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Melatonin protects against fluoride-induced developmental neurotoxicity by alleviating abnormal mitophagy and apoptosis via the PINK1/Parkin pathway

Chun Wang [a,b,c], Runjiang Ma [a,b,c], Wenqi Qin [a,b,c], Chulin Yan [a,b,c], Meng Zhang [a,b,c], Yajie Li [a,b,c], Yongkang Liang [a,b,c], Huayong Wu [d,*], Jingjing Zhang [a,b,c,**], Qiang Niu [a,b,c,**]

[a] Shihezi university school of public health, Shihezi, Xinjiang, PR China
[b] Key Laboratory for Prevention and Control of Emerging Infectious Diseases and Public Health Security, Xinjiang, PR China
[c] Key Laboratory of Xinjiang Endemic and Ethnic Diseases (Ministry of Education), School of Medicine, Shihezi University, Shihezi, Xinjiang, PR China
[d] Center for Disease Control and Prevention of The Seventh Division Hu Yanghe City, PR China

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ABSTRACT

Fluoride induces developmental neurotoxicity, although the underlying mechanisms remain unclear. This study aimed to elucidate the roles of mitophagy and apoptosis mediated by the PTEN-induced kinase 1 (PINK1)/E3 ubiquitin-protein ligase Parkin (Parkin) pathway in fluoride-induced developmental neurotoxicity, as well as the protective effects of melatonin. A sodium fluoride (NaF) exposure model with melatonin intervention was established in F_1-generation Sprague-Dawley (SD) rats. Exposure to NaF impaired spatial learning and memory performance in offspring rats, promoted mitophagy initiation, but disrupted autophagic flux, resulting in accumulation of autophagosomes and subsequent neuronal apoptosis—evidenced by elevated levels of PINK1, Parkin, translocase of outer mitochondrial membrane 20 (TOMM20), Voltage-dependent anion channel (VDAC1), OMA1 zinc metallopeptidase (OMA1), microtubule-associated protein 1 light chain 3-II (LC3-II), sequestosome 1 (SQSTM1/p62), cleaved poly (ADP-ribose) polymerase (cleaved PARP), and BCL-2 Associated X protein (BAX), reduced levels of B-cell lymphoma 2 (Bcl-2) in brain tissues. Notably, melatonin treatment attenuated NaF-induced neurotoxicity by enhancing mitophagic clearance via activation of the PINK1/Parkin pathway, thereby restoring autophagic flux and suppressing apoptotic cell death. Collectively, our findings demonstrate that NaF activates the PINK1/Parkin-mediated mitophagy pathway; however, incomplete autophagic degradation leads to mitochondrial dysfunction and neuronal apoptosis, contributing to developmental neurotoxicity. Importantly, melatonin mitigates these adverse effects, suggesting its potential as a therapeutic agent for preventing fluoride-induced neurodevelopmental impairment through modulation of the PINK1/Parkin signaling axis.

1. Introduction

Fluorine is one of the commonly recognized elements in the human environment and can enter the human body through media such as water, food, and air (Wan et al., 2021). A trace intake of fluoride is beneficial for dental and bone health. However, overexposure to fluoride may result in health issues, including dental fluorosis and skeletal fluorosis, etc (Fujiwara et al., 2021), and can also cause damage to other non-skeletal systems, such as the cardiovascular system, nervous system, blood glucose homeostasis, as well as liver and kidney function, thyroid function, reproductive system, and immune system (Yan et al., 2021). Due to the non-renewable nature of neurons, the nervous system is particularly vulnerable to fluoride-induced damage (Chen et al., 2018). Research demonstrates that prolonged excessive fluoride exposure may result in its accumulation in brain tissue via the blood-brain barrier (BBB), thereby causing neurotoxicity and damaging brain function,

* Correspondence to: Department of Center for Disease Control and Prevention of The Seventh Division Hu Yanghe City, Xinjiang 834034, PR China.
** Correspondence to: Department of Preventive Medicine, School of Medicine, Shihezi University, North 2th Road, Shihezi, Xinjiang 832000, PR China.
E-mail addresses: 371539317@qq.com (H. Wu), 463739163@qq.com (J. Zhang), niuqiang@shzu.edu.cn (Q. Niu).

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which contributes to changes in cognitive function and mental symptoms (Ren et al., 2022).According to the World Health Organization, the permissible limit of fluoride in drinking water is 1.5 mg/L(Organization, World Health., 2022). However, chronic exposure to levels exceeding this limit, commonly observed in endemic fluorosis areas, poses a significant health risk. Epidemiological studies have found that mother's exposure to fluoride during gestation is associated with intellectual disabilities in their offspring (Farmus et al., 2021). Both cross-sectional studies and cohort studies indicated that prenatal exposure to fluoride could result in intellectual and cognitive impairment in offspring (Prabhakar et al., 2021; Goodman et al., 2022). Animal experiments have further demonstrated that exposure to fluoride during the gestational and lactation periods can damage the hippocampal tissue of rats and affect their cognitive function (Ferreira et al., 2021). Therefore, these studies collectively indicate that fluoride exposure during the early phase of growth can cause neurotoxicity. However, the mechanisms by which fluoride induces developmental neurotoxicity are not yet fully understood.

The stability of the mitochondrial environment is essential for neuronal development, function, and survival (Dawson and Dawson, 2017). Mitophagy can specifically remove damaged mitochondria, thereby maintaining the homeostasis of the mitochondrial internal environment and promoting cell survival. In neurons, the inability to fully clear damaged mitochondria through mitophagy induces the generation of reactive oxygen species, other oxidative substances, and reactive nitrogen species, which may contribute to the development of various neurodegenerative diseases (Shefa et al., 2019). Current research has discovered that the mechanisms of mitophagy can be broadly categorized into two major types: ubiquitin (Ub) -dependent pathways and non-ubiquitin -dependent pathways. Among these mechanisms, the PINK1/Parkin pathway is the most extensively studied. Under conditions of impaired mitochondrial membrane potential, PINK1 fails to be imported into the mitochondrial inner membrane, leading to its stable accumulation on the outer mitochondrial membrane. Parkin, an E3 ubiquitin ligase, then catalyzes the attachment of Ub molecules to substrate proteins, targeting them for proteasomal recognition and degradation. By ubiquitinating multiple mitochondrial membrane proteins, Parkin recruits cargo receptors (such as SQSTM1), thereby facilitating the clearance of damaged mitochondria through mitophagy (Dong et al., 2023). After mitochondrial damage, PINK1 interacts with Parkin to cooperatively regulate the mitophagy process, thereby maintaining mitochondrial quality control (Lu et al., 2023). Accumulating research indicates that defects in PINK1/Parkin-dependent mitophagy play a key role in the development of several neurological diseases (Li et al., 2022) (Mao et al., 2022). Previous research has revealed that perinatal exposure to fluoride during perinatal may cause autophagy defects, leading to learning and memory impairments (Zhao et al., 2019). Dong et al. (Dong et al., 2024) proposed that chronic fluorosis can cause brain damage by affecting mitophagy. However, the specific mechanism by which fluoride participates in the mitophagy pathway through PINK1/Parkin to produce neurotoxicity remains incompletely understood.

Research has demonstrated that melatonin participates in mitophagy, mitochondrial function and apoptosis processes, and can improve hypoxia-induced autophagy and cell death (Zhang et al., 2024), restore redox homeostasis and reduce excessive autophagy (Wang, Wang et al., 2024). Based on the research report by (Alghamdi, 2018), melatonin can significantly improve neuronal damage caused by $HgCl_2$. The research found that melatonin pretreatment weakened TOCP-induced autophagy by inhibiting oxidative stress and sustaining ERK1/2 phosphorylation (Said et al., 2021). Additionally, it is (Potes et al., 2023) reported that melatonin can reduce oxidative stress and significantly alleviate neuronal damage. Melatonin is an endogenous circadian rhythm indoleamine (Amaral and Cipolla-Neto, 2018), secreted primarily by the pineal gland, that exhibits diverse biological functions, including anti-inflammatory, antioxidant, and anti-tumor properties. It also easily crosses the BBB and has neuroprotective effects (Zhou et al., 2019). However, the precise mechanism through which melatonin exerts its protective role against fluoride-induced neurotoxicity remains incompletely understood.

