

The regulatory role of lncRNA-LOC680225 in fluoride-induced neurotoxicity: Insights into the miR-194–5p/Slc5a7 signaling axis

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ABSTRACT

Our previous epidemiological studies showed that low-to-moderate fluoride exposure induces cholinergic dysfunction in children, but the underlying mechanisms remain unclear. Here, transcriptome sequencing of fluoride-exposed rat hippocampal tissues identified altered expression of solute carrier family 5 member 7 (Slc5a7), which encodes choline transporter 1 (Cht1), long non-coding RNA (lncRNA) LOC680225, and microRNA (miRNA) miR-194–5p. *In vivo* and *in vitro* assays confirmed that fluoride disrupted the cholinergic system and synaptic plasticity, decreased acetylcholine (ACh) content, downregulated Slc5a7, choline acetyltransferase (ChAT), postsynaptic density protein-95 (PSD-95), and synapsin-1, and increased acetylcholinesterase (AChE) expression. Fluoride also suppressed LOC680225 while elevating miR-194–5p in rat hippocampus and PC12 cells. LOC680225 overexpression or miR-194–5p inhibition partially rescued fluoride-mediated Cht1 reduction, neuronal apoptosis, and cell viability loss, whereas miR-194–5p overexpression aggravated cellular injury. Luciferase assays validated that LOC680225 and Slc5a7 are direct targets of miR-194–5p. Collectively, our findings support a potential ceRNA regulatory network, in which LOC680225 sponges miR-194–5p to modulate Slc5a7 expression. The LOC680225/miR-194–5p/Slc5a7 signaling axis represents one critical regulatory pathway underlying fluoride-induced neurotoxicity rather than the exclusive mechanism, providing novel insights into the molecular basis of fluoride-mediated cholinergic and synaptic dysfunction.

1. Introduction

Fluorine is a chemically active halogen element that exists in the environment in the form of fluoride. In addition to causing dental fluorosis and skeletal fluorosis (Chang et al., 2026), excessive fluoride exposure can penetrate the blood–brain barrier and induce irreversible developmental neurotoxicity (Krzeczkowski et al., 2024; Zhao et al., 2024; Zhu et al., 2024). Epidemiological evidence has linked fluoride exposure to reduced IQ and increased internalizing behaviors in children (Riddell et al., 2019), as well as a higher prevalence of ADHD symptoms and clinically diagnosed ADHD among Canadian adolescents (Adkins et al., 2022). *In vivo* studies, fluoride has been shown to elicit pathological damage and neuronal loss in the hippocampus, thereby impairing learning and memory function. Worldwide, an estimated 180 million people remain at risk of excessive fluoride exposure through drinking water, especially in Asia and Africa (Fernando et al., 2020; Podgorski

and Berg, 2022). Despite these observations, the precise molecular mechanisms underlying fluoride-induced neurotoxicity remain inadequately understood.

Accumulating evidence indicates that excessive fluoride exposure induces synaptic injury, impairs plasticity, and disrupts neurotransmission (Lingli et al., 2023). Acetylcholine (ACh) is synthesized from choline and acetyl-CoA via a one-step reaction catalyzed by choline acetyltransferase (ChAT). When an action potential reaches the presynaptic terminal, ACh binds transiently to its receptors; following dissociation, it is hydrolyzed by acetylcholinesterase (AChE) into choline and acetate. Choline is then taken up into the presynaptic terminal via the choline transporter (Cht1). Transcriptome sequencing revealed downregulated Slc5a7 expression in the hippocampus of Sprague–Dawley rats subjected to fluoride exposure. Yet the exact mechanisms by which Slc5a7 and the cholinergic system contribute to fluoride neurotoxicity remain incompletely elucidated.

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Non-coding RNAs (ncRNAs) represent a diverse class of RNA molecules that lack functional open reading frames (ORFs) and are not translated into proteins. The major classes of ncRNAs include long non-coding RNAs (lncRNAs) and microRNAs (miRNAs). Research indicates that these genomic elements are crucial for physiological processes and tissue homeostasis both in health and disease (Aliperti et al., 2021; Soutschek Schratt, 2023). lncRNAs are essential for neurodevelopment and the maintenance of neuronal function (Srinivas et al., 2023). For instance, the combined toxicity of carbon nanoparticle–Cr–Pb complexes in PM2.5 triggers cell cycle arrest via upregulating the novel lncRNA NONHSAT074301.2 (Pan et al., 2021). lncRNA dysregulation and aberrant DNA methylation have also been linked to dental fluorosis (Hu et al., 2024). Through transcriptome sequencing, we identified a panel of fluoride-induced differentially expressed lncRNAs, and bioinformatic analysis predicted that LOC680225 may interact with Slc5a7.

miRNAs negatively regulate gene expression and play critical roles in nervous system differentiation and development, synaptic plasticity, and the maintenance of learning and memory (Gu et al., 2023; Ma et al., 2024). MiR-742-3p, miR-141-5p, miR-878-3p, and miR-143-5p have been implicated in fluoride-induced epididymal damage (Yu et al., 2024). Fluoride can also trigger endothelial dysfunction by inducing oxidative stress and activating the p53/miR-200c-3p pathway (Zhang et al., 2025). Furthermore, miR-296-5p may enhance the release of autophagy-related mediators by activating AMPK/ULK1 signaling in fluorosis (Luo et al., 2022). Our deep sequencing analysis also identified 42 up-regulated and 9 down-regulated miRNAs in hippocampus of fluoride-exposed SD rats.

Recent studies have demonstrated that lncRNAs not only directly regulate gene expression but also function as competing endogenous RNAs (ceRNAs) by interacting with miRNAs. CeRNAs are RNA molecules that construct regulatory networks through shared microRNA response elements (MREs), competitively binding to miRNAs and thus attenuating their inhibitory effects on target mRNAs. However, the regulatory mechanism involving LOC680225, miRNAs, and Slc5a7 in fluoride-induced neurotoxicity has not yet been reported.

Based on the above evidence, we herein investigated the roles of LOC680225, miRNAs, and Slc5a7 in the cholinergic system underlying fluoride-induced developmental neurotoxicity. Using PC12 cells for *in vitro* assays and Sprague–Dawley rats for *in vivo* exposure, we established neuronal injury models to explore the molecular mechanisms and potential therapeutic targets of fluoride neurotoxicity.

2. Materials and methods

2.1. Chemicals and reagents

The chemical reagents used in this study were as follows: sodium fluoride (NaF; Sigma-Aldrich, USA) and fetal bovine serum (FBS; Gibco, USA). Primary antibodies included Cht1 (Abcam, UK), PSD-95 (Abcam, UK), Synapsin-1 (Abcam, UK), ChAT (Proteintech, USA), AChE (Boster, China), and GAPDH (Bioworld Technology, USA). All other chemicals and reagents were of analytical grade and obtained from local commercial sources.

2.2. Animals and treatment

Forty SPF female Sprague–Dawley rats (8 weeks old, weighing 180–220 g) were housed under controlled conditions: temperature 22–25 °C and humidity 50%–60%. Rat grouping and fluoride exposure doses were set according to our previously published study (Chen et al., 2018). Briefly, rats were randomly divided into a control group and three NaF treatment groups (10, 50, and 100 mg/L). After 6 months of exposure, all rats were sacrificed within 24 h. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC approval number:3570) of Huazhong University of Science and Technology and performed in accordance with the NIH Guide for the

Care and Use of Laboratory Animals.

2.3. Morris Water Maze (MWM) test

MWM test was conducted as follows. Place navigation test (PNT) was conducted for 4 consecutive days, with each rat undergoing 4 trials per day. Rats were released from fixed starting points in each quadrant, and the latency to locate the platform was recorded. Rats failing to find the platform within 60 s were guided to it and allowed to stay for 20 s. After each trial, rats were returned to their cages for a 15–20 min inter-trial interval. On day 5, a spatial probe test (SPT) was carried out with the platform removed. Rats were released from the quadrant opposite the original platform position and allowed to swim freely for 60 s. The number of platform site crossings, the percentage of time spent in the target quadrant, and the percentage of distance traveled in the target quadrant were all recorded.

