Journal of Integrative Neuroscience

J. Integr. Neurosci. **2025**; 24(11): 44394
https://doi.org/10.31083/JIN44394

Original Research

Deficiency of *Kdm1a* Induces Locomotor Abnormalities and Learning and Memory Deficits in Zebrafish Larvae

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Academic Editors: Vincent Lelievre and Bettina Platt
Submitted: 30 June 2025 Revised: 19 September 2025 Accepted: 15 October 2025 Published: 25 November 2025

Abstract

Background: Lysine-Specific Demethylase 1A (*Kdm1a*) is the first discovered histone lysine-specific demethylase, and mutations in *kdm1a* have been detected in neurodevelopmental disorders. However, the effect of *kdm1a* on neurobehaviors and the underlying mechanisms remain largely unknown. **Methods**: In this study, *kdm1a* deficient zebrafish were constructed using (clustered regularly interspaced short palindromic repeat) Clustered Regularly Interspaced Short Palindromic Repeats/CRISPRassociated protein 9 (CRISPR/Cas9) and the neurodevelopment was systematically assessed by a series of behavioral tests. **Results**: We found that *kdm1a* knockout zebrafish exhibited developmental toxicity and abnormal neurobehaviors, including locomotor abnormalities, and learning and memory deficits. *Kdm1a* deficiency suppressed central nervous system (CNS) neurogenesis in Tg (*HuC:egfp*) zebrafish, reduced motor neuron axon length in Tg (*hb9:egfp*) zebrafish and downregulated the expression of neurodevelopment related genes at 96 hours post fertilization (hpf). In addition, the expression of genes related to autophagy and apoptosis increased significantly in *kdm1a* knockout zebrafish. **Conclusions**: These results indicated that *kdm1a* deficiency induced locomotor abnormalities and learning and memory deficits in zebrafish larvae accompanied by activation of autophagy and apoptosis. These findings indicate a key role of *kdm1a* in neurodevelopment, providing novel insights into the mechanisms underlying the neurodevelopmental disorders.

Keywords: histone demethylases; neurodevelopmental disorders; autophagy; apoptosis; zebrafish

1. Introduction

Lysine-Specific Demethylase 1A (*Kdm1a*) (also known as *LSD1*, or *BHC110*) is the first discovered histone lysine-specific demethylase, which is an amine oxidase histone demethylase. *kdm1a* maps to 1p36.12, which encodes a nuclear protein containing a Swi3p, Rsc8p, and Moira (SWIRM) domain, a flavin adenine dinucleotide (FAD)-binding motif, and an amine oxidase domain. *Kdm1a* is a component of several histone deacetylase complexes. It mono-methylates and di-methylates histone H3K4 or H3K9 via a FAD-dependent amine oxidation reaction [1–3]. Previous studies have shown that *kdm1a* plays an important role in a variety of physiological processes, such as the cell cycle, chromosome segregation, cell differentiation, cell proliferation, stem cell self-renewal, spermatogenesis, the epithelial-mesenchymal transition and tumorigenesis [4–6]. *Kdm1a* is also required for neurogenesis, and plays a role in neuron progenitor cell proliferation [7–9] and terminal differentiation [10,11].

Mutations in *kdm1a* have been recently identified in a new neurodevelopmental disorder, which phenotypically resembles Kabuki syndrome but with distinctive facial features, skeletal anomalies and cognitive impairment [12,13]. Additionally, several studies have shown that *kdm1a* is involved in neurological disorders. Christopher *et al.* [14] reported that deletion the *kdm1a* gene in adult mice leads to paralysis, along with widespread neuronal cell death in the hippocampus and cortex, and associated learning and memory deficits. However, studies on the potential molecular mechanisms of the neuronal damage mediated by loss of *kdm1a* are still limited.

A study has indicated that *kdm1a* is an essential regulator of autophagy [15]. Autophagy is accompanied by increases in microtubule-associated protein 1 light chain 3, lipidated LC3-II and cytosolic LC3-I (*LC3II/LC3I*) and decreases in sequestosome 1 (*p62*) [16]. Some studies have found *kdm1a* depletion triggers autophagy in neuroblastoma cells through the Sestrin 2-Mechanistic Target of Rapamycin Complex1(SESN2-MTORC1) pathway [17]. Autophagy is involved in neuronal damage [18]. Prostate cancer cells are suppressed by inducing apoptosis and autophagy can be induced with the specific *kdm1a* inhibitor N-[(1S,2R)-2-Phenylcyclopropyl]-1H-pyrrolo[2,3-b]pyridin-4-amine (NCL-1) [19]. Therefore, we hypothe-

sized that loss of *kdm1a* may induce abnormal autophagy and apoptosis, which results in neurodevelopmental disorders.