Therefore, we constructed a NaF exposure model and a melatonin intervention model in SD rats, aiming to elucidate the specific neuroprotective actions of melatonin against fluoride exposure and its potential regulatory mechanism on fluoride-induced abnormal mitophagy, thus providing a theoretical foundation for understanding fluorosis and the therapeutic potential of melatonin.

2. Materials and methods

2.1. Antibodies, reagents, chemicals

NaF and melatonin were provided by Sigma Corporation (USA). The RIPA cell lysate was obtained from Beijing Solaibao Technology Co., LTD. Horseradinase-labeled rabbit anti-goat IgG, horseradinase-labeled mouse anti-goat IgG, purchased from Beijing Zhongshan Jinqiao Biotechnology Co., LTD. GAPDH, PINK1, Parkin, LC3, SQSTM1, cleaved PARP, TOMM20, VDAC1, OMA1, BAX, Bcl-2 antibodies were provided by Proteintech (USA).

2.2. Animals and treatments

The SD rats were served by Sibefu Biotechnology Co., LTD., with the license number: SYXK (Beijing) 2024–0010. Rats were subjected to a controlled 12:12 h light:dark photoperiod, humidity (50 %–60 %), and constant temperature (20–25°C). All rats were raised in plastic cages, drank double-distilled water and were fed standardized pellet feed. All animal experiments involved in the present study were approved by the Animal Research Ethics Committee of Shihezi University School of Medicine and were administered in strict accordance with the "Guidelines for the Care and Use of Laboratory Animals" issued by the Ministry of Health of the People's Republic of China.This study has been approved by the Animal Experiment Ethics Review Committee of the First Affiliated Hospital of the Medical College of Shihezi University, and the relevant approval documents are available upon request.

Thirty-six SPF-grade SD rats (180–220 g), with a ratio of male to female of 1:2. During the experiment, rats were randomly assigned to 6 groups (control group (double-distilled water), 10 mg/kg NaF group, 20 mg/kg NaF group, 40 mg/kg NaF group, melatonin alone group (10 mg/kg), and co-treatment group (40 mg/kg NaF+10 mg/kg melatonin). Males and females were housed together at a 1:2 ratio (2 males and 4 females per group). After the cage was closed, the rats were observed at 9 a.m. every day. Conception was determined by the detachment of the vaginal plug or the detection of sperm through vaginal smear microscopy. Then, the pregnant female rats were raised separately. NaF was administered to female rats by gavage from the date of conception. The exposure doses were as follows: control group (double-distilled water), 10 mg/kg NaF group, 20 mg/kg NaF group, and 40 mg/kg NaF group. The NaF exposure dose was determined on the basis of the fluoride ion metabolism level of SD rats, residents' fluoride exposure in endemic fluorosis regions, and some related literature (Araujo et al., 2019; Jiang et al., 2019).After the F_1 generation was born, litter sizes were adjusted within the same dose group to be approximately equal to minimize the litter effect. On postnatal day (PND) 8, the melatonin alone group and the co-treatment group began to receive melatonin intervention. On PND 10, the parental exposure dose was continued for intragastric administration of NaF to the offspring. Upon weaning (PND 21), the F_1 generation were separated from their dams and housed by sex. At this point, maternal exposure ceased, and the F_1 generation received the same NaF exposure regimen as their dams until 2 months of age. After that, 6 F_1 generation rats (1:1 male and female) were chosen from each cohort utilizing randomization for the Morris water maze (MWM) experiment. Following the MWM experiment, F_1

generation rats were sacrificed within 8 h, and hippocampus samples were taken on ice. The remaining samples should be covered in Tinfoill, then rapidly frozen in liquefied nitrogen for 15 s, marked with a marker pen, and finally transferred to an −80℃ refrigerator for storage and future use.

2.3. Morris water maze test (MWM)

The MWM (XR-XM101, China) instrument is comprised of a circular water pool (Its dimensions are 1.8 m in diameter and 0.5 m in height), an underwater platform (A black column, its diameter is 8 cm and its height is 30 cm.) a camera was fixed in a stationary position above the center of the pool in the target quadrant to take images of the swimming rats and was attached to a monitoring system. The pool was filled with water (maintained at 24 ± 1℃), with the water level 2 centimeters above the platform. Non-toxic black ink was added to render the water opaque, thereby hiding the platform.

MWM testing includes Positioning and Navigation Test (PNT) and Space Detection Test (SPT). In PNT, each rat was oriented towards the wall and gently introduced into the water to begin a free-swimming trial. If the platform was found within 60 s, the escape latency was recorded and the rat was allowed to remain on the platform for 10 s. If not found, the rat was guided to the platform and allowed to stay for 15 s, with the escape latency recorded as 60 s. For four successive days, each rat will be placed in each of the quadrants once per day. The following parameters were documented: escape latency, swimming distance, swimming speed and swimming path. In the spatial position test (SPT), remove the platform and allow the rat to freely swim in the pool for 60 s. The following parameters were documented: number of platform crossings, time spent in the target quadrant, swimming distance, and swimming path.

2.4. Nissl staining

The hippocampal tissue was dehydrated at a gradient of 75~85 % ~ 90~95 % ~ 100~100 % alcohol, with each stage lasting for 4 h, 2 h, 1.5 h, 1 h, 0.5 h and 0.5 h respectively. After dehydration was completed, the hippocampal tissue was transferred to a mixed solution of anhydrous ethanol and xylene (with a mixing ratio of 1:1) for soaking for 10 min, followed by soaking in xylene for 10 min and 7 min respectively. Finally, soak the wax in 60℃ paraffin three times, each time for one hour. All the above steps were completed using a dehydrator. Fix the prepared paraffin embedding blocks on the specimen holder, cut them into 4 μm thick sections with an ultra-thin sectioning machine, and bake them at 60℃ for 3 h. Slice and add xylene twice, each time for 10 min. Twice in anhydrous ethanol, for 5 min each time. Then, add alcohol with a gradient of 95~90 % ~ 80~70 % in sequence, for 3 min, 3 min, 2 min, and 2 min respectively. Finally, perform water washing for 2 min. The staining was carried out with 1 % toluidine blue staining solution at 60℃ for 40 min and then washed with water three times. Dehydrate with 95 % alcohol until the background is clearly visible under the microscope. Finally, the water is successively dehydrated and made transparent with anhydrous ethanol and xylene. After air-drying, the plates are sealed.

2.5. Western blotting analysis

The hippocampus tissues were homogenized in RIPA buffer containing with 1 % protease inhibitor. The overall protein content was determined. The proteins were resolved via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. Then, incubate the PVDF membrane at room temperature contains 5 % skim milk lasting 2 h. Incubate with the primary antibody at 4℃ for 14 ~ 16 h, including GAPDH (1:50000), cleaved PARP (1:1000), PINK1 (1:5000), Parkin (1:4000), SQSTM1 (1:5000), LC3 (1:5000), TOMM20 (1:5000), VDAC1 (1:5000), OMA1 (1:5000), BAX (1:3000), Bcl-2 (1:5000). Subsequently, the membranes were added to the corresponding secondary antibody (1:20,000) and incubated for 2 h at room temperature. The membrane was developed with enhanced chemiluminescence (ECL) reagents on a chemiluminescent analyzer.