2.4. Full-length transcriptome sequencing

Three SD rats were randomly selected from both the control group and the 100 mg/L NaF-exposed group, respectively. Total RNA was extracted from the hippocampal tissues. For lncRNA and mRNA sequencing, the library was constructed via the following sequential steps: total RNA extraction, ribosomal RNA depletion, RNA fragmentation, reverse transcription, end repair and adapter ligation of cDNA, PCR amplification, and high-throughput sequencing. For miRNA sequencing, the steps of ribosomal RNA depletion and RNA fragmentation were omitted, while all other procedures were performed identically.

2.5. Cell culture and treatments

PC12 cells are derived from rat adrenal medullary pheochromocytoma and serve as a classic *in vitro* surrogate neuronal model in neurotoxicology, which is suitable for exploring the mechanism of fluoride-induced cholinergic neuronal injury in this study. They exhibit typical neuronal characteristics. After differentiation induced by nerve growth factor (NGF), PC12 cells develop neurites and abundantly express neuronal markers, choline transporter protein CHT1, as well as synapse-related molecules. Equipped with intact cholinergic signaling pathways, these cells can recapitulate the *in vivo* hippocampal injury phenotypes triggered by fluoride exposure, including impaired cholinergic function, reduced synaptic plasticity, and cell apoptosis (Greene and Tischler, 1976).

PC12 cells were cultured in RPMI 1640 medium (Hyclone) supplemented with 10% FBS (Gibco), while 293 T cells were maintained in DMEM (Gibco) containing 10% FBS. All cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂. For NaF treatment, PC12 cells were exposed to NaF solutions at concentrations of 20, 40, and 80 mg/L for 24 h.

PC12 cells were transfected with 50 nM miR-194-5p mimic, miR-194-5p inhibitor, or negative control. In addition, PC12 cells were transfected with adenovirus overexpressing LOC680225 (Ad-LOC680225) at a multiplicity of infection (MOI) of 200. All cells were incubated for an additional 48 h post-transfection.

2.6. Scanning electron microscope (SEM)

PC12 cells were incubated with 50 ng/mL nerve growth factor (NGF) to induce neurite outgrowth. After 6 days of induction, cells were seeded at a density of 6×10^3 cells per well in 24-well plates and exposed to NaF for 24 h. Subsequently, cells were fixed with glutaraldehyde for 2 h and rinsed three times with PBS. Secondary fixation was performed at 4 °C for 12 h. Samples were dehydrated in a graded ethanol series followed by tertiary butyl alcohol, and then dried in a desiccator for 12 h. The dried samples were mounted on scanning SEM copper stubs, carbon-coated, and gold-sputtered. Images were captured using a HITACHI

SU8010 scanning electron microscope

2.7. Fluorescence in situ hybridization (FISH)

PC12 cells were seeded and fixed as described above. Prehybridization solution was added, and cells were incubated at 37 °C for 1 h. The prehybridization solution was then removed, and hybridization solution containing the LOC680225 (or miR-194-5p) probe was added dropwise. Cells were incubated overnight at 37 °C. Subsequently, the Cht1 primary antibody (1:500) was applied, and cells were incubated overnight at 4 °C, followed by three washes with PBS. All subsequent steps were performed in the dark. Alexa Fluor 594/488 goat anti-rabbit IgG (H+L) secondary antibody (1:500) was added and incubated for 2 h, followed by another three washes with PBS. Nuclei were counterstained with DAPI, and anti-fade mounting medium was added to seal the coverslips. Images were acquired using a confocal laser scanning microscope.

2.8. Luciferase reporter assay

293 T cells were seeded in 12-well plates and transfected at 60–70% confluence with the following plasmid/miRNA combinations: wild-type (WT) Slc5a7 3'UTR + mimic NC; WT Slc5a7 3'UTR + miR-194-5p mimics; mutant (Mut) Slc5a7 3'UTR + mimic NC; Mut Slc5a7 3'UTR + miR-194-5p mimics; WT LOC680225 3'UTR + mimic NC; WT LOC680225 3'UTR + miR-194-5p mimics; Mut LOC680225 3'UTR + mimic NC; and Mut LOC680225 3'UTR + miR-194-5p mimics. Cells were harvested 48 h post-transfection. Luciferase activity was detected using the Dual-Luciferase Reporter Assay System (GenePharma, Shanghai, China) in accordance with the manufacturer's instructions.

2.9. Statistical analysis

All experiments were performed in at least three independent biological replicates. Representative data are shown and presented as the mean $\pm$ standard deviation (SD). Normality and homogeneity-of-variance tests were strictly performed prior to all statistical comparisons. (1) Normality testing: The Shapiro–Wilk test was applied to verify whether each dataset conformed to a normal distribution. (2) Homogeneity of variance testing: Levene's test was used to assess the homogeneity of variances among groups for one-way ANOVA. For data satisfying both normal distribution and equal variances, independent sample *t*-test or one-way ANOVA followed by Tukey's post-hoc test were adopted.

Statistical analysis was conducted using SPSS 20.0 software. One-way ANOVA was applied for comparisons among multiple groups, followed by Dunnett's post hoc test. Student's *t*-test was used to determine significant differences between two groups. Graphs were generated using GraphPad Prism 8.0, and figures were assembled using Adobe Photoshop CS6. A value of $P < 0.05$ was considered statistically significant.

More experimental methods can be found in the [supplementary materials](#).

3. Results

3.1. NaF exposure impaired learning and memory with hippocampal neuronal loss in female rats

In the SPT of the MWM test, the number of platform crossings was significantly decreased in the 50 and 100 mg/L NaF-treated groups compared with the control group (Fig. 1A). In addition, rats exposed to 50 and 100 mg/L NaF spent significantly less swimming time in the target quadrant than control rats (Fig. 1B). Meanwhile, the swimming distance traveled in the target quadrant was reduced in all NaF-treated groups relative to the control group, but the difference was not

statistically significant (Fig. 1C). Collectively, these results indicate that NaF exposure impairs learning and memory in rats.

As shown in Fig. 1E, neurons in the control group exhibited abundant, darkly stained Nissl bodies arranged in a dense and orderly pattern. With increasing NaF exposure dose, the arrangement of neurons gradually became loose, and the staining intensity of Nissl bodies was reduced, indicating that NaF exposure decreases the viability of hippocampus neurons in female rats.