Zebrafish (*Danio rerio*) are an ideal model for developmental and neurological studies due to their rapid external development, efficient reproduction, optical transparency and genetic similarities to humans [20]. Larval zebrafish begin to swim freely at 5 days post-fertilization (dpf) and the emergent patterns of development and movement are well-described [21]. Two transgenic (Tg) zebrafish models (*HuC:egfp* and *hb9:egfp*) provide visualization and analysis for neurogenesis and axonogenesis *in vivo*. Previous studies mostly focused on the effect of *kdm1a* on the developmental and behavioral characteristics in adult animal models [14,22], while the effect during early life stages has been less studied. Hence, zebrafish were used to help elucidate the potential neurotoxicity induced by *kdm1a* deficiency and the potential mechanisms.

In this study, we established the inaugural *kdm1a* knockout zebrafish model, achieved through Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR/Cas9) genome editing. The *kdm1a*-deficient zebrafish larvae exhibited multiple behavioral abnormalities, such as locomotor abnormalities and learning and memory deficits during the early stages of development. *Kdm1a* deficiency also affected central nervous system (CNS) neurogenesis and reduced motor neuron axon length. Moreover, depleting *kdm1a* activated autophagy and apoptosis through abnormal gene transcription. These results strengthen our understanding of the role of *kdm1a* during early neurodevelopment, providing a potential new target for neurodevelopmental disorders in the future.

2. Materials and Methods

2.1 Chemicals and Reagents

Rabbit anti-NeuN (1:1000, ab104225, Abcam, Cambridge, MA, USA), mouse anti-Microtubule-Associated Protein 2 (MAP2) (ab11267, Abcam, 1:1000), donkey polyclonal secondary antibody to rabbit IgG (1:1000, Alexa Fluor488, ab150061, Abcam), donkey polyclonal secondary antibody to mouse IgG (1:1000, Alexa Fluor594, ab150108, Abcam). Acridine orange (AO) stain (A9231) and tricaine methanesulfonate (MS-222) (E10521, 98% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hematoxylin and eosin (HE) (C0105M) and Nissl staining (C0117) were performed using commercial kits from the Beyotime Institute of Biotechnology (Shanghai, China). Trizol reagent (9109), reverse transcription reagent kits (RR037A), and SYBR-green RT-PCR kits (RR420A) were obtained from TaKaRa (TaKaRa, Dalian, Liaoning, China).

2.2 Animal Husbandry

The Tg zebrafish (*HuC:egfp* and*hb9:egfp*) and wild-type zebrafish (TU strain) were purchased from the model animal research center of Nanjing University, China. All experiments were performed following the Guidelines for Laboratory Animals. The zebrafish were maintained in a recirculating culture system at 28.5 ℃ with a 10/14 h dark/light cycle according to standard conditions. The water circulating in the system was filtered by reverse osmosis (pH 7.5). The zebrafish were fed twice daily with brine shrimp. The zebrafish larvae used in the behavioral tests were 5–10 days old. The fish were randomly assigned to groups using a computer-generated randomization sequence. The experimenter was unblinded to group allocation during data collection and analysis to minimize bias. Blinding of the experimenter was not feasible; however, steps were taken to mitigate bias, such as objective outcome measures and independent assessors. After the experiment is completed, zebrafish larvae were euthanized at the designated time points by an overdose of tricaine methanesulfonate (MS-222, 300 mg/L) buffered with sodium bicarbonate (pH 7.0), followed by prolonged immersion (≥10 min after cessation of opercular movement) to ensure death prior to disposal. This method complies with AVMA guidelines for euthanasia of zebrafish and was approved by the Animal Care Committee of Nanjing Medical University.

2.3 Generation of Kdm1a Deficient Zebrafish by CRISPR/Cas9

The detailed procedure for zebrafish CRISPR/Cas9 editing was described previously [23]. The *kdm1a* target in this study was 5′-CAAAACCAAGCAGGACAACTT-3′. A solution containing 400 pg of Cas9 mRNA and 250 pg of gRNA was prepared for microinjection. Mutation sites were verified by comparing on the unaffected wild-type sequences (chimerism). To generate heterozygous *kdm1a*$^{+/-}$ mutants, chimeric founders were outcrossed to wild-type TU strain zebrafish for three consecutive generations. Then, *kdm1a*$^{+/-}$ males and *kdm1a*$^{+/-}$ females were crossed to obtain *kdm1a*$^{-/-}$ littermates.

2.4 Assessment of Embryonic Development

Embryos were collected at 2 hours post-fertilization (hpf) and normally fertilized and developed embryos were selected for the subsequent experiment (n = 50 in each group). Hatching and survival rates were manually counted every 24 hpf. The malformation rate was quantified at 96 hpf, while heart rate was assessed at 48 hpf. After anesthetizing the fish in MS-222 (168 mg/L), the abnormally developing embryos during different periods were observed and captured by stereoscopic microscopy (SMZ18, Nikon, Tokyo, Japan). The fluorescence intensity of green fluorescent protein (GFP) in *HuC:egfp* zebrafish larvae (n = 10) and the axonal length of motor neurons in *hb9:egfp* zebrafish larvae (n = 10 in each group) were quantified using ImageJ software (version 1.53k; National Institutes of Health, Bethesda, MD, USA)

2.5 Behavioral Assessment

Zebrafish larvae were subjected to four behavioral tests, including a spontaneous locomotor activity test, the open field test, the mirror image attack test, and the Y-maze test. Previous studies reported that 12–15 larvae per group is suitable for assessing behavior [24]. To facilitate adaptation, zebrafish were allowed a 2-minute period for tank acclimation prior to experimentation. All tests were monitored and evaluated with the Zebralab ViewPoint system (version 3.90; manufactured by ViewPoint Life Sciences, Lyon, France) from 10 AM. to 5 PM. All experiments were performed at least three times independently.