2.6. Statistical analysis

All data were analyzed using SPSS software (version 26.0). Repeated-measures ANOVA was used to analyze the water maze data. Perform multiple comparisons using one-way analysis of variance, succeeded by SNK multiple comparison tests. Values are mean ± SD. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. NaF exposure impairs the abilities in learning, memory, and retention of F_1 generation rats

We used the MWM method to evaluate the effects of NaF on learning and memory in rats. In the PNT experiment, the escape latency in the NaF treatment group and the control group showed a downward trend over the training days. However, the escape latency of rats in the 40 mg/kg NaF group from the second day to the fourth day was remarkably longer than that of the controls ($P < 0.05$; Fig. 1A). Rats in the 40 mg/kg NaF group exhibited remarkably greater swimming distances on days 2 and 4 compared to the control group ($P < 0.05$; Fig. 1B). On the first and third days, the 40 mg/kg NaF treatment group exhibited significantly lower swimming speeds than those of the control group ($P < 0.05$; Fig. 1C).

In the SPT, rats in all NaF-treated groups crossed the platform significantly less frequently than the controls. ($P < 0.05$; Fig. 1E). The percentage of time and the ratio of distance of rats that received with 40 mg/kg NaF treatment were remarkably lower than those of the controls ($P < 0.05$; Fig. 1F-G). Fig. D and Fig. H respectively showed the representative paths of PNT and SPT.

3.2. NaF exposure leads to neurons damage and induces apoptosis

To investigate the impact of NaF on neural injury in rats, we analyzed hippocampal neurons using Nissl staining to reveal the organization and number of Nissl bodies. In the control group, the Nissl bodies are more regularly arranged and present in greater numbers. The quantity of Nissl bodies hippocampal tissue decreased progressively in rat, their arrangement became irregular and indistinct as the NaF exposure dose increased. (Fig. 2A).

We also detected relevant indicators through Western blotting. Our results showed that, versus the control group, the cleaved PARP protein level in the 40 mg/kg NaF treatment group was remarkably increased. ($P < 0.05$; Fig. 2B-C). Bcl-2 protein level was significantly decreased in the 40 mg/kg NaF group ($P < 0.05$; Fig. 2B, D), while BAX protein expression was significantly increased in rats treated with 20 and 40 mg/kg NaF ($P < 0.05$; Fig. 2B, E).

3.3. NaF exposure causes abnormal mitophagy but impairs autophagic degradation

For the purpose of investigating the role of NaF in mitophagy, we detected mitophagy indicators by Western blotting. The findings demonstrated that the PINK1 protein level was substantially elevated in the 40 mg/kg NaF-treated group compared to the control group, and the Parkin protein level in the 20 and 40 mg/kg NaF-treated groups was also markedly increased ($P < 0.05$; Fig. 3A-B). Compared to the controls, the protein levels of SQSTM1 in the 20 and 40 mg/kg NaF treatment groups and LC3-II in the 40 mg/kg NaF treatment groups were notably increased ($P < 0.05$; Fig. 3C-D). In the 40 mg/kg NaF-treated group,

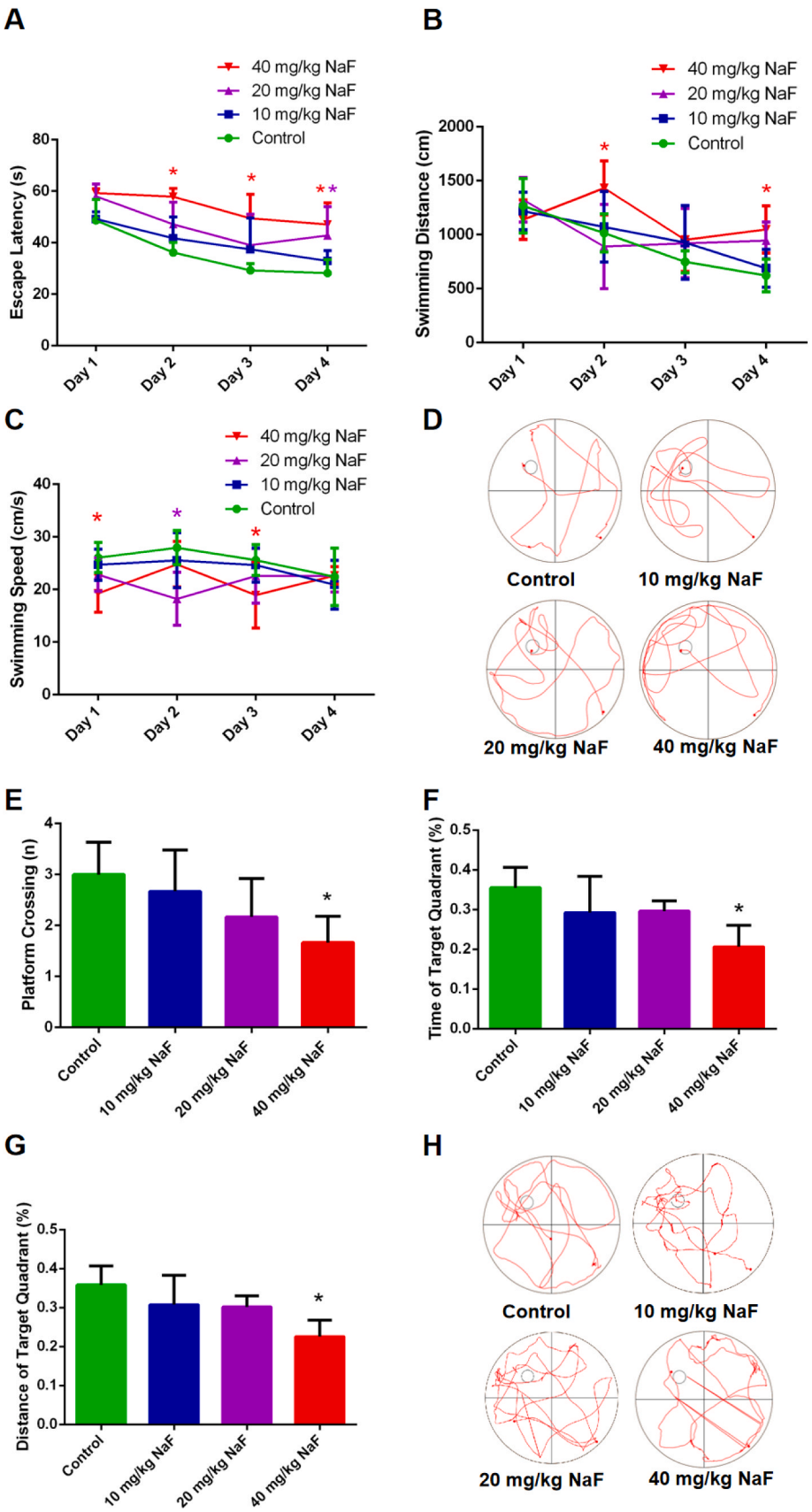

Fig. 1. NaF exposure impairs the abilities in learning, memory, and retention of F1 generation rats. (A) The mean escape latency to the platform. (B) The mean swimming distance to the platform. (C) The mean swimming speed to the platform. (D) Representative traces in the PNT. (E) The number of platform crossings. (F) Time spent in the target quadrant. (G) Distance spent in the target quadrant. (H) Representative traces in the SPT. The data are presented for six rats in each group. *$P < 0.05$ versus the control group.