3.2. NaF exposure reduced synaptic connectivity in PC12 cells

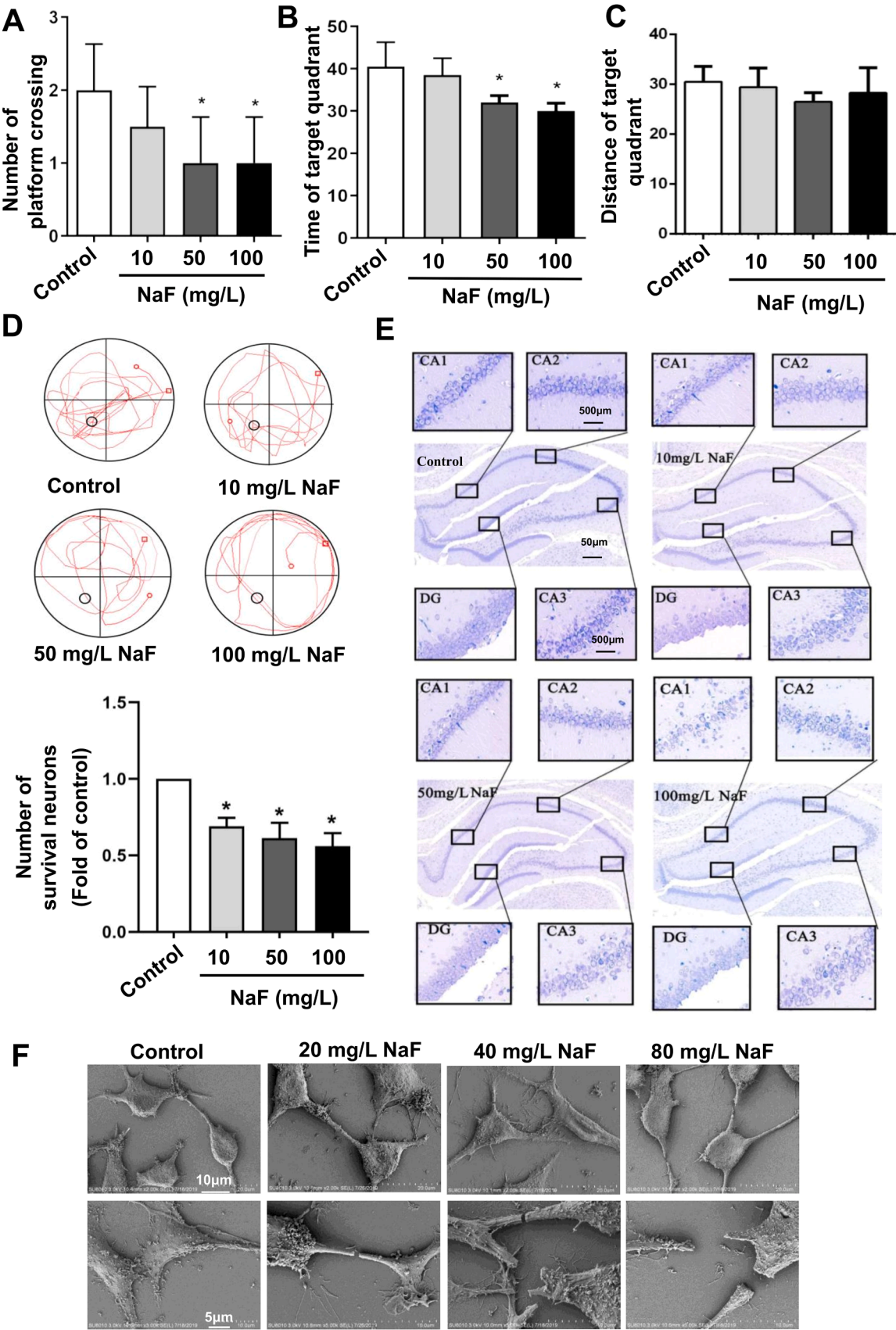
In vitro observations revealed that PC12 cells in the control group were morphologically intact, with abundant neurites and dense synaptic connections between cells. In the 20 mg/L NaF-treated group, synaptic length was shortened, synaptic number was reduced, and partial neurite fragmentation was observed, although cells remained relatively intact. In the 40 mg/L NaF-treated group, synaptic number was further decreased, neurite fragmentation was aggravated, and cell shrinkage began to appear. In the 80 mg/L NaF-treated group, normal synaptic connections were completely lost, extensive neurite fragmentation occurred, and severe cell shrinkage was observed (Fig. 1F). These findings demonstrate that NaF exposure damages synaptic connectivity in PC12 cells in a dose-dependent manner.

3.3. NaF exposure induced cholinergic system dysfunction in both SD rats and PC12 cells

Compared with the control group, the 100 mg/L NaF-treated group exhibited 7 upregulated and 37 downregulated mRNAs (Fig. 2A). KEGG pathway enrichment analysis identified multiple signaling pathways associated with these differentially expressed genes (Table S3). Notably, the cholinergic synapse pathway, which is known to be closely associated with nervous system function, was among the enriched pathways. Based on our previous findings that synaptic damage is involved in fluoride-induced neurotoxicity, further analysis of differentially expressed genes in the cholinergic synapse KEGG pathway revealed that Slc5a7 (Cht1) was significantly downregulated in the hippocampus of NaF-treated rats.

As shown in Fig. 2, compared with the control group, Cht1 and ChAT protein levels were decreased and AChE protein levels were increased in NaF-treated groups (Fig. 2D). In addition, Slc5a7 and ChAT mRNA levels were downregulated in the 50 and 100 mg/L NaF-treated groups, and AChE mRNA levels were upregulated in the 100 mg/L NaF-treated group (Fig. S1A–C). ELISA results showed that ACh levels in rat serum were significantly decreased in the 50 and 100 mg/L NaF-treated groups compared with the control group (Fig. 2B). Furthermore, compared with the control group, the mRNA expression levels of M1 muscarinic acetylcholine receptor (M1 mAChR), M3 muscarinic acetylcholine receptor (M3 mAChR) and $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) were significantly decreased in the 100 mg/L NaF-treated group (Fig. S2D–F).

PC12 cells exposed to NaF exhibited significantly downregulated protein levels of ChAT and Cht1 compared with the control group. Meanwhile, AChE protein expression was elevated in the 20 and 80 mg/L NaF-treated groups (Fig. 2E). Slc5a7 and ChAT mRNA levels were decreased and AChE was increased in the 80 mg/L NaF-treated group relative to controls (Fig. S1G–I). In addition, ACh levels in PC12 cell supernatants were markedly diminished in the 80 mg/L NaF-treated group (Fig. 2C). These findings indicate that NaF exposure disrupts the cholinergic system by reducing ACh synthesis and increased hydrolysis of ACh, ultimately leading to reduced ACh levels. Likewise, mRNA levels of M1 mAChR, M3 mAChR, and $\alpha 7$ nAChR were significantly decreased in NaF-treated cells compared with the control group (Fig. S2J–L). Taken together, the above findings suggest that NaF exposure downregulates cholinergic receptor mRNA expression, thereby impairing ACh transmission.



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Fig. 1. NaF induced learning and memory impairment, decreased neuronal viability in hippocampus, and reduced synaptic connectivity in PC12 cells. Female SD rats were randomly divided into four groups: one control group, and the other three groups were treated with 10, 50, and 100 mg/L NaF, respectively, for 6 consecutive months. PC12 cells were treated with different concentrations of NaF (0, 20, 40, 80 mg/L) for 24 h (A) Number of platform crossings in the SPT. (B) Percentage of time spent in the target quadrant in the SPT. (C) Percentage of distance traveled in the target quadrant in the SPT. (D) Representative swimming trajectories in the SPT. (E) Representative Nissl-stained images and quantitative analysis of Nissl-positive neurons in rat hippocampi from control and NaF-treated groups. Middle row: scale bar = 50 μ m; upper and lower rows: scale bar = 500 μ m. (F) SEM images of PC12 cells from the control and NaF treated groups (20, 40, 80 mg/L NaF). The Upper row: scale bar = 10 μ m; the lower row: scale bar = 5 μ m. The data are presented as the mean $\pm$ S.D. of six independent experiments * $P < 0.05$ vs the control group.

3.4. NaF impaired synaptic plasticity and promoted excessive apoptosis in PC12 cells

As shown in Fig. 3, compared with the control group, the protein levels of PSD-95 and synapsin-1 were significantly downregulated in the 80 mg/L NaF-treated group (Fig. 3A). Meanwhile, mRNA levels of PSD-95 and Synapsin-1 were also decreased (Fig. 3B). Furthermore, the expression of cleaved caspase-3, a key executioner of apoptosis, was upregulated (Fig. 3A). Collectively, these results indicate that NaF exposure impairs synaptic plasticity and triggers excessive apoptosis.

