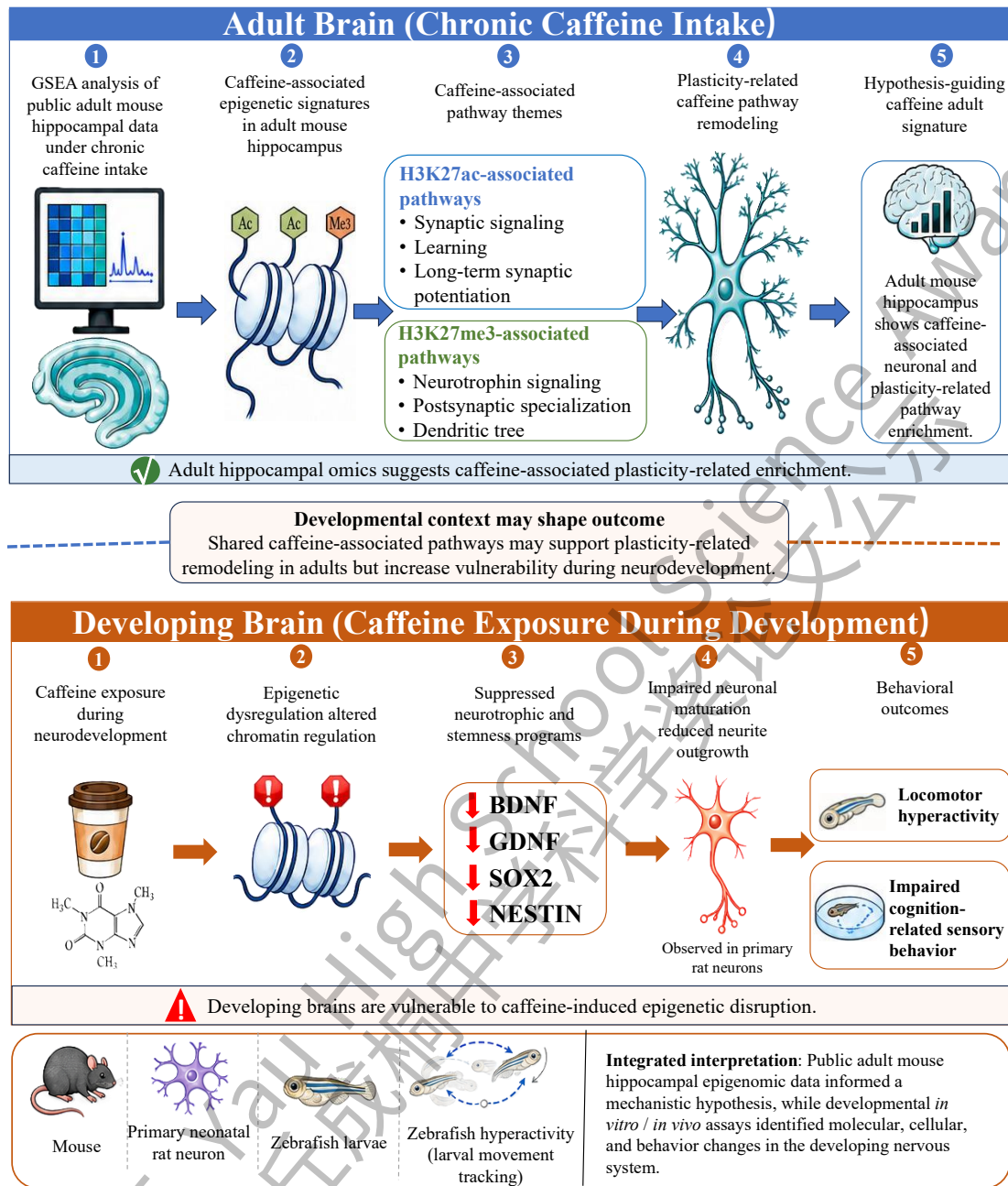


Figure 8. Proposed stage-dependent epigenetic model of caffeine effects on brain development and function.

5. Conclusion

Caffeine exposure was associated with changes across several levels of neural organization in this study. In adult mouse hippocampal epigenomic data, caffeine was linked to enrichment of neuronal, synaptic, and plasticity-related pathways, including neurotrophin signaling. In developmental models, caffeine exposure reduced neurite complexity in primary neonatal rat neurons and decreased expression of *sox2*, *nestin*, *bdnf*, and *gdnf* in zebrafish larvae. The

behavioral effects were also clear, with locomotor hyperactivity and impaired cognition-related sensory behavior observed after exposure.