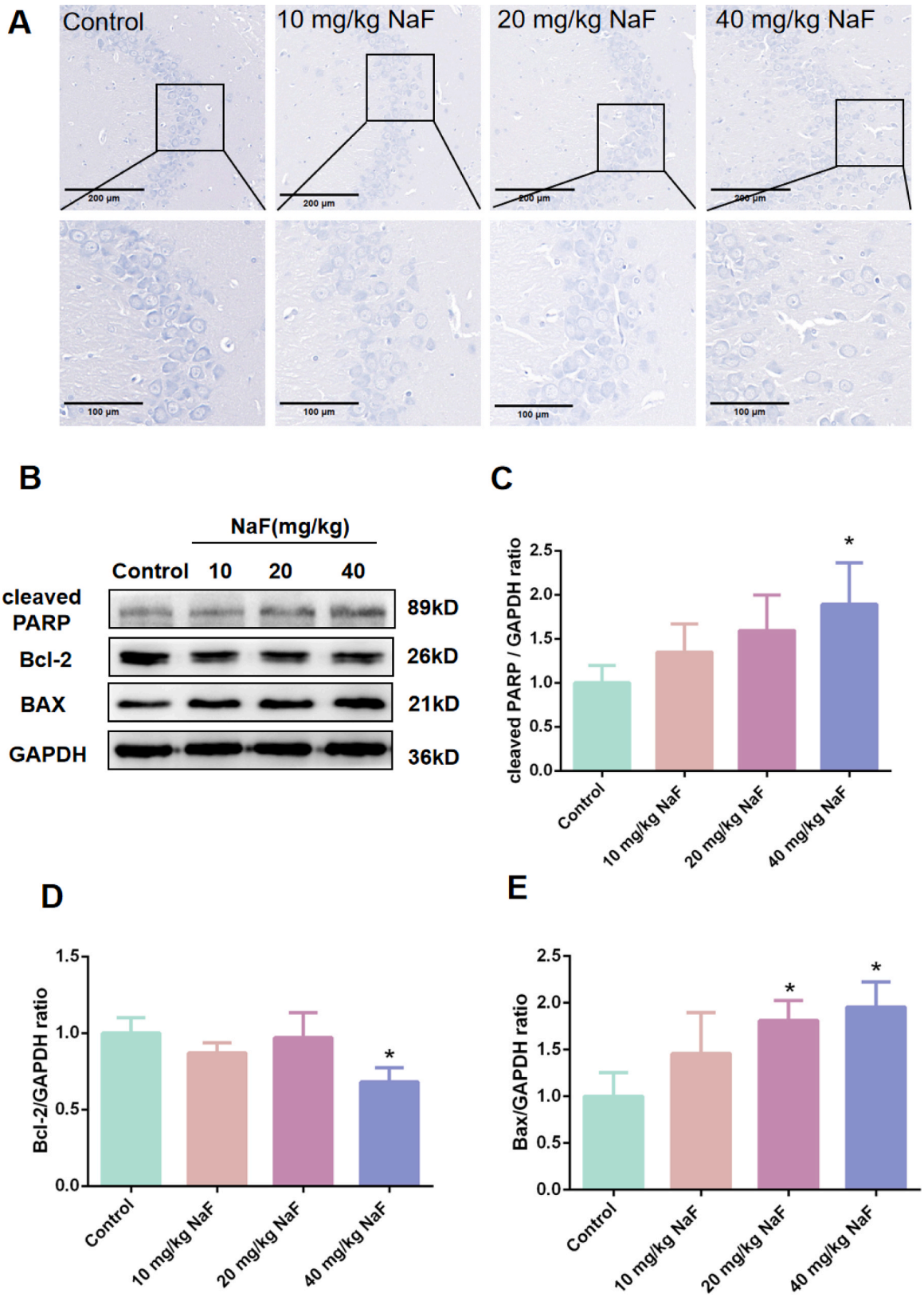

Fig. 2. NaF exposure leads to neurons damage and induces apoptosis. (A)The Nissl staining in rat hippocampus. (B) Representative images of western blot for apoptosis marker cleaved PARP, Bcl-2 and BAX in hippocampal tissues of adult rats. (C) Quantitative analyses of the apoptosis marker cleaved PARP in hippocampal tissues of adult rats. (D) Quantitative analyses of the apoptosis marker Bcl-2 in hippocampal tissues of adult rats. (E) Quantitative analyses of the apoptosis marker BAX in hippocampal tissues of adult rats. The data are presented as the means ± S.D. for three different experiments. The scale bar represents 20 μm. $^{*}P < 0.05$ versus the control group. $^{**}P < 0.01$ versus the control group. $^{***}P < 0.001$ versus the control group.

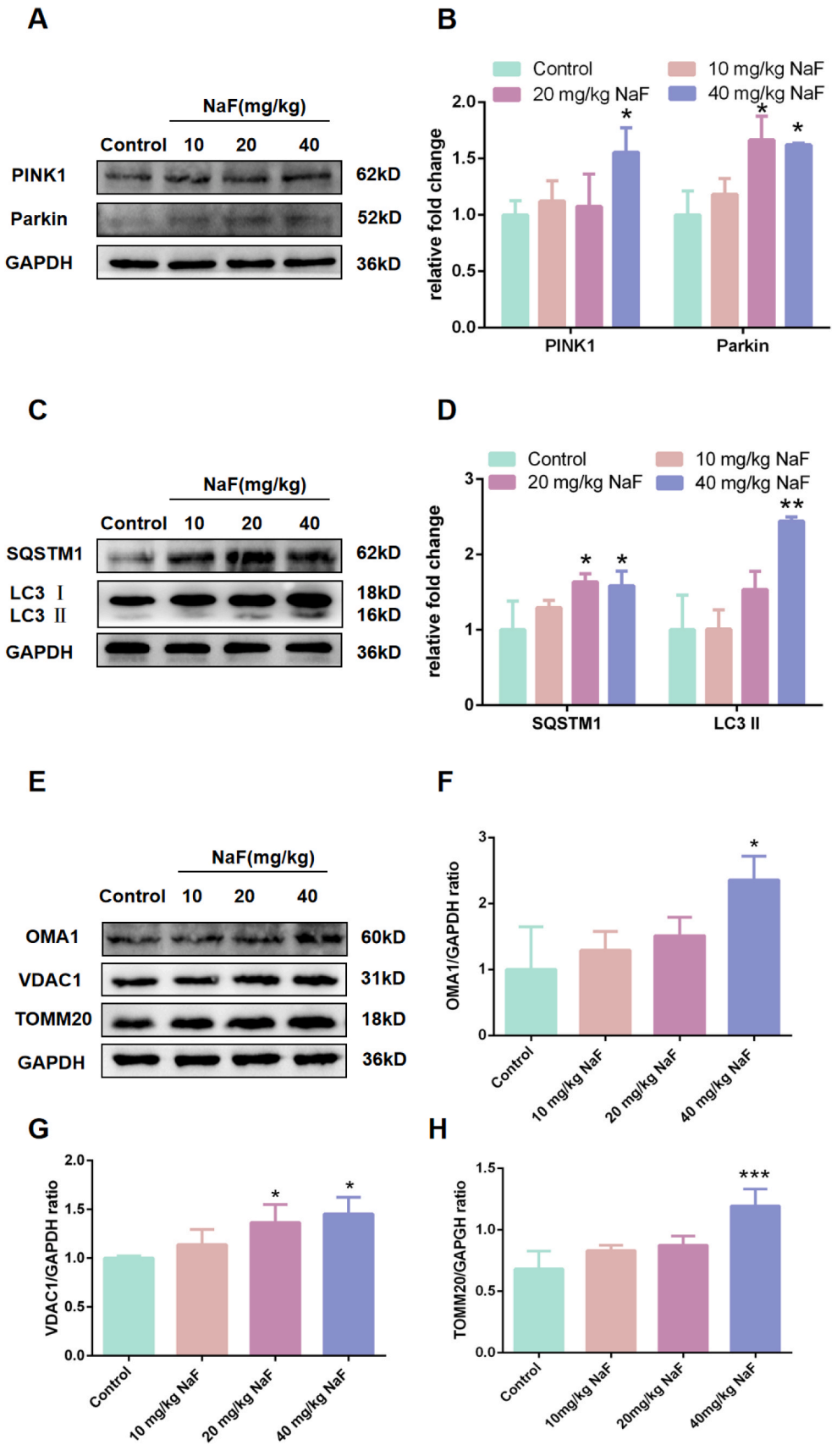

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Fig. 3. NaF exposure causes abnormal mitophagy but impairs autophagic degradation. (A) Representative images of western blot for mitophagy markers PINK1 and Parkin in hippocampal tissues of adult rats. (B) Quantitative analyses of the mitophagy marker PINK1 and Parkin in hippocampal tissues of adult rats. (C) Representative images of western blot for autophagy markers SQSTM1 and LC3-II in hippocampal tissues of adult rats. (D) Quantitative analyses of the autophagy markers SQSTM1 and LC3-II in hippocampal tissues of adult rats. (E) Representative images of western blot for OMA1, VDAC1 and TOMM20 in hippocampal tissues of adult rats.(F) Quantitative analyses of OMA1 in hippocampal tissues of adult rats. (G) Quantitative analyses of VDAC1 in hippocampal tissues of adult rats. (H) Quantitative analyses of TOMM20 in hippocampal tissues of adult rats. The data are presented as the means ± S.D. for three different experiments. $^{*}P < 0.05$ versus the control group. $^{**}P < 0.01$ versus the control group. $^{***}P < 0.001$ versus the control group.