2.5.1 Locomotion Test

A larval locomotion test was performed using a previously published method [25]. Zebrafish larvae (5 days post-fertilization [dpf], n = 12 in each group) were randomly selected from each group and added to a 24-well plate with a single animal in each well. Videos were recorded for 10 min by a camera on top of the tank. The swimming speed of the larvae was analyzed using ZebraLab software (version 5.10; manufactured by ViewPoint Life Sciences, Lyon, France), and the active times were quantified by the locomotor activity assay.

2.5.2 Open Field Test

The open field test was conducted as described previously [26]. The experimental arena was partitioned automatically into 16 identical sectors, with the innermost four sectors designated as the central area. Zebrafish larvae (n = 12 in each group) were allowed to swim freely inside the tank for 15 min. The swimming distance and time spent in the central zone were calculated.

2.5.3 Mirror Attack Test

The mirror test was performed following the protocol of a previous study [27]. The transparent 5 × 3 × 2 cm acrylic tank was used with a 3 × 3 cm mirror on one side of the tank. The region in which the zebrafish (n = 12 in each group) could touch the mirror was designated as the "mirror area" (2.5 cm in width). The distance moved and the time spent in the "mirror area" were calculated.

2.5.4 Y-maze Test

The Y-maze task was conducted with 7–8 dpf fish to assess learning and memory ability as described in previous studies [28]. The Y-maze was composed of three arms at 120° to each other (6 × 2 × 2 cm). The outer surface of each arm was covered with a layer of black adhesive film to block external stimuli. Each arm had a white square, triangle, or circular incision (one shape per arm). During the first trial (training, 5 min), the zebrafish (n = 12 in each group) were allowed to explore only two arms (start and open arm), with the third arm (novel arm) closed. For the second trial (1 hour later), zebrafish were placed back in the same starting arm, with free access to all three arms for 5 min. The training and test sections were recorded and the distance moved and time spent in the "novel arm" were assessed.

To minimize potential experimenter bias during behavioral data collection and analysis, key experiments were conducted with the assistance of independent assessors who were not involved in the experimental treatment groups' daily management and were blinded to the genotype/treatment conditions. For all larval behavioral tests, video recordings were scored automatically using automated tracking software where possible. For parameters requiring manual scoring, the videos were randomized and assessed by two independent researchers who were blinded to sample identity. Their scored results were then compared, and any discrepancies were re-evaluated jointly to reach a consensus. This approach ensured that the quantification of behavioral phenotypes was objective and unbiased.

2.6 Assessment of Apoptosis

To visualize apoptotic cells within 96 hpf larvae, acridine orange staining (AO) staining was carried out. Briefly, live larvae (n = 10 in each group) were cultured with AO solution (5 μg/mL) for 30 min in the dark at room temperature, and then washed three times. Stained larvae were photographed by a stereoscopic microscope following the manufacturer's instructions (model Stemi 508; Carl Zeiss, Tokyo, Japan) after anesthesia (0.01% MS-222).

2.7 Histopathological Evaluation of Brain Tissue

Zebrafish larvae (n = 10 in each group) were fixed in 4% paraformaldehyde solution (P0099, Beyotime) for 24 h and then transferred to a graded ethanol series. After dehydration in ethanol, the tissues were embedded in paraffin-wax. Then the brain tissue blocks were sectioned at 5 μm thickness, and stained with HE for microscopic examination.

2.8 Nissl Staining

Paraffin sections were obtained by the method described above. The brain sections (n = 10 in each group) were dewaxed in a microwave with an antigen repair solution for 30 min. The slices were fixed in 4% paraformaldehyde at room temperature for 20 min and rinsed in water for 2 min. The treated samples were stained with Nissl staining solution for 5 min. The Nissl-positive cells were visualized under a light microscope (Product No.: C1791, Millipore-Sigma, Burlington, MA, USA).