3.5. We screened lncRNAs and miRNAs in the hippocampus of female rats exposed to NaF that may interact with Slc5a7

Bioinformatics analysis was performed to screen lncRNAs and miRNAs with potential regulatory interactions with *Slc5a7*. Compared with the control group, 3 lncRNAs were upregulated and 13 lncRNAs were downregulated in hippocampus from 100 mg/L NaF-treated rats. Among these differentially expressed lncRNAs, lncRNA LOC680225 was identified as a candidate regulator targeting *Slc5a7*. The co-expression correlation coefficient between LOC680225 and all *Slc5a7* transcripts exceeded 0.85 and was statistically significant (Table S4). This strong positive correlation suggests a reciprocal regulatory relationship between LOC680225 and *Slc5a7*.

MiRNA sequencing results revealed that 42 miRNAs were upregulated and 9 miRNAs were downregulated in hippocampus from the 100 mg/L NaF-treated rat group (Fig. 3C). To explore the potential ceRNA regulatory network, cytoscape was used to visualize lncRNA-miRNA-mRNA interactions (Fig. 3D). This analysis identified two miRNAs—rno-miR-194-5p and rno-miR-511-3p—that were predicted to target both LOC680225 and *Slc5a7*. Sequence alignment via the miRBase database showed that the sequence of miR-194-5p is identical between humans and rats, exhibiting high evolutionary homology. Given its high evolutionary conservation, miR-194-5p was selected as the candidate miRNA for subsequent mechanistic studies.

3.6. Ad-LOC680225 mediated overexpression of LOC680225 alleviated the NaF-induced downregulation of Cht1 in PC12 cells

The expression of LOC680225 was significantly downregulated in hippocampus of rats and PC12 cells from NaF-treated groups compared with the control (Fig. 4A,B). FISH analysis revealed that both LOC680225 and Cht1 were distributed in the cytoplasm and nucleus (predominantly in the cytoplasm) and showed spatial co-localization. With increasing NaF concentration, the expression of both molecules gradually decreased, supporting their co-regulation (Fig. 4C). Transfection with Ad-LOC680225 significantly elevated LOC680225 expression (Fig. 4D). Compared with the control group, Cht1 expression was decreased in the NaF-treated group but significantly upregulated in the Ad-LOC680225 group. Moreover, Cht1 protein levels were higher in the NaF + Ad-LOC680225 group than in the 80 mg/L NaF-treated group (Fig. 4E). Collectively, these results demonstrate that overexpression of LOC680225 rescues NaF-induced downregulation of Cht1 in PC12 cells.

3.7. Inhibition of miR-194-5p partially reversed the NaF-induced downregulation of Cht1 in PC12 cells

NaF exposure upregulated the expression of miR-194-5p in hippocampus of rats and PC12 cells (Fig. 5A). FISH analysis showed that miR-194-5p was distributed in both the cytoplasm and nucleus. With increasing NaF concentration, miR-194-5p expression increased progressively (Fig. 5C). Transfection with the miR-194-5p mimic significantly upregulated miR-194-5p expression (Fig. 5B), whereas treatment with the miR-194-5p inhibitor significantly reduced its expression (Fig. 5E). Compared with the control group, Cht1 expression was decreased in both the NaF-treated group and the miR-194-5p mimic group. Notably, Cht1 protein levels were further reduced in the NaF + miR-194-5p mimic group compared with the 80 mg/L NaF-treated group (Fig. 5D). Conversely, the miR-194-5p inhibitor upregulated Cht1 expression relative to the control, and Cht1 levels in the NaF + miR-194-5p inhibitor group were higher than those in the NaF-treated group (Fig. 5F). Collectively, these results demonstrate that miR-194-5p negatively regulates Cht1 expression in NaF-treated PC12 cells.

3.8. Both LOC680225 and Slc5a7 are targeted by miR-194-5p

To validate the direct interactions between miR-194-5p and its predicted targets, luciferase reporter assays were performed. Co-transfection of pmirGLO-LOC680225-wild-type (WT) with the miR-194-5p mimic significantly reduced luciferase activity ($P < 0.01$) compared with the mimic negative control (Fig. 6A). Mutation of the predicted miR-194-5p binding site in LOC680225 abolished this inhibitory effect, with no significant difference in luciferase activity between the pmirGLO-LOC680225-Mut + miR-194-5p mimic group and the Mut + mimic NC group. Consistent results were obtained for *Slc5a7* and miR-194-5p (Fig. 6B). These results confirm that both LOC680225 and *Slc5a7* are direct targets of miR-194-5p, providing mechanistic insight into their regulatory interactions during fluoride-induced neurotoxicity.

3.9. LOC680225 overexpression or miR-194-5p inhibition rescues fluoride-suppressed cell viability

To investigate whether LOC680225 and miR-194-5p influence downstream apoptotic pathways and cell viability, further functional assays were conducted in PC12 cells. Overexpression of LOC680225 decreased cleaved caspase-3 levels and enhanced cell viability relative to the control group. Consistently, in NaF-treated PC12 cells, LOC680225 overexpression attenuated cleaved caspase-3 expression and improved cell viability (Fig. 6C). Compared with the control group, both the miR-194-5p mimic group and the NaF-treated group showed elevated cleaved caspase-3 expression and decreased cell viability. Notably, in the miR-194-5p mimic + NaF group, cleaved caspase-3 levels were further increased and cell viability further reduced compared with the NaF-treated group (Fig. 6D, E), indicating that miR-194-5p exacerbates NaF-induced apoptosis and cellular injury. By contrast, transfection with miR-194-5p inhibitor reduced cleaved caspase-3 expression and restored cell viability compared with the NaF-treated group (Fig. 6F). Collectively, these results suggest that inhibition of miR-194-5p or overexpression of LOC680225 can partially