OMA1 and VDAC1 protein expression significantly increased ($P < 0.05$; Fig. 3E-G).The level of TOMM20 was substantially elevated in the 40 mg/kg NaF treatment group compared to the control group. ($P < 0.05$; Fig. 3E, H).

3.4. Melatonin improves the abilities to learning, memory, and retention impaired by NaF exposure in F_1 generation rats

Evaluating the role of melatonin using the MWM method on improving NaF on learning and memory in F_1 generation rats. In the PNT experiment, the escape latency of the 40 mg/kg NaF-treated group and the Mel+NaF group showed a downward trend. Particularly, the 40 mg/kg NaF group exhibited a marked increase in escape latency on days 3–4 compared to the Mel+NaF group ($P < 0.05$; Fig. 4A). The rats in the 40 mg/kg NaF group on the second and fourth days exhibited significantly greater swimming distances than that in the Mel+NaF group ($P < 0.05$; Fig. 4B). The swimming speed of the 40 mg/kg NaF group was lower than that of the Mel+NaF group, but the variation is not statistically significant (Fig. 4C). Rats in every NaF group crossed the platform markedly less often than those in the Mel+NaF group during the SPT. ($P < 0.05$; Fig. 4E). Rats treated with 40 mg/kg NaF showed significantly lower time and distance percentages in the target quadrant compared to the Mel+NaF group ($P < 0.05$; Fig. 4F-G). Fig. D and Fig. H showed the typical paths of PNT and SPT, sequentially.

3.5. Melatonin alleviates abnormal mitophagy by NaF exposure and restores autophagic degradation ability

To explore whether melatonin improves the effect of NaF on mitophagy, we detected mitophagy indicators by Western blotting. The findings confirmed that, compared to the 40 mg/kg NaF treatment group, PINK1 and Parkin protein levels in the Mel+NaF group were significantly reduced ($P < 0.05$; Fig. 5A-B). Compared with 40 mg/kg NaF, SQSTM1 and LC3-II protein levels in the Mel+NaF group were markedly reduced ($P < 0.05$; Fig. 5C-D). Compared to the 40 mg/kg NaF group, OMA1 and VDAC1 protein expression were significantly reduced ($P < 0.05$; Fig. 5E-G) , the mitochondrial outer membrane protein TOMM20 protein level in the Mel+NaF treatment group of rats reduced significantly ($P < 0.05$; Fig. 5E, H).

3.6. Melatonin alleviates neurons damage and apoptosis caused by NaF exposure

To explore the improving effect of melatonin, hippocampal neurons were analyzed to reveal the organization and number of Nissl bodies by Nissl staining. In the Mel+NaF group, the neurons exhibited a high density and orderly arrangement of Nissl bodies. The 40 mg/kg NaF treatment group had fewer Nissl bodies in the hippocampus than the Mel+NaF group, the staining became lighter and indistinct (Fig. 6A)

We also detected associated indicators through Western blotting. Our observations indicate that, compared to the Mel+NaF group, the cleaved PARP protein level in the 40 mg/kg NaF treatment group was notably increased ($P < 0.05$; Fig. 6B-C). Bcl-2 protein expression was significantly increased, while BAX protein expression was significantly decreased ($P < 0.05$; Fig. 6B, D-E).

4. Discussion

This study indicates that NaF activates mitophagy via the PINK1/Parkin pathway, but impairs autophagic degradation, leading to apoptosis and consequently developmental fluoride neurotoxicity. It is worth noting that melatonin mitigated abnormal mitophagy via the PINK1/Parkin pathway, restored autophagic degradation capacity, and suppressed apoptosis, thereby reducing fluoride-induced developmental neurotoxicity in rats.

Previous findings have demonstrated that fluoride is recognized as a developmental neurotoxicant (Zhu et al., 2024; Qiu et al., 2025). Evidence from research shown that exposure to fluoride can affect serotonin (5-HT) and gamma-aminobutyric acid (GABA) Levels in rats, leading to neurological symptoms such as motor disorders, memory impairment, depression and anxiety (Cao et al., 2022). This effect may be attributed to the fact that fluoride can cross the BBB. It affects different brain regions and leads to behavioral, molecular and metabolic disorders (Li et al., 2019). Adkins EA et al. (Adkins and Brunst, 2021)found that fluoride, as a neurotoxicant, can impede mitochondrial complexes and alter intracellular homeostasis. Studies have shown that NaF exposure reduces Nrf-2 activity, thereby increasing oxidative stress, neuroinflammation and apoptosis in rats (Owumi et al., 2024). There is also epidemiological evidence implying that long-term fluoride exposure may cause interruptions in brain development, alter neural transmission and hormonal regulation, thereby leading to autism spectrum disorder (Strunecka and Strunecky, 2019). A study conducted in Mexico demonstrated that increased maternal fluoride intake during pregnancy was linked to reduced non-verbal ability in offspring at 24 months of age. (Cantoral et al., 2021). A review summarized the studies related to fluoride exposure and neurotoxicity and found that most epidemiological studies have demonstrated that lower intelligence indicatorsis associated with high fluoride exposure (Guth et al., 2020). Our findings are consistent with these reports, demonstrating that NaF exposure impaired learning, memory and memory-retention abilities in rats, which is in agreement with the research results of Xiang et al. (Xiang et al., 2023), further supporting the role of fluoride as a developmental neurotoxicant.

Yang et al. (Yang et al., 2024) discovered that aluminum exposure may lead to damage neuronal function, resulting in a lower amount of Nissl bodies and neuronal apoptosis. Yu et al. (Yu et al., 2023)also proposed that excessive copper exposure could lead to neurotoxicity in rats, with specific manifestations including a dramatic loss of Nissl bodies was noted in the hippocampal region. This is similar to our research results. Apoptosis, as a programmed form of cell death, it is crucial for sustaining neuronal numbers (Zhou et al., 2021). According to reports, NaF exposure can trigger the activation pathway of mitochondrial apoptosis (Yang, Zhu et al., 2021). Li et al. (Li et al., 2021) discovered that Lactobacillus rhamnosus GR-1 can prevent Escherichia coli-initiated apoptosis via PINK1/Parkin-mediated mitophagy. Yang et al. (Yang, Liao et al., 2021) found that copper exposure can induce mitophagy via the PINK1/Parkin pathway, and mitophagy may attenuate the mitochondrial apoptosis induced by copper. Research has found that copper oxides can elevate the BAX/Bcl-2 ratio, thereby inducing neurotoxicity (Yang et al., 2025). Wang et al. (Wang, Wang et al., 2025) has also found that the pollutant 6-PPD reduces Bcl-2 expression while increasing Bax expression. Following 6-PPD exposure, mitochondrial membrane potential decreases and apoptosis rates increase, suggesting activation of the mitochondrial apoptosis pathway. Our results also