2.9 Immunofluorescence

Paraffin sections were obtained by the method described above. The brain sections (n = 10 in each group) were dewaxed for 30 min in a microwave with an antigen repair solution, permeabilized for 5 min with 0.3% Triton X-100 (P0096, Beyotime) in PBS, and blocked for 30 min with

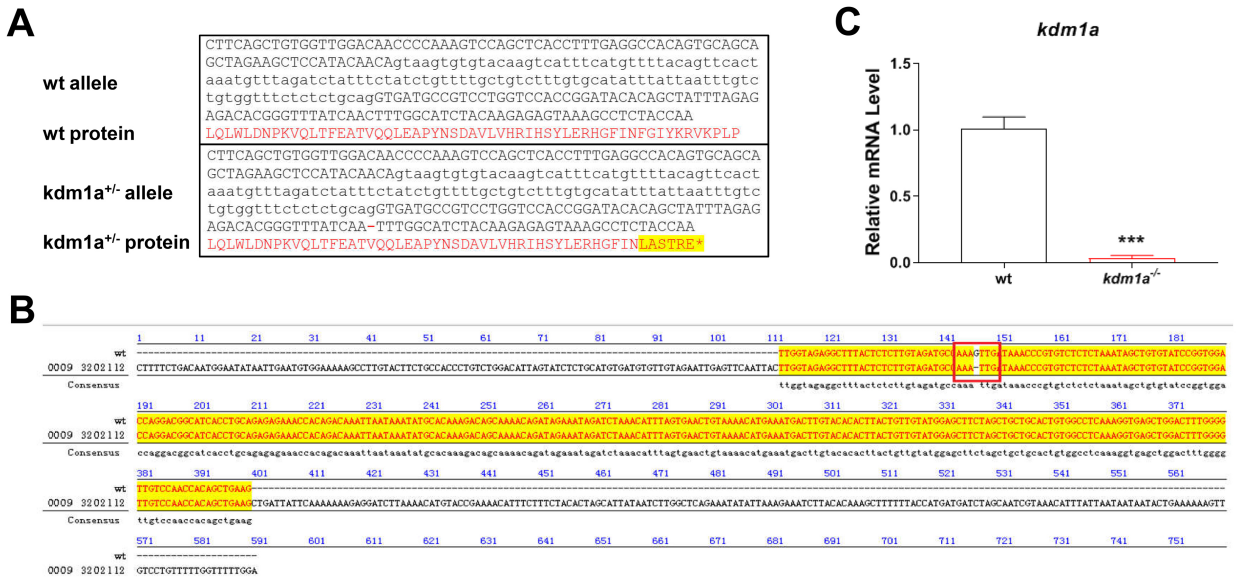

Fig. 1. Generation of *kdm1a* deficient zebrafish. (A) The genomic and protein architecture of zebrafish *kdm1a*. The nucleotides in black are gene sequences. The translated amino acid sequences are marked with red words, and the amino acid which change resulting from frame shift mutations are highlighted by yellow. (B) Sequence alignment of the WT and *kdm1a*$^{-/-}$ zebrafish line, including the –1 bp deletion in homozygotes. (C) Relative mRNA level of *kdm1a* in the *kdm1a* deficient zebrafish larvae (n = 20 in each group). Data were presented as mean ± SD of at least three independent experiments. $^{***}p < 0.001$, compared to wt group. WT, wild type; SD, standard deviation; *kdm1a*, Lysine-Specific Demethylase 1A.

3% bovine serum albumin (ST023, Beyotime). The brain tissues were incubated with anti-MAP2 and anti-NeuN antibodies (1:200) overnight at 4 °C and stained with 4′,6-Diamidino-2-Phenylindole (DAPI) solution (C1006, Beyotime). Images were captured with a Nikon Eclipse Ti2 inverted fluorescence microscope (Serial N12345, Nikon Instruments Inc., Melville, NY, USA).

2.10 Gene Expression Profiling

Total RNA was isolated from larvae sample (about 20 tails/group) with Trizol reagent. First-strand cDNA was synthesized with Avian Myeloblastosis Virus (AMV) reverse transcriptase, followed by SYBR Green-based qPCR analysis. Primer sequences for genes related to neurodevelopment, autophagy and apoptosis are provided in **Supplementary Table 1**. Gene expression levels were normalized to *β-actin* and determined using the $2^{-\Delta\Delta Ct}$ method.

2.11 Statistical Analysis

Data are presented as mean ± standard deviation (SD) of at least three independent biological replicates. Statistical analyses were conducted using SPSS 25.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 9.5.1 (GraphPad Software, San Diego, CA, USA). Differences among groups were evaluated by one-way analysis of variance (ANOVA) with Duncan's post-hoc test. Differences were considered statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1 Generation of Kdm1a$^{-/-}$ *Zebrafish*

A 21-nucleotide guide RNA (gRNA) targeting exons 5–6 of the zebrafish *kdm1a* gene was designed to enable sequence-specific editing. To obtain *kdm1a* knockout (KO) zebrafish, the Cas9 mRNA and gRNA were injected into embryos. DNA sequencing of target-specific PCR products confirmed that the *kdm1a* targeted allele carried a deletion of one base, resulting in a frame shift mutation and premature translational termination (Fig. 1A and **Supplementary Fig. 1**). Homozygous *kdm1a* (*kdm1a*$^{-/-}$) mutants were obtained from a heterozygous cross between *kdm1a*$^{+/-}$ males and *kdm1a*$^{+/-}$ females. Subsequently, homozygous *kdm1a* (*kdm1a*$^{-/-}$) mutants were identified by DNA sequencing (Fig. 1B). *Kdm1a*$^{-/-}$ zebrafish exhibited a substantial decrease in *kdm1a* mRNA expression by 4 dpf (Fig. 1C). These results indicated that *kdm1a* KO zebrafish were successfully generated.