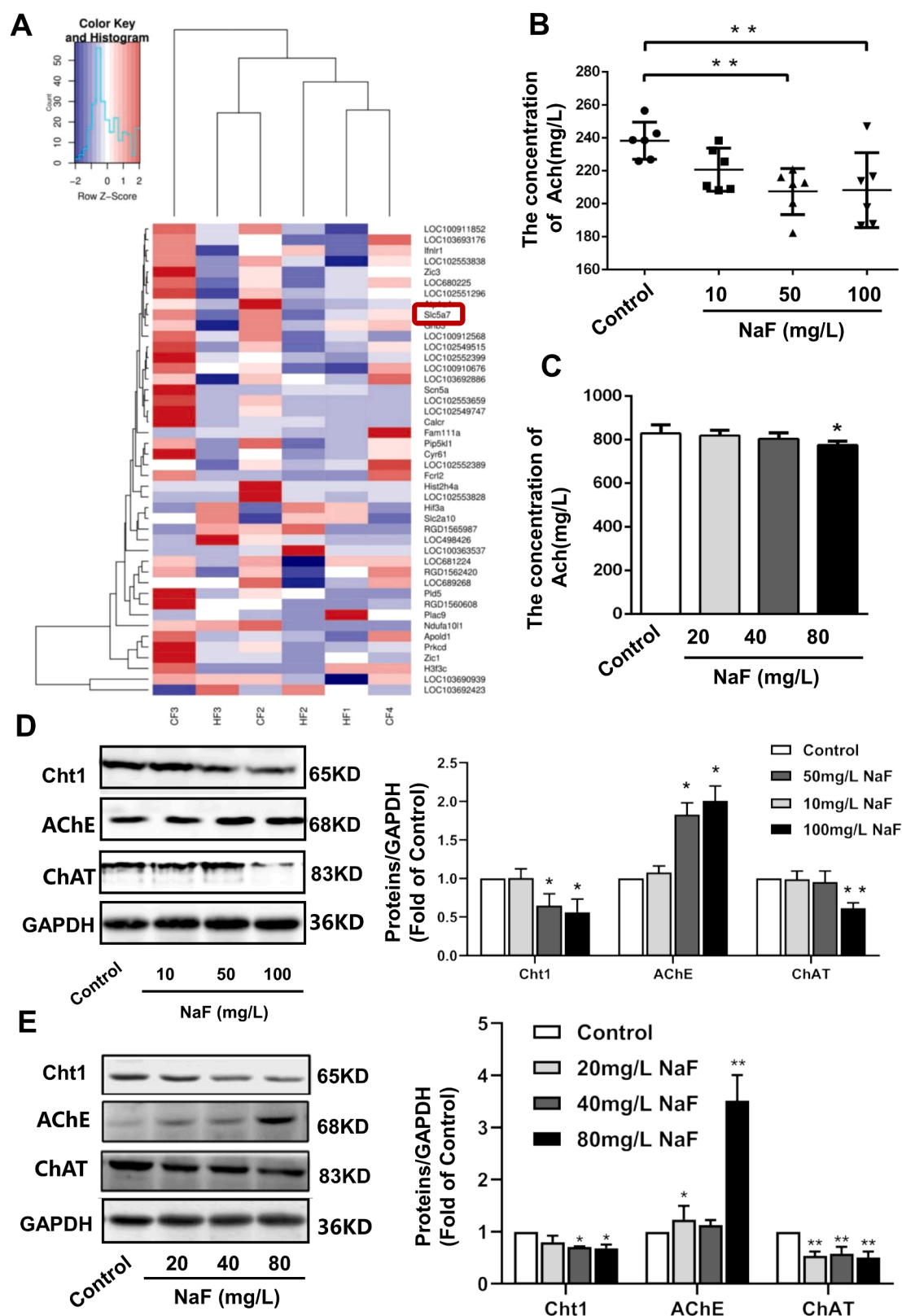


Fig. 2. NaF led to cholinergic system dysfunction in SD rats and PC12 cells. (A) Heatmap of differentially expressed genes in rat hippocampus from the control and NaF-treated (100 mg/L) groups. (n = 3) (B) ACh levels in rat serum detected by ELISA (C) ACh levels in the supernatant of PC12 cells detected by ELISA. (D) Representative western blotting images and quantification of ChAT, Cht1 and AChE protein levels in the hippocampus of SD rats. (E) Representative western blotting images and quantification of ChAT, Cht1 and AChE protein levels in PC12 cells. The data are presented as the mean $\pm$ S.D. of six independent experiments for animals independent and three independent experiments for cells. * $P < 0.05$ vs. the control group; ** $P < 0.01$ vs. the control group.

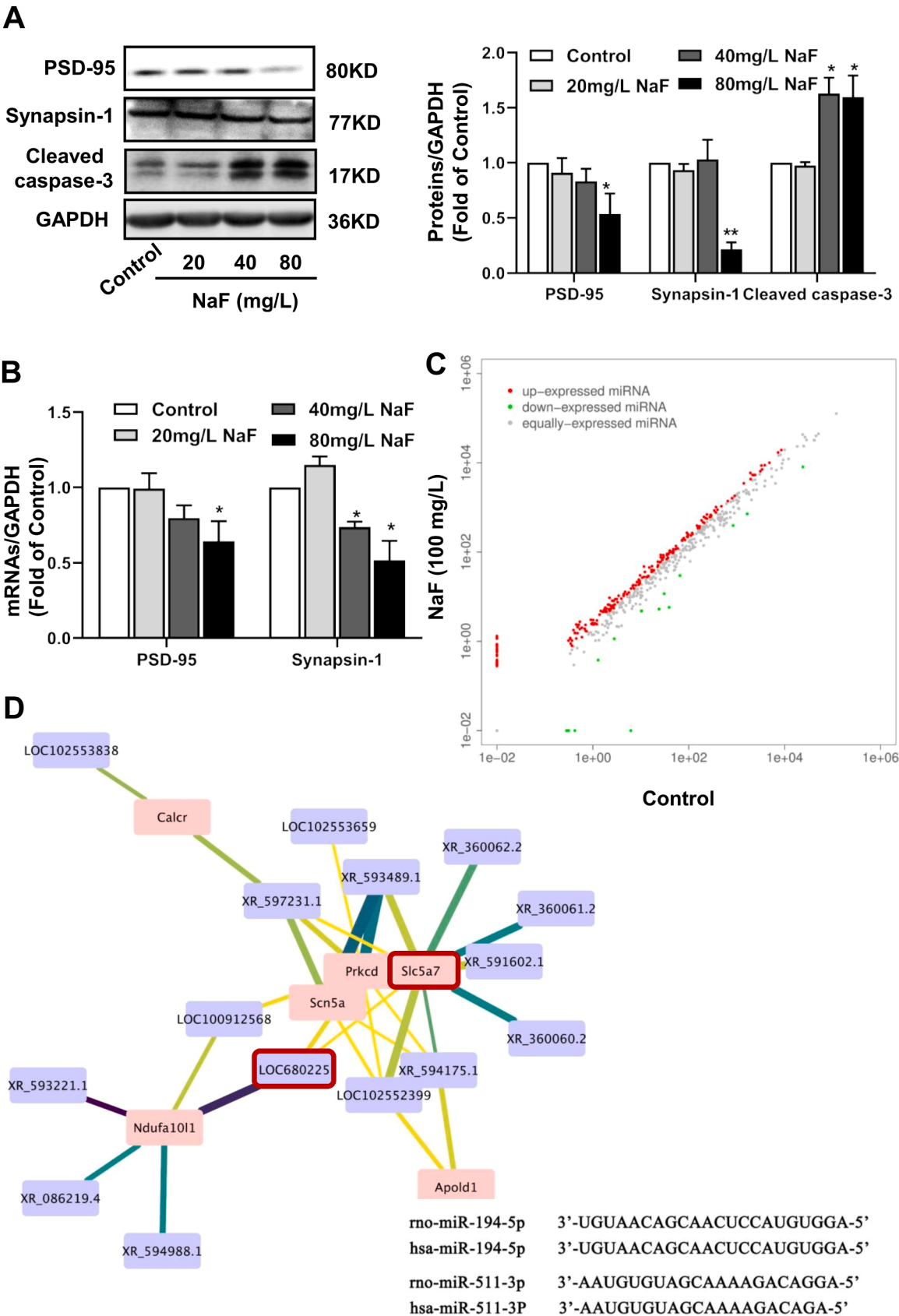


Fig. 3. NaF impaired synaptic plasticity and induced excessive apoptosis in PC12 cells. Differentially expressed lncRNAs and miRNAs were screened in the hippocampus of female rats exposed to NaF, which were predicted to interact with *Slc5a7*. (A) Representative western blotting images and quantification of PSD-95, Synapsin-1, and cleaved caspase-3 in PC12 cells. (B) RT-qPCR analysis of Synapsin-1 and PSD-95 in PC12 cells. (C) Differentially expressed miRNAs induced by fluoride in rat hippocampus. (D) Cytoscape network diagram showing the binding relationships among differentially expressed lncRNAs, miRNAs and mRNAs. The data are presented as the mean $\pm$ S.D. of three independent experiments. * $P < 0.05$ vs. the control group; ** $P < 0.01$ vs. the control group.