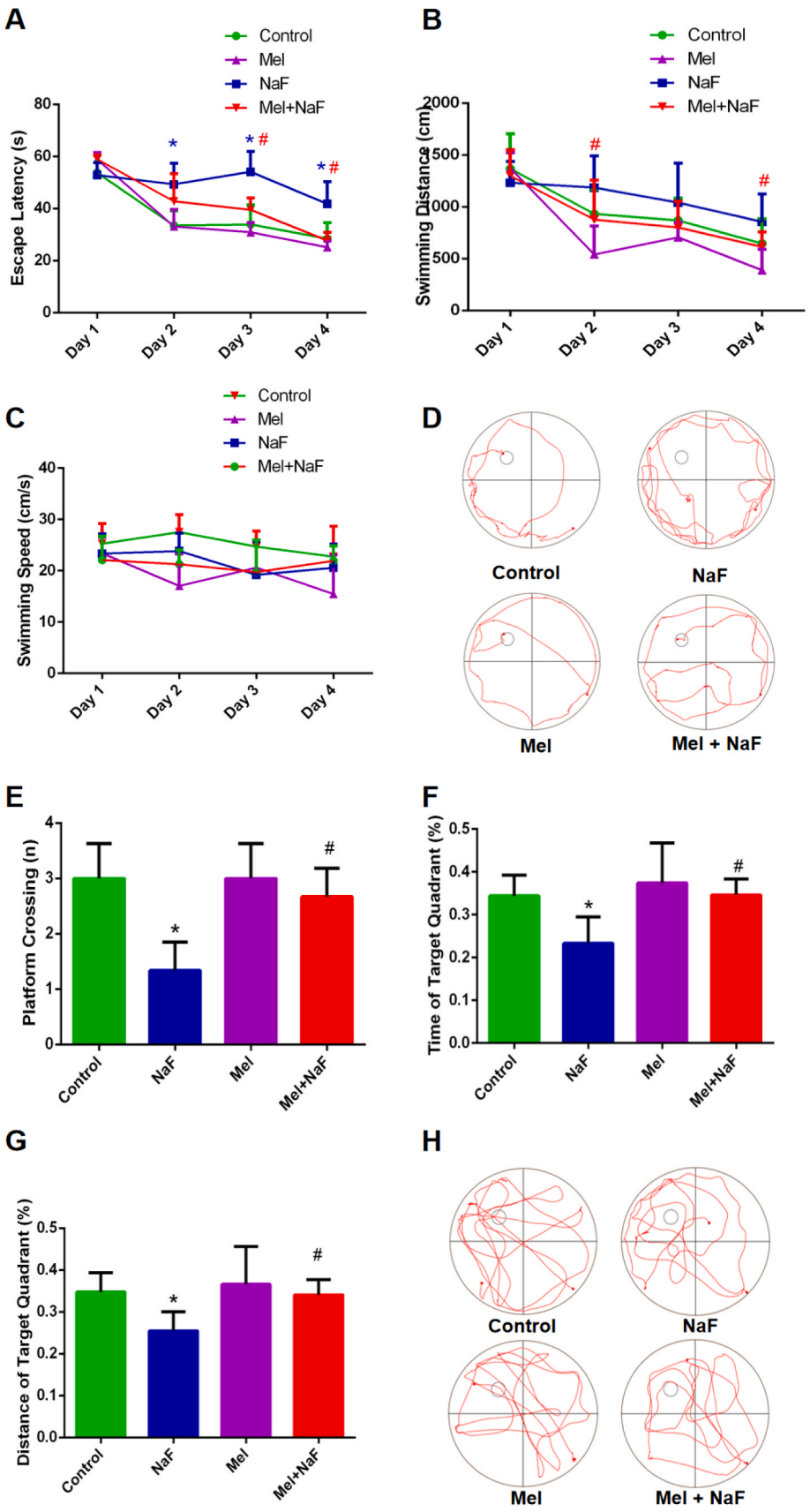

Fig. 4. Melatonin improves the abilities to learning, memory, and retention impaired by NaF exposure in F1 generation rats. (A) The mean escape latency to the platform. (B) The mean swimming distance to the platform. (C) The mean swimming speed to the platform. (D) Representative traces in the PNT. (E) The number of platform crossings. (F) Time spent in the target quadrant. (G) Distance spent in the target quadrant. (H) Representative traces in the SPT. The data are presented for six rats in each group. *$P < 0.05$ versus the control group.

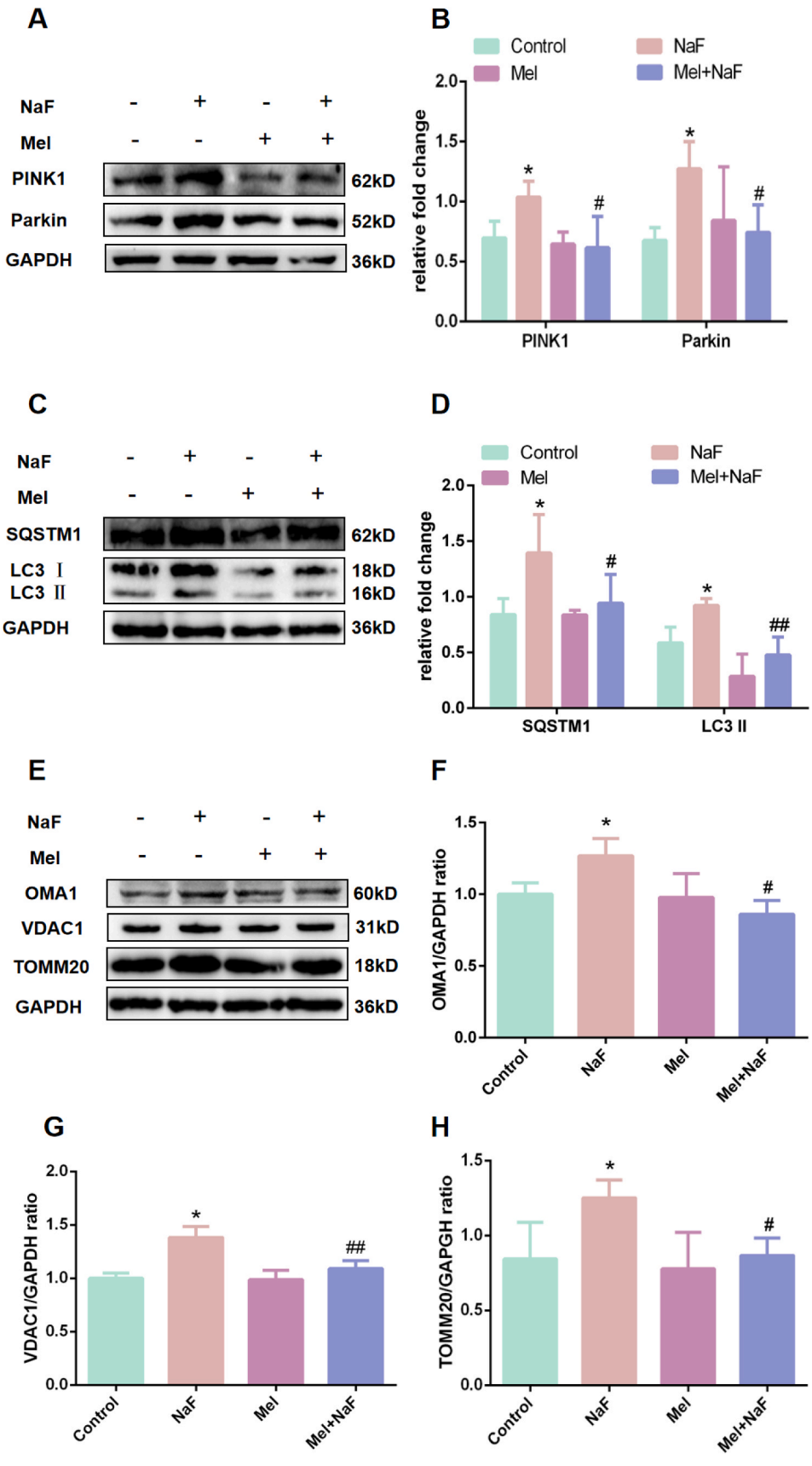

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Fig. 5. Melatonin alleviates abnormal mitophagy by NaF exposure and restores autophagic degradation ability. (A) Representative images of western blot for mitophagy markers PINK1 and Parkin in hippocampal tissues of adult rats after NaF combined with melatonin treatment. (B) Quantitative analyses of the mitophagy marker PINK1 and Parkin in hippocampal tissues of adult rats after NaF combined with melatonin treatment. (C) Representative images of western blot for autophagy markers SQSTM1 and LC3-II in hippocampal tissues of adult rats after NaF combined with melatonin treatment. (D) Quantitative analyses of the autophagy markers SQSTM1 and LC3-II in hippocampal tissues of adult rats after NaF combined with melatonin treatment. (E) Representative images of western blot for OMA1, VDAC1 and TOMM20 in hippocampal tissues of adult rats after NaF combined with melatonin treatment. (F) Quantitative analyses of OMA1 in hippocampal tissues of adult rats after NaF combined with melatonin treatment. (G) Quantitative analyses of VDAC1 in hippocampal tissues of adult rats after NaF combined with melatonin treatment. (H) Quantitative analyses of TOMM20 in hippocampal tissues of adult rats after NaF combined with melatonin treatment. The data are presented as the means ± S.D. for three different experiments. $^{*}P < 0.05$ versus the control group. $^{**}P < 0.01$ versus the control group. $^{***}P < 0.001$ versus the control group.