3.2 Developmental Toxicity of Kdm1a$^{-/-}$ *Zebrafish Larvae*

To investigate the role of *kdm1a* in developmental abnormalities, we firstly analyzed the hatching rate, survival rate, heart rate and malformation rate. The hatching and survival rates of *kdm1a*$^{-/-}$ zebrafish decreased significantly after 48 hpf (Fig. 2A,B). While the malformation rate increased in *kdm1a*$^{-/-}$ zebrafish (Fig. 2C). No significant changes in the heart rate were observed in the

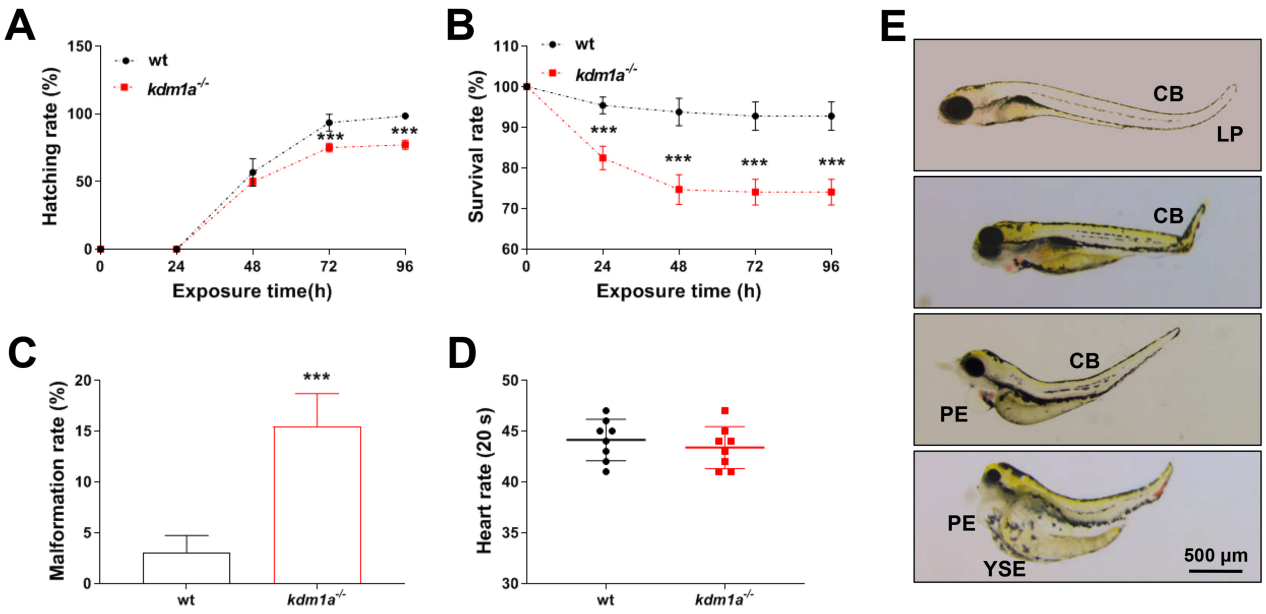

Fig. 2. Developmental toxicity in *kdm1a* deficiency zebrafish larvae. (A) Hatching rate. (B) Survival rate. (C) Malformation rate at 96 hpf. (D) Heart rate. (E) Typically morphological alterations. The scale bar = 500 μm. PE, pericardial edema; YSE, yolk sac edema; CB, curved body; LP, low pigment. Data were presented as mean ± SD of at least three independent experiments (n = 50 in each group). $^{***}p < 0.001$, compared to wt group.

kdm1a$^{-/-}$ group at 72 hpf (Fig. 2D). The typical morphological alterations mainly included pericardial edema (PE), yolk sac edema (YSE), curved body (CB) and low pigment (LP) (Fig. 2E).

3.3 Kdm1a$^{-/-}$ *Zebrafish Displayed Impaired Locomotor Behavior*

We used behavioral assays to analyze *kdm1a* deficiency in zebrafish and detected some abnormal behaviors. Spontaneous locomotor activity of individual larvae (5 dpf) was measured in a 24-well plate for 10 min. Active time decreased significantly in *kdm1a*$^{-/-}$ zebrafish (**Supplementary Fig. 2A,B**), indicating that the *kdm1a* deficiency impaired locomotion.