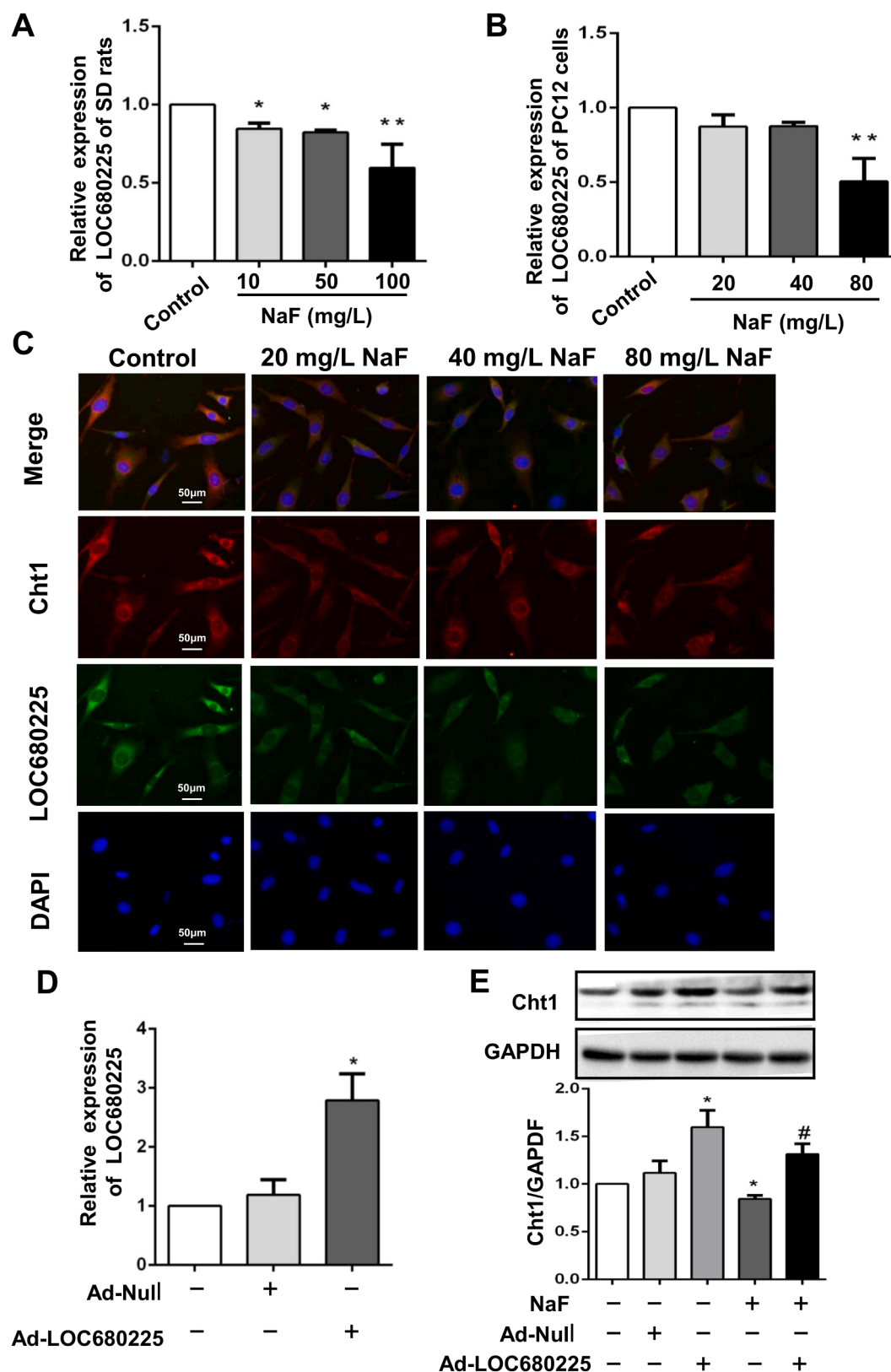
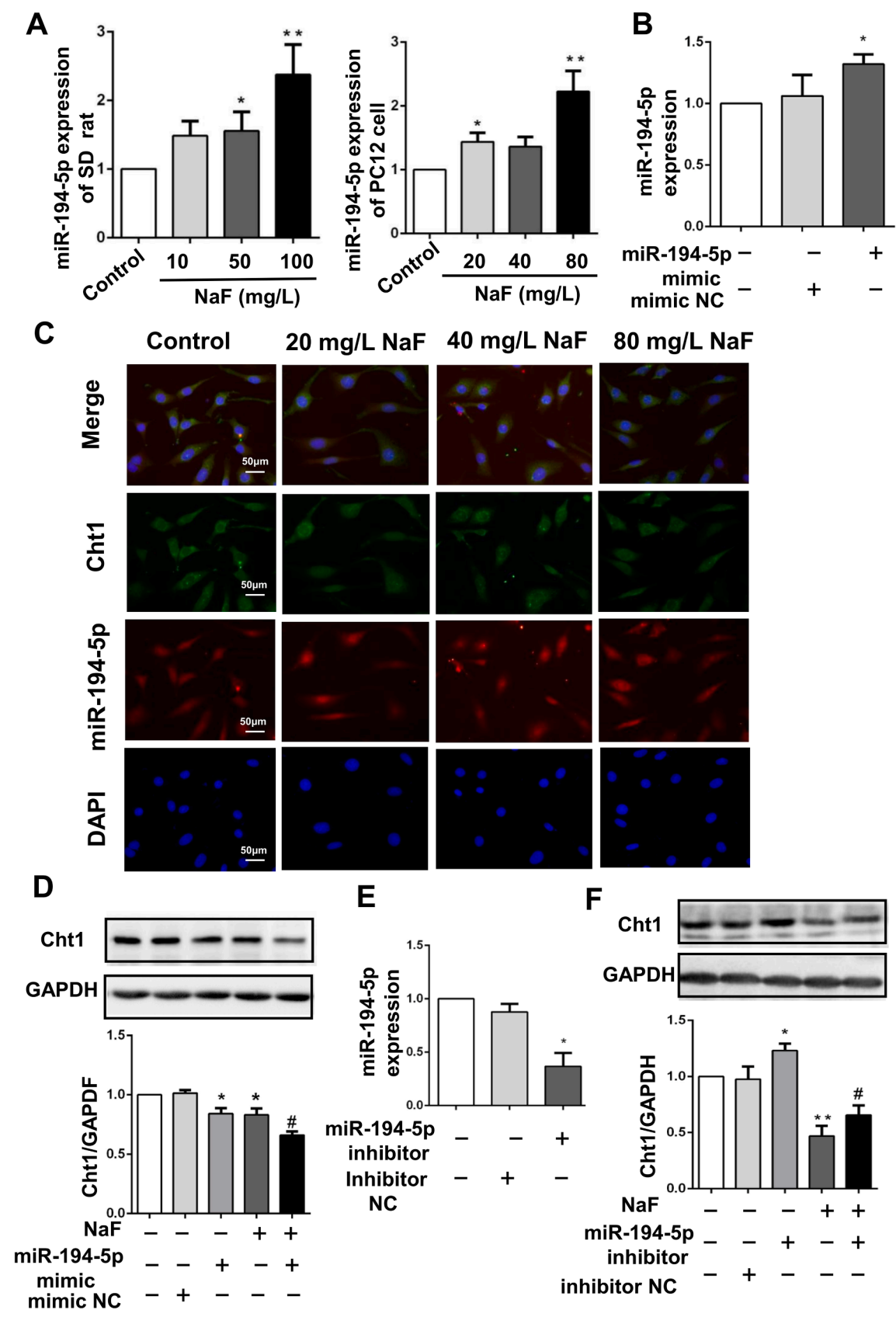


Fig. 4. LOC680225 is involved in fluoride-induced downregulation of Slc5a7 expression. (A) Expression of LOC680225 in the hippocampus of female SD rats. (B) Expression of LOC680225 in PC12 cells. (C) Representative FISH images showing the expression of LOC680225 and Cht1; scale bar = 50 μ m. (D) Relative expression of LOC680225 after transfection with Ad-LOC680225. (E) After infection with Ad-LOC680225 or Ad-Null (all at an MOI of 200, pretreatment for 24 h), PC12 cells were treated with 80 mg/L NaF for 24 h. Representative Western blotting image and quantification of Cht1 after transfection with Ad-LOC680225 and NaF treatment. The data are presented as the mean $\pm$ S.D. of three independent experiments. * $P < 0.05$ vs. the control group; ** $P < 0.01$ vs. the control group; # $P < 0.05$ vs. the NaF-treated group.