support this evidence that fluoride exposure can reduce the amount of Nissl bodies in the hippocampal tissue of rats, increase the expression of cleaved PARP and BAX protein and decrease the expression of Bcl-2. It is suggested that NaF-induced apoptosis participates in fluoride-induced developmental neurotoxicity.

Zhang et al.'s research showed that fluoride impairs the learning capability in rats, possibly via the induction of autophagy in rat hippocampal neurons. (Zhang et al., 2020). LC3 is the most vital indicator of autophagosomes. SQSTM1 is ubiquitin-conjugating protein that mediates the conjugation of ubiquitylated proteins to autophagosomes, which are ultimately degraded by autophagolysosomes. When autophagy is initiated, the SQSTM1 protein is normally degraded. (Lamark, Svenning, and Johansen, 2017). However, when the degradation of SQSTM1 is blocked, the SQSTM1 protein accumulates with the rise of LC3 levels, thereby leading to autophagy dysfunction (Yao et al., 2023). Consistent with these expression patterns, the observed increase in SQSTM1 and LC3 levels in the hippocampal tissue of rats in this study suggests that fluoride exposure causes impaired autophagy-mediated degradation. Mitophagy is a specialized form of selective autophagy. Previous studies have found that mitophagy dysfunction may lead to many neuronal diseases (Ejma et al., 2020). In the normal physiological situation, PINK1 is translocates to the inner mitochondrial membrane, where its degradation is observed. In the event of mitochondrial dysfunction, the loss of the mitochondrial membrane may impairs the translocation of PINK1, resulting in its stabilization and subsequent accumulation on the mitochondrial outer membrane. The pool of accumulated PINK1 facilitates the recruitment of Parkin. (Lamark, Svenning, and Johansen, 2017). Parkin initiates the ubiquitylation process, thereby recruiting receptor proteins such as SQSTM1, which associate with LC3 on the phagocytic bubble membrane. The phagocytic vesicles then mature into autophagosomes, which engulf damaged mitochondria destined for degradation through fusion with lysosomes. (Li et al., 2023). Research have demonstrated that fluoride causes injury to testicular cell mitochondria and overactivates PINK1/Parkin-mediated mitophagy. (Liang et al., 2020). Zhao et al. also revealed that fluoride can induce mitochondrial injury and mitophagy in liver cells (Zhao et al., 2022). Hu et al. (Hu et al., 2024) has been clarified that fluoride exposure induces mitochondrial injury in bones, which subsequently activates PINK1/Parkin-mediated mitophagy and leads to bone damage. Wang et al. (Wang, Li et al., 2024) also proposed that fluoride causes liver inflammatory damage by stimulating Parkin mediated mitophagy in vivo in mice. OMA1 is a metalloprotease on the inner mitochondrial membrane that activates when the mitochondrial membrane potential decreases. Activated OMA1 cleaves mitochondrial fusion GTPases and inhibits mitochondrial fusion (Yamada et al., 2025). As a mitochondrial governor, VDAC1 is critical for cell survival and death signals and implicated in neurodegenerative diseases (Zhao et al., 2024). Gao et al. (Gao et al., 2024) found that exposure to cyclosporine A upregulates the mRNA and protein expression levels of VDAC1, thereby inducing apoptosis in rat hippocampal neurons. Elsherbini et al. (Elsherbini et al., 2020) revealed that Aβ directly interacts with mitochondrial VDAC1, whose elevated expression disrupts mitochondrial protein transport and promotes the release of pro-apoptotic factors, leading to mitochondrial dysfunction in Alzheimer's disease. Wu et al. (Wu et al., 2021) proposed that OMA1 is activated through self-cleavage in response to mitochondrial membrane depolarization and other cellular stresses, thereby influencing mitochondrial protein quality control. Our findings demonstrate that fluoride exposure promotes the activation of the PINK1/Parkin pathway and the expression of OMA1 and VDAC1 also increased. What's more, TOMM20 expression also increased. TOMM20 is situated on the mitochondrial membrane and can indicate the abundance of mitochondria. As TOMM20 is normally degraded upon successful mitophagy, its accumulation, alongside increased PINK1/Parkin, suggests that although mitophagy is initiated, the final degradative step is impaired. This phenomenon of impaired autophagic flux is implicated in various neurodegenerative contexts. For instance, research has found that impairment of the autophagic-lysosomal pathway leads to the accumulation of undegraded autophagic vesicles and proteins, exacerbating pathology in Alzheimer's disease (Yi et al., 2024). Research has found that when the autophagic lysosomal pathway is impaired, it leads to the accumulation of autophagic vesicles and tau proteins that cannot be properly degraded, thereby exacerbating the symptoms of Alzheimer's disease. (Li et al., 2024). Similarly, defective lysosomal degradation in neurons results in the accumulation of undegraded autophagic structures (Wang, Sooram et al., 2025). Thus, our results show that mitophagy is promoted at the initiation stage, but the ultimate degradation is blocked, indicating aberrant mitophagic flux.

Melatonin can easily cross the BBB and possesses neuroprotective properties (Wang, Wang et al., 2024). Mansouri et al. (Mansouri et al., 2022) found that melatonin treatment effectively improved cognitive impairment in mice resulting from a diet high in fat. It is reported that melatonin can enhance the cognitive function in rats with vascular dementia (Thangwong et al., 2023). Consistent the evidence of the objective of this study, after melatonin intervention, the escape latency and swimming distance of fluoride-exposed rats decreased, and the count of platform crossings increased, indicating that melatonin can mitigate memory learning and memory retention ability impairment in rats caused by NaF exposure.

Studies have revealed that melatonin treatment can reduce the incidence of apoptosis in dorsal root ganglion cells, promote mitophagy and inhibit inflammasomes in dorsal root ganglion cells (Xie et al., 2021). Wang et al. (Wang et al., 2022) suggested that melatonin inhibits the ROS-dependent HIF-1α/BNIP3/LC3B axis to promote mitophagy and reduce apoptosis in both in vitro and in vivo. Melatonin can diminish the level of PINK1/Parkin in granulosa cells and simultaneously improve mitochondrial dysfunction, thereby alleviating polycystic ovary syndrome (Yi et al., 2020). Some studies have also revealed that melatonin diminishes the PINK1/Parkin-mediated mitophagy activated by PM2.5 in liver, consequently ameliorating particulate matter-triggered hepatic fibrosis (Zhu et al., 2023). Shi et al. (Shi et al., 2024) indicated that melatonin suppresses dysfunctional mitophagy in vivo and in vitro, decreases PINK1 and Parkin levels, improved cognitive deficits after stroke in mice and reduced neuronal loss. This is in accordance with the results of this research, indicating that melatonin corrects the abnormal mitophagy induced by NaF exposure in the PINK1/Parkin pathway and restores the autophagic degradation ability. Ying et al. (Yingying et al., 2025) found that stinging nettle effectively downregulates VDAC1 expression, upregulates the anti-apoptotic protein Bcl-2, and downregulates the pro-apoptotic protein Bax, indicating that the mitochondrial apoptosis pathway is inhibited. Wei et al. (Wei et al., 2024) revealed that melatonin can protect mitochondrial function by