Taken together, these findings support a stage-dependent view of caffeine action on the nervous system. Shared caffeine-associated pathways may contribute to plasticity-related remodeling in the mature brain, while disruption of the same or related processes during neurodevelopment may have less adaptive consequences. The proposed mechanism remains to be tested within a single developmental system, but the present data identify neurotrophic and developmental pathways as plausible points of vulnerability and provide a basis for further work on how caffeine affects the developing nervous system.

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Supplementary Information

Sensitivity analysis of the GSEA ranking method

To examine whether the pathway-level results depended strongly on the gene-ranking metric, we repeated the GSEA using $\log_2\text{FC}$ alone instead of the primary ranking score, $\log_2\text{FC} \times [-\log_{10}(\text{FDR})]$. The major neuronal and synaptic pathways retained the same enrichment direction under the alternative ranking scheme, although the magnitude of the NES changed for some pathways.

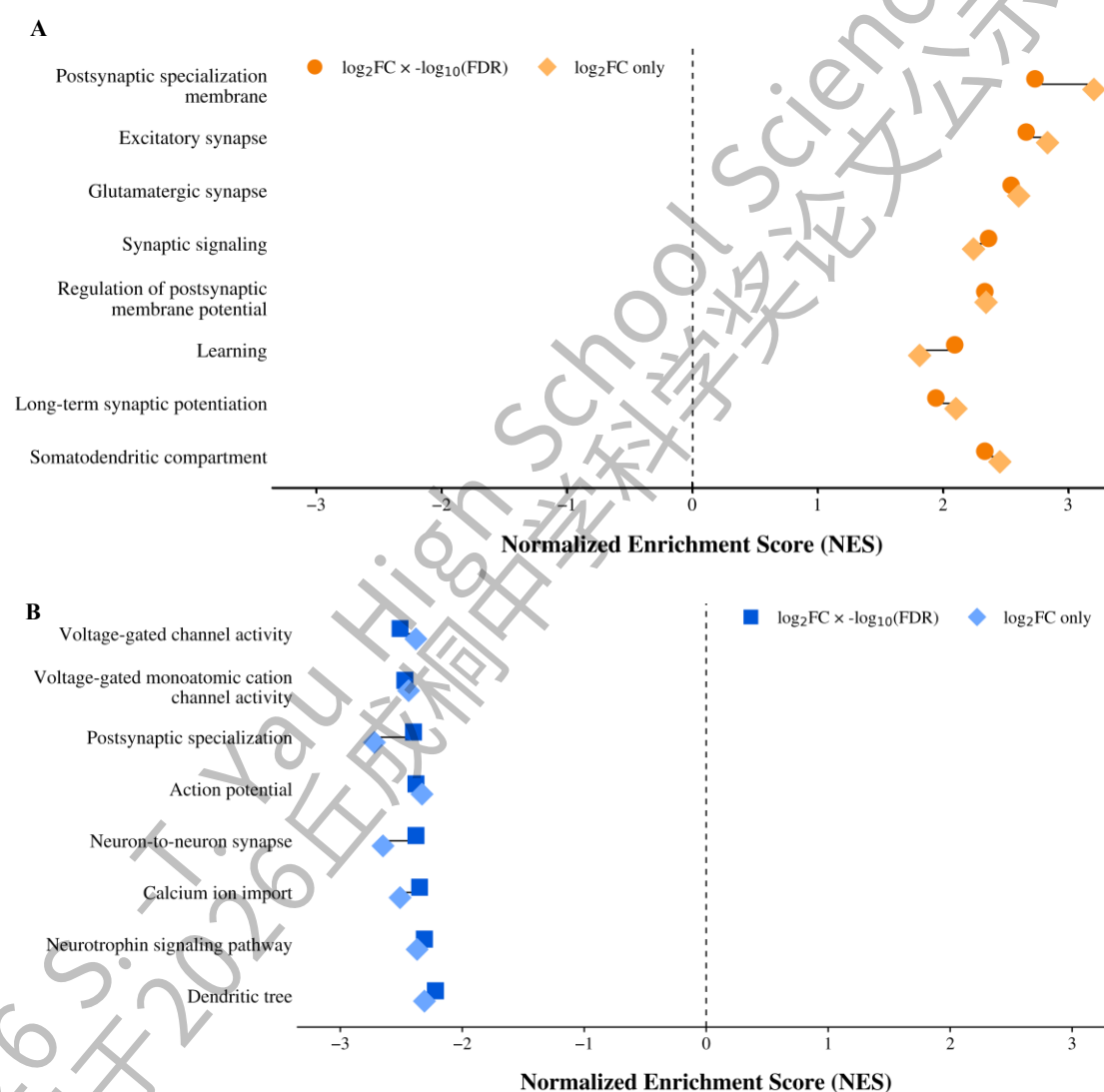


Figure S1. Sensitivity analysis of GSEA results to the gene-ranking metric. (A) H3K27ac-positive pathways and (B) H3K27me3-negative pathways displayed in Figure 3A were reanalyzed using $\log_2$ fold change ($\log_2\text{FC}$) alone as the gene-ranking metric and compared with the primary ranking score, $\log_2\text{FC} \times [-\log_{10}(\text{FDR})]$. Paired symbols connected by horizontal

lines represent the NES obtained for the same pathway under the two ranking schemes. When multiple differential peaks mapped to the same gene, the peak with the largest absolute value of the corresponding ranking metric was retained while preserving its sign. All other GSEAPreranked parameters and gene-set definitions were kept unchanged. The major synaptic and neuronal enrichment patterns retained their direction under the alternative ranking scheme, although the magnitude of enrichment varied among individual pathways.

Acknowledgements

I would like to express my sincere gratitude to all the teachers and lab staff members who have provided indispensable guidance and assistance to help me complete my project.

Due to my father's work situation, since kindergarten years, I have been traveling back and forth across the Pacific Ocean, attending schools in both U.S. and China. As I finally settled in the Chinese school system since Grade 8, I had been under a lot of pressure and stress for a couple of semesters because the U.S. schools underprepared me for the rigorous Chinese curricula. During that time period, I grew fond of bubble tea and became a frequent consumer, which helped me deal with the stress. But drinking bubble tea soon came with a cost for me: very so often, I would get a headache and agitated. My frustration grew even more when I noticed that my mother had a completely reversed reaction to tea drinking. She would enjoy sharper mind and feel energized after consuming some strong