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Fig. 5. miR-194-5p is involved in fluoride-induced downregulation of Slc5a7 expression. (A) The expression of miR-194-5p in the hippocampus of female SD rats and PC12 cells. (B) PC12 cells were transfected with 50 nM miR-194-5p mimic or mimic NC for 48 h. Relative expression of miR-194-5p after transfection with miR-194-5p mimic or mimic NC. (C) Representative FISH images showing the expression of miR-194-5p and Cht1; scale bar = 50 μ m. (D) After infection with miR-194-5p mimic or mimic NC (pretreatment for 24 h), PC12 cells were treated with 80 mg/L NaF for 24 h. Representative Western blotting image and quantification of Cht1 after transfection with miR-194-5p mimic/mimic NC and NaF treatment. (E) Relative expression of miR-194-5p after transfection with miR-194-5p inhibitor or inhibitor NC. (F) Representative Western blotting images and quantification of Cht1 in PC12 cells transfected with miR-194-5p inhibitor or inhibitor NC, followed by NaF treatment. The data are presented as the mean $\pm$ S.D. of three independent experiments. * $P < 0.05$ vs. the control group; ** $P < 0.01$ vs. the control group; # $P < 0.05$ vs. the NaF-treated group.

reverse fluoride-induced upregulation of cleaved caspase-3 and the decline in cell viability, further supporting the role of the LOC680225/miR-194-5p/Slc5a7 axis in mediating fluoride neurotoxicity.

4. Discussion

Epidemiological investigations, as well as in vitro and in vivo experimental studies, have shown that excessive fluoride exposure can induce neurotoxicity, manifested as reduced IQ in children, impaired learning and memory in rats, and disrupted neurodevelopment (Till et al., 2020; Veneri et al., 2023; Zhao et al., 2024, 2023). Previous studies on fluoride exposure have predominantly focused on the gestational period in female or neonatal animals. In addition, our study used an adolescent rat model and found that long-term fluoride exposure during puberty can also cause cognitive impairment, which is consistent with our previous findings (Tang et al., 2024). Bittencourt LO et al. also reported that sustained high-fluoride exposure from adolescence through adulthood can induce comprehensive hippocampal damage manifested by molecular, morphological, and functional impairments (Bittencourt et al., 2023). These observations point to puberty as another susceptible developmental window to fluoride-triggered neurological impairments. Neither human epidemiological investigations nor animal studies have identified significant sex-specific differences in fluoride-induced neurotoxicity (Green et al., 2020), which is also supported by our preliminary epidemiological findings (Wang et al., 2020). In addition, male rats are innately aggressive and prone to fighting and biting one another during group housing. Considering this confounding factor, female SD rats were selected as experimental subjects in the current study.

Fluoride exposure has been shown to increase hippocampus synaptic cleft width, reduce dendritic spine density, and downregulate the expression of PSD-95 (Chen et al., 2018). Through whole transcriptome sequencing we identified that the cholinergic system is involved in fluoride-induced neurotoxicity. Consistent with this, low-to-moderate fluoride exposure has been associated with cholinergic system alterations in school-aged children (Wang et al., 2021). Specifically, fluoride inhibited the mRNA and protein expression of Slc5a7 and ChAT. Downregulation of Slc5a7 leads to insufficient precursor supply for ACh synthesis, while reduced ChAT further impairs ACh production. Unexpectedly, both mRNA and protein levels of AChE were significantly increased, accompanied by decreased serum ACh content. Collectively, NaF exposure results in reduced ACh synthesis and enhanced ACh hydrolysis, ultimately lowering ACh levels and weakening signal transmission between neurons.

Cholinergic interneurons act as “gatekeepers” of striatal circuitry and play pivotal roles in attention, goal-directed behavior, habit formation, and behavioral flexibility (Poppi et al., 2021). Dysfunction of cholinergic interneurons has been linked to numerous neurodevelopmental, neurodegenerative, and neuropsychiatric disorders (He et al., 2023; Schumacher et al., 2022; Wu et al., 2023). In SD rats with chronic fluorosis, downregulation of M₁ mAChR and M₃ mAChR was significantly associated with learning and memory deficits and excessive oxidative stress (Dong et al., 2015). In contrast, Dondossola reported that prolonged NaF exposure significantly decreased AChE level in zebrafish brain (Dondossola et al., 2022). Notably, in PC12 cells exposed to NaF, we observed cholinergic system changes consistent with those in SD rats, supporting that prolonged fluoride exposure exerts adverse

neurological effects via disrupting the cholinergic system.

Meanwhile, we also found that fluoride reduced both mRNA and protein levels of the synaptic plasticity-related proteins PSD-95 and synapsin-1 in PC12 cells. A previous study reported that exposure to fluoride during critical neurodevelopmental periods may compromise excitatory synapses, leading to deficits in synaptic integrity and neuronal function (Qiu et al., 2025). As reported by Wang et al., honokiol could modulate mtROS homeostasis, partially through the AMPK / PGC-1 α / Sirt3 signaling cascade, to counter NaF-triggered oxidative-mitochondrial damage and ameliorate synaptic injury-associated cognitive impairments (Wang et al., 2022). In parallel, we found that fluoride exposure promoted apoptosis in PC12 cells. Also, fluoride can induce apoptosis in the ovaries of mice (Ommati et al., 2025). Existing evidence indicates that fluoride promotes spermatocyte apoptosis: MAMs facilitate IP3R1/MCU-driven mitochondrial calcium overload to drive cell death (Guo et al., 2025). Together, these data highlight that synaptic damage and apoptosis represent two key cellular manifestations of fluoride neurotoxicity.

LncRNAs function as key modulators in multiple neurobiological processes and are critically involved in neurotoxicity. Long-term cadmium exposure impairs cognitive function by activating lnc-Gm10532/m6A/FIS1 axis-mediated mitochondrial fission and dysfunction (Deng et al., 2023). lncRNA Neat1 aggravates sevoflurane-provoked neurotoxicity via sponging miR-298-5p and consequently elevating Srpkl expression (Wei et al., 2021). We found significant down-regulation of lncRNA LOC680225 and its interactive regulatory relationship with Slc5a7 in the cholinergic synaptic pathway. LOC680225, located on rat chromosome 17, exhibits sequence similarity with tubulin alpha-3 chain; to our knowledge, its biological function has not been previously reported. Overexpression of LOC680225 partially reversed fluoride-induced downregulation of Cht1, upregulation of cleaved caspase-3, and the reduction in cell viability. A dual-luciferase reporter assay verified the direct target binding interaction between LOC680225 and Slc5a7. Previous studies have indicated that lncRNAs are also involved in the pathogenesis of dental fluorosis (Hu et al., 2024). For the first time, we identified the role of LOC680225 in fluoride-induced neurotoxicity. Nevertheless, we only assessed the effect of LOC680225 overexpression in this study, and complementary knockdown studies are needed to validate these findings.