In the open field test, the typical locomotion tracking pattern illustrated differences in the exploration of the central and peripheral zones (Fig. 3A). *Kdm1a*$^{-/-}$ zebrafish exhibited less time and shorter distances in the periphery of the field (Fig. 3B,C), suggesting that *kdm1a* deficient zebrafish had a weakened ability to adapt to new environments.

3.4 Kdm1a$^{-/-}$ *Zebrafish Displayed Learning and Memory Deficits*

The mirror attack test was used to study social behavior and response to novelty in zebrafish. The locomotion tracking patterns illustrated the differences between the mirror zone and the non-mirror zone during swimming traces (Fig. 3D). The time and distance traveled in the mirror area decreased significantly in *kdm1a*$^{-/-}$ zebrafish (Fig. 3E,F). In other words, the *kdm1a*-deficient zebrafish displayed less perception and interactive behavior, suggesting reduced cognitive ability.

To better understand the cognitive abilities of *kdm1a*$^{-/-}$ zebrafish, the Y-maze test was employed to analyze the time and distance in the novel arm. The locomotion tracking pattern illustrated the differences in swimming traces in Y-maze arms (Fig. 3G). As a result, significant decreases in the time and distance spent in the novel arm were observed in *kdm1a*$^{-/-}$ zebrafish (Fig. 3H,I), suggesting that cognitive ability, particularly learning and memory, was impaired in *kdm1a*$^{-/-}$ zebrafish.

3.5 Kdm1a Deficiency Suppressed Neurodevelopment in Zebrafish Larvae

To determine whether neurobehavioral dysfunction of *kdm1a*$^{-/-}$ zebrafish is closely associated with neurogenic impairment, we analyzed the brain structure and function of zebrafish. The HE and Nissl stained brain tissue revealed that the density of brain cells and Nissl bodies decreased in *kdm1a*$^{-/-}$ zebrafish (Fig. 4A,B). To investigate the effects of *kdm1a* on nervous system development, *HuC:egfp* and *hb9:egfp* zebrafish lines were used to determine the neurotoxic effects of *kdm1a* deficiency. As shown in Fig. 4C, *kdm1a* deficiency significantly decreased GFP intensity in the brain at 96 hpf. Similarly, the motor neuron axon length was significantly reduced in Tg (*hb9:egfp*) zebrafish (Fig. 4D). Moreover, immunofluorescent staining indicated significantly lower levels of NeuN and MAP2 (neuron markers) in *kdm1a*$^{-/-}$ zebrafish (Fig. 4E). The genes in-