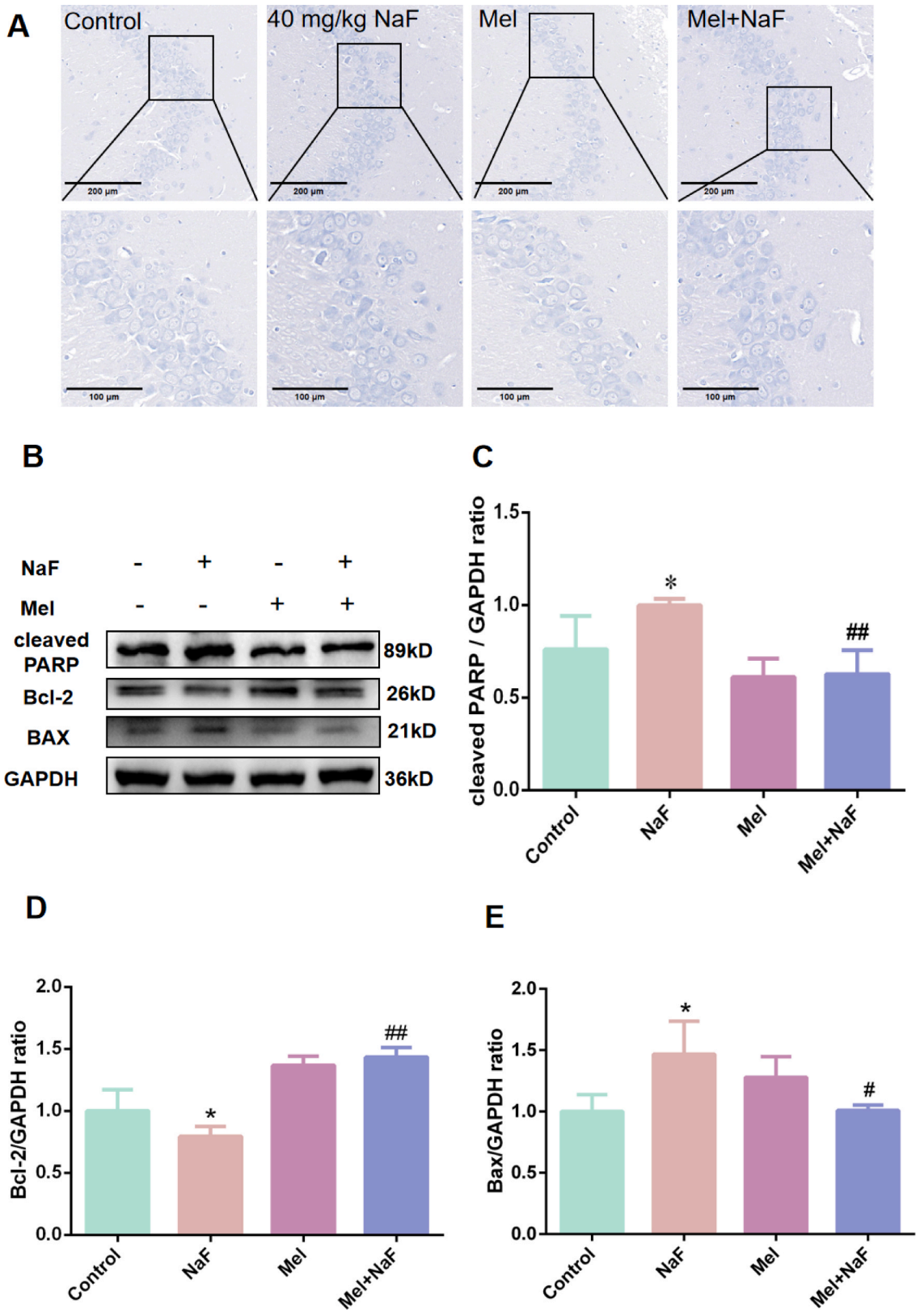

Fig. 6. Melatonin alleviates neurons damage and apoptosis caused by NaF exposure. (A)The Nissl staining in rat hippocampus. (B) Representative images of western blot for apoptosis marker cleaved PARP, Bcl-2 and BAX in hippocampal tissues of adult rats after NaF combined with melatonin treatment. (C) Quantitative analyses of the apoptosis marker cleaved PARP in hippocampal tissues of adult rats after NaF combined with melatonin treatment. (D) Quantitative analyses of the apoptosis marker Bcl-2 in hippocampal tissues of adult rats after NaF combined with melatonin treatment. (E) Quantitative analyses of the apoptosis marker BAX in hippocampal tissues of adult rats after NaF combined with melatonin treatment.The data are presented as the means ± S.D. for three different experiments. The scale bar represents 20 μm. $^{*}P < 0.05$ versus the control group. $^{**}P < 0.01$ versus the control group. $^{***}P < 0.001$ versus the control group.

regulating OMA1 levels to inhibit mitochondrial dysfunction, thereby safeguarding against chronic obstructive pulmonary disease induced by viral infection. Furthermore, our results indicated that OMA1, VDAC1 and TOMM20 expression levels also diminished after the combined intervention of melatonin and NaF. As a mitochondrial membrane protein, the reduction of TOMM20 reflects the restoration of mitophagic degradation and the clearance of damaged mitochondria.

Autophagic degradation disorder is an important cause of apoptosis. Melatonin has been shown to ameliorate cellular apoptosis (Rafiyian et al., 2024). Hong et al. (Hong et al., 2024) demonstrated that melatonin suppressed calcineurin activity and autophagy levels, weakened amyloid protein-induced apoptosis in neuroblastoma cells, and thereby alleviated neurotoxicity. Melatonin dramatically enhanced the abundance of Nissl bodies in neurons in a rat model of nerve damage caused by spinal cord injury (Jing et al., 2017). The findings of this research also demonstrate that melatonin can increase the number of Nissl bodies in neurons and reduce the expression of the apoptotic protein cleaved PARP. This aligns with Zeng's (Zeng et al., 2022) research findings. They demonstrated that melatonin suppresses excessive the PINK1/Parkin-dependent mitophagy and alleviates ropivacaine-induced apoptosis of PC 12 and HT 22 cells. Qin et al. (Qin et al., 2021) revealed that melatonin can restore mitochondrial function, reduce oxidative stress, decrease expression of the pro-apoptotic gene Bax, and inhibit cell apoptosis. Gao et al. (Gao et al., 2021) also found that melatonin supplementation reduces BAX expression, thereby improving inflammatory bowel disease by mitigating oxidative stress, mitochondrial dysfunction, and apoptosis. In short, our current research results indicated that melatonin improves NaF-induced apoptosis and thereby alleviated the developmental neurotoxicity caused by fluoride.

5. Conclusion

Our results indicated that NaF exposure induced abnormal mitophagy via the PINK1/Parkin pathway but inhibited autophagic degradation, resulting in the accumulation of damaged mitochondria and subsequently apoptosis, ultimately leading to developmental neurotoxicity. In contrast, melatonin ameliorated abnormal mitophagy through the PINK1/Parkin pathway, restored autophagic degradation capacity, and reduced apoptosis. This further alleviated developmental fluoride neurotoxicity.

CRediT authorship contribution statement

Yongkang Liang: Visualization, Investigation. **Meng Zhang:** Visualization, Investigation. **Yajie Li:** Visualization, Investigation. **Wenqi Qin:** Writing – review & editing. **Chulin Yan:** Visualization, Investigation. **Chun Wang:** Writing – original draft, Methodology, Formal analysis. **Runjiang Ma:** Investigation. **Qiang Niu:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Huayong Wu:** Visualization, Funding acquisition. **Jingjing Zhang:** Visualization, Funding acquisition.

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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Data availability

The data that has been used is confidential.

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