MiR-124, miR-182, and miR-10a-5p are involved in fluoride-induced neurotoxicity (Manoharan et al., 2024). miR-296-5p promotes autophagy in mouse LS8 cells under excessive fluoride exposure via the AMPK/ULK1 pathway (Luo et al., 2022). Moreover, our previous study demonstrated that miR-708-3p/SIRT1 signaling alleviates fluoride-induced neuronal damage by regulating mitochondrial network dynamics (Zhao et al., 2023). Bioinformatics analysis revealed that LOC680225 functions as a ceRNA and co-regulates Slc5a7 expression by competitively binding to miR-194-5p. miR-194-5p has been reported to modulate the proliferation and apoptosis of hippocampus neurons in pediatric epilepsy (Niu et al., 2021). Furthermore, we observed that inhibition of miR-194-5p alleviated fluoride-induced reductions in cell viability and elevated expression of cleaved caspase-3, and vice versa. Direct target binding between miR-194-5p and Slc5a7 was verified using a dual-luciferase reporter assay. Based on these findings, our study demonstrated that LOC680225 might function as a ceRNA to regulate Slc5a7 expression by competitively binding to miR-194-5p, thereby mediating cholinergic system dysregulation during fluoride

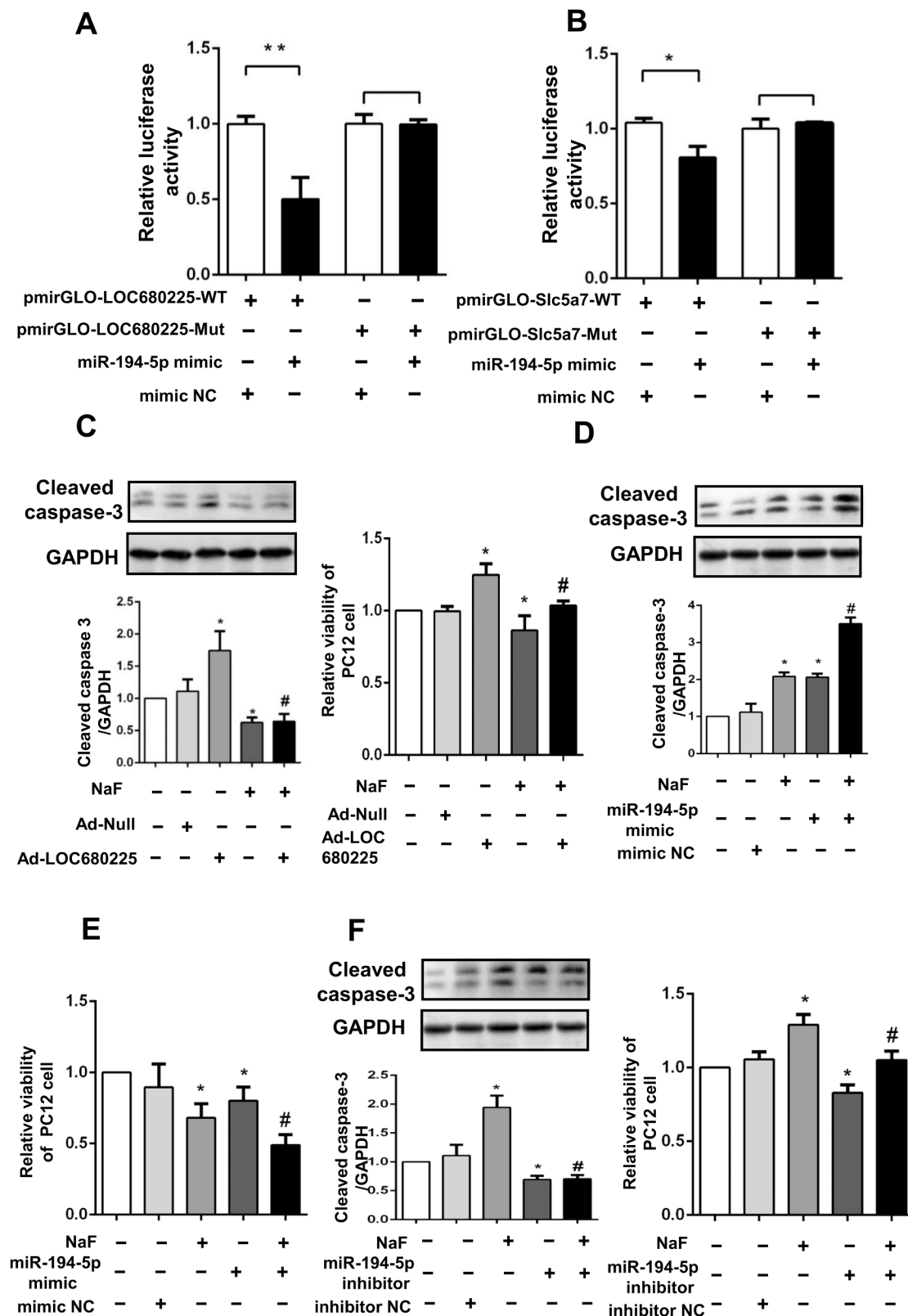


Fig. 6. miR-194-5p targets both LOC680225 and *Slc5a7*, and LOC680225 together with miR-194-5p modulates cleaved caspase-3 expression and cell viability in NaF-treated PC12 cells. (A) 293 T cells were co-transfected with miR-194-5p or mimic NC with WT or MUT-UTR of LOC680225, Relative luciferase activity was assayed. (B) 293 T cells were co-transfected with miR-194-5p or mimic NC with WT or MUT-UTR of *Slc5a7*, Relative luciferase activity was assayed. (C) Western blotting analysis of cleaved caspase-3 and cell viability in PC12 cells after transfection with Ad-LOC680225 and NaF treatment. (D) Western blotting analysis of cleaved caspase-3 and cell viability in PC12 cells after transfection with miR-194-5p mimic and NaF treatment. (E) Western blotting analysis of cleaved caspase-3 and cell viability in PC12 cells after transfection with miR-194-5p inhibitor and NaF treatment. The data are presented as the mean $\pm$ S.D. of three independent experiments. * $P < 0.05$ vs the control group, ** $P < 0.01$ vs control group. # $P < 0.05$ vs the NaF treated group.

neurotoxicity. To our knowledge, this is the first report describing a ceRNA mechanism in fluoride neurotoxicity. But, in the present study, we only conducted a preliminary investigation into the ceRNA mechanism underlying LOC680225/miR-194-5p interaction, and further *in vivo* experiments are required to validate this regulatory mechanism. Also, in male offspring, BPB potentially targets key transcription factors (Tcf4/Neurog2/Sp9) to dysregulate lncRNA NON-MMUT096884.1. Acting as a ceRNA, this lncRNA further perturbs the miR-139-3p/-miR-7118-5p-Galr1/Oxt axis and neuropeptide-dependent synaptic transmission, contributing to depression- and anxiety-like behavioral phenotypes(Xue et al., 2025).

Our study has several limitations. First, only female SD rats were included in this study; cohorts with equal male-to-female ratios were not established. Second, we did not manipulate the expression of LOC680225 and miR-194-5p *in vivo* to investigate their potential as intervention targets for fluoride neurotoxicity. In addition, we did not perform LOC680225 knockdown experiments, leaving unanswered whether knockdown of LOC680225 alone could reproduce fluoride-mediated neurotoxic phenotypes. At last, we did not measure the cellular miR-194-5p levels following LOC680225 overexpression. Further comprehensive studies are required to clarify the regulatory relationship among LOC680225, miR-194-5p, and *Slc5a7*.

5. Conclusions

In conclusion, by whole-transcriptome sequencing, we identified *Slc5a7* as a differentially expressed gene, along with the noncoding RNAs LOC680225 and miR-194-5p, in the hippocampus of fluoride-exposed rats. Based on the putative ceRNA regulatory hypothesis, our data suggest that LOC680225 may function as a ceRNA to modulate *Slc5a7* expression via competitive binding to miR-194-5p, thereby alleviating cholinergic system dysregulation under fluoride-induced neurotoxicity. Our findings may help establish a novel theoretical foundation for developing optimized preventive and therapeutic strategies targeting fluoride-induced neurotoxicity.

CRedit authorship contribution statement

Li Xie: Validation, Methodology. **Tao Xia:** Investigation. **Aiguo Wang:** Writing – review & editing, Visualization, Supervision, Resources, Funding acquisition, Conceptualization. **Jingwen Chen:** Validation, Investigation. **Guoyu Zhou:** Methodology, Formal analysis. **Sumei Wang:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Qiang Niu:** Methodology, Investigation.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2026.120724](https://doi.org/10.1016/j.ecoenv.2026.120724).

Data availability

The authors do not have permission to share data.

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