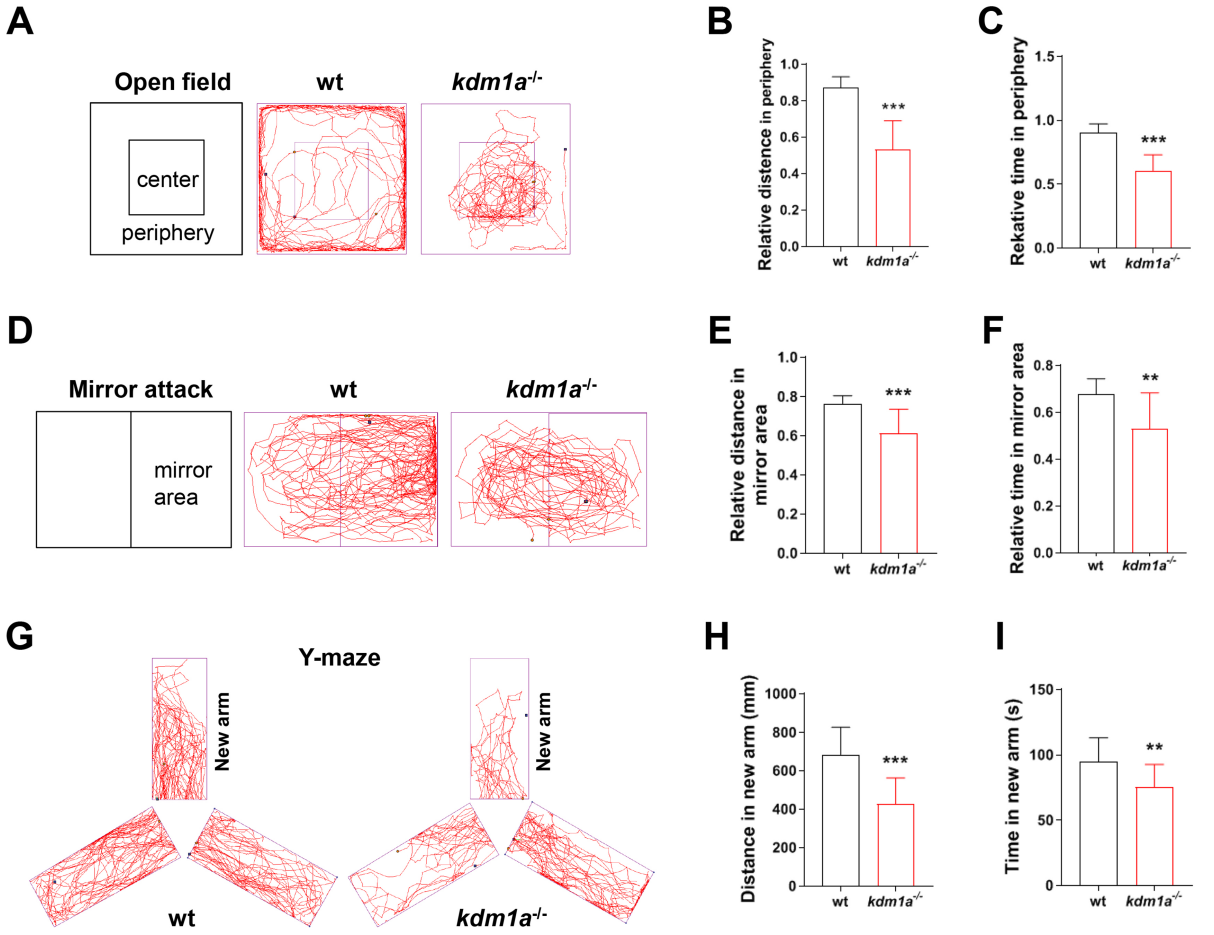

Fig. 3. Neurobehavioral alteration in *kdm1a* deficiency zebrafish larvae. (A) The motion trail recording, (B) total distance, (C) time in the peripheral zone in the open field test at 120 hpf (15 min). (D) The motion trail recording, (E) relative distance and (F) time in mirror area in the mirror attack test at 120 hpf (15 min). (G) The motion trail recording, (H) distance and (I) time in new arm in Y-maze test at 8 dpf (5 min). Data were presented as mean ± SD of at least three independent experiments (n = 12 in each group). $^{**}p < 0.01$, $^{***}p < 0.001$, compared to wt group.

volved in early neurogenesis (*neurod1*, *neurog1* and *elavl3*) and neural maturation (*tuba1*, *gfap*, *gap43*, and *syn2a*) were downregulated in *kdm1a*$^{-/-}$ zebrafish (Fig. 4F). These results illustrate that loss of *kdm1a* could induce significant neuronal impairment, which may be associated with abnormal behavior.

3.6 Kdm1a Deficiency Induced Cell Autophagy and Apoptosis in Zebrafish Larvae

To elucidate the mechanisms of neuronal damage in *kdm1a*$^{-/-}$ zebrafish, the expression of genes related to autophagy and apoptotic signaling was determined. As shown in Fig. 5A–G, the expression levels of *caspase 3*, *caspase 8*, *caspase 9*, *lc3*, and *beclin1* increased, but the levels of *p62* and *bcl2/bax* decreased in *kdm1a*$^{-/-}$ zebrafish. *Kdm1a* deficiency induced marked signs of neuronal apoptosis (Fig. 5H), indicating that loss of *kdm1a* triggered abnormal autophagy and apoptosis.

4. Discussion

Neurodevelopmental disorders (NDDs), including syndromes characterized by abnormal CNS development, affect learning, cognition, emotion, and memory. Environmental and genetic factors contribute to neuronal impairment, resulting in NDDs [29]. Increasing evidence suggests that genetic factors play a major role in NDDs [30]. For example, a recent study reported that causal variants were identified in 36% of NDD individuals, and 23% of NDD individuals had uncertain significant variants [31]. KDM1A (also designated as LSD1), discovered in 2004, was the first histone demethylase to be identified. This enzyme utilizes FAD as a cofactor to catalyze the demethylation of histone marks, including H3K4me1/2 and H3K9me1/2 [32]. KDM1A is frequently overexpressed in a wide array of human cancers, such as acute myeloid leukemia (AML), prostate cancer, lung cancer, bladder cancer, lymphoid neoplasms, and breast cancer [33–36]. Its oncogenic func-

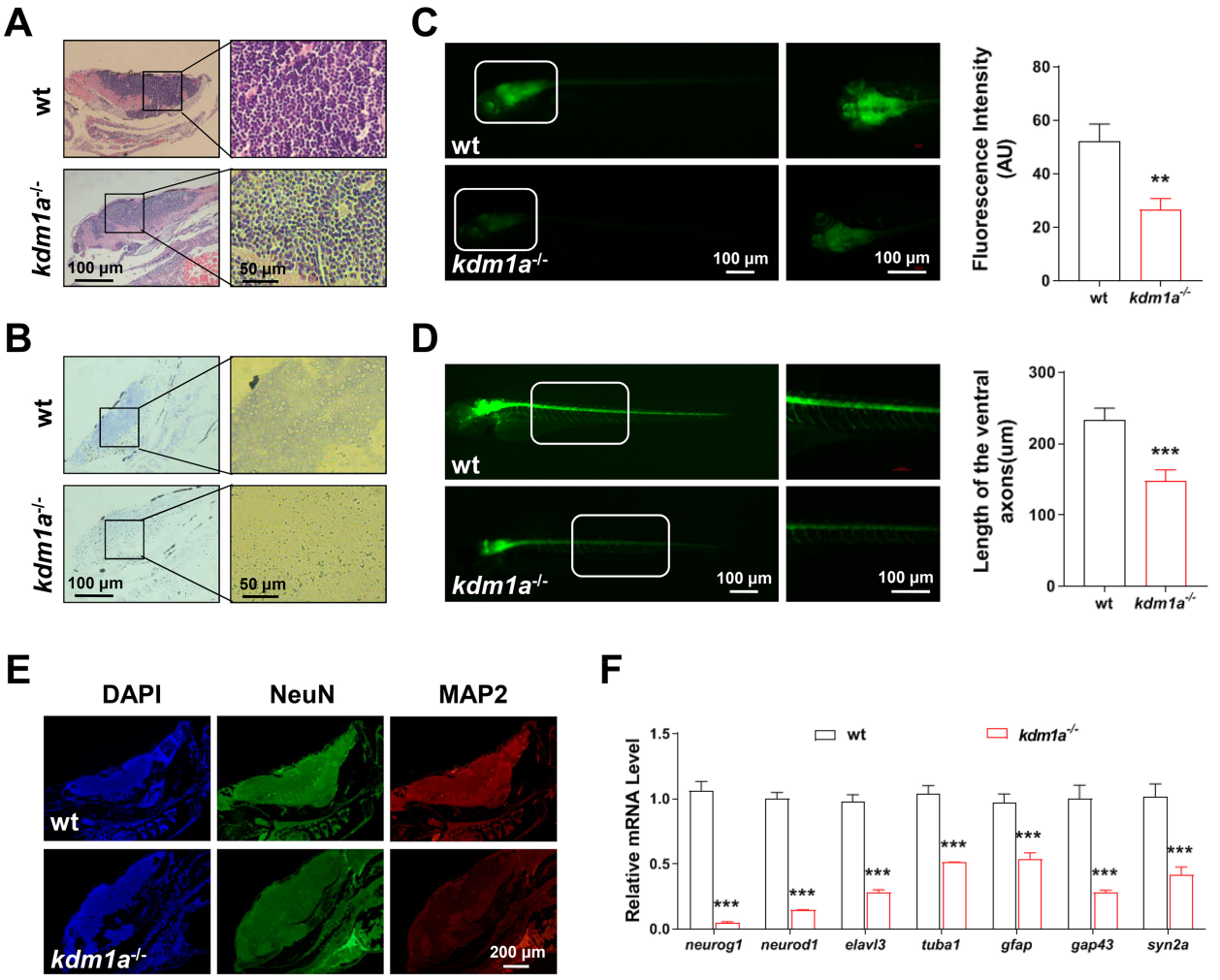

Fig. 4. *Kdm1a* deficiency inhibited neurodevelopment in zebrafish larvae. (A) HE and (B) Nissl staining of zebrafish brain in zebrafish larvae (n = 10 in each group). Scale bar: 100 μm (left) and 50 μm (right). (C) Representative fluorescence of neurogenesis in the CNS for whole zebrafish (left) and a magnified view of the corresponding cerebral regions (right) (n = 10 in each group). Scale bars: 100 μm. (D) Representative fluorescence of motor neuron for whole zebrafish (left) and a magnified view of the corresponding spinal regions (right) (n = 10 in each group). Scale bars: 100 μm. (E) Representative immune-stained images of MAP2 and NeuN in the brain (n = 10 in each group). Scale bar: 200 μm. (F) Relative mRNA level of early neurogenesis and neural maturation related genes (n = 20 in each group). Data were presented as mean ± SD of at least three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, compared to wt group. HE, Hematoxylin and eosin; CNS, Central Nervous System.

tions are mediated through diverse mechanisms: for instance, it regulates hematopoietic differentiation and promotes AML progression via H3K4 demethylation, while in breast cancer, it operates within the SIN3A/HDAC complex to support cell survival and tumorigenesis. Consequently, both genetic knockout and pharmacological inhibition of LSD1 have been demonstrated to effectively suppress tumor growth [37–39]. More recently, emerging evidence has revealed that KDM1A is not only implicated in tumorigenesis but also associated with NDDs. Pilotto *et al.* [40] found that three human patients with mutations in the *kdm1a* gene exhibited neurodevelopmental delay and mental retardation. Another study reported that deleting *kdm1a* in adult mutant mice induces severe paralysis, significantly reducing spatial learning and reference memory capacity, while hippocampal and cortical neurons appeared cell death [14]. Similarly, *kdm1a* knock-in (KI) mice exhibited short-term and long-term contextual fear memory as well as spatial memory deficits [22]. However, few studies have investigated the effects and mechanisms of *kdm1a* on neuronal development during the early life stages. Our study, which identifies a critical requirement for *kdm1a* in zebrafish neurogenesis, directly addresses this gap in knowledge and underscores the multifaceted nature of this epigenetic regulator.