tea. I had to cut back my bubble tea consumption but this mystical contrast had really spiked my interest. As I learned more about biology, this seemingly naïve curiosity gradually morphed into a specific research question, which I found later on even bothered the authoritative public health agencies and scientists. That is, are intakes of caffeine, the main substance of these different tea variations, impacting adults and youths in significantly different ways? I feel very fortunate that in the past three semesters, I have been able to investigate this interesting question using the neuroscience concepts and experimental methods I learned in classrooms, from the school mentorship program, and through interactions with my advisor and lab staff members.

First and foremost, I would like to thank Professor Qingpeng Zhang from Hong Kong University for his insightful guidance and unwavering patience. Professor Zhang is my main advisor for this project and has been a constant, significant, and reliable presence from the beginning and throughout the entire process. He guided me tirelessly in the design of the research questions, the understanding of the related literature, the setup of the experiments, the software needed to process the experimental data, and the iterative process of discussing the findings and finalizing the writing. Without Professor Zhang's guidance, I would have zero chance in completing this project.

I wish to sincerely thank Professor Yichang Jia from Tsinghua University. I joined Professor Jia's group through my school's research mentorship program. In Professor Jia's lab, I was exposed to several neuroscience experimental methods key to my project. I learned about experimental procedurals and protocols, as well as data collection methods. This lab training experience has been integral to my understanding of research in general in addition to specific techniques and preparation steps learned.

I also would like to thank the lab staff at Hunter Biotech. They provided excellent experimental platforms, and ensured standards-compliant and safe conducting of the experiments and collection of data.

Lastly, I would like to express gratitude to Tsinghua International School for providing all the intellectual, instructional, administrative, and spiritual supports needed to pursue self-driving research of my own interests. In particular, the research mentorship program played a key role in preparing me to design and execute a rigorous research study.

Aside from necessary experimental expenses, no paid tutoring or fee-based academic guidance was involved in the conduct of this project or the preparation of this manuscript.

AI Use

When carrying out the project, I have used the Google's Gemini system and OpenAI's GPT system for the following tasks:

- Gaining better understanding of certain neuroscience technical terms by directly asking the LLMs and interacting with these LLMs with follow-up questions
- Providing summaries of several non-essential past references
- Checking for grammatical errors of the final report I wrote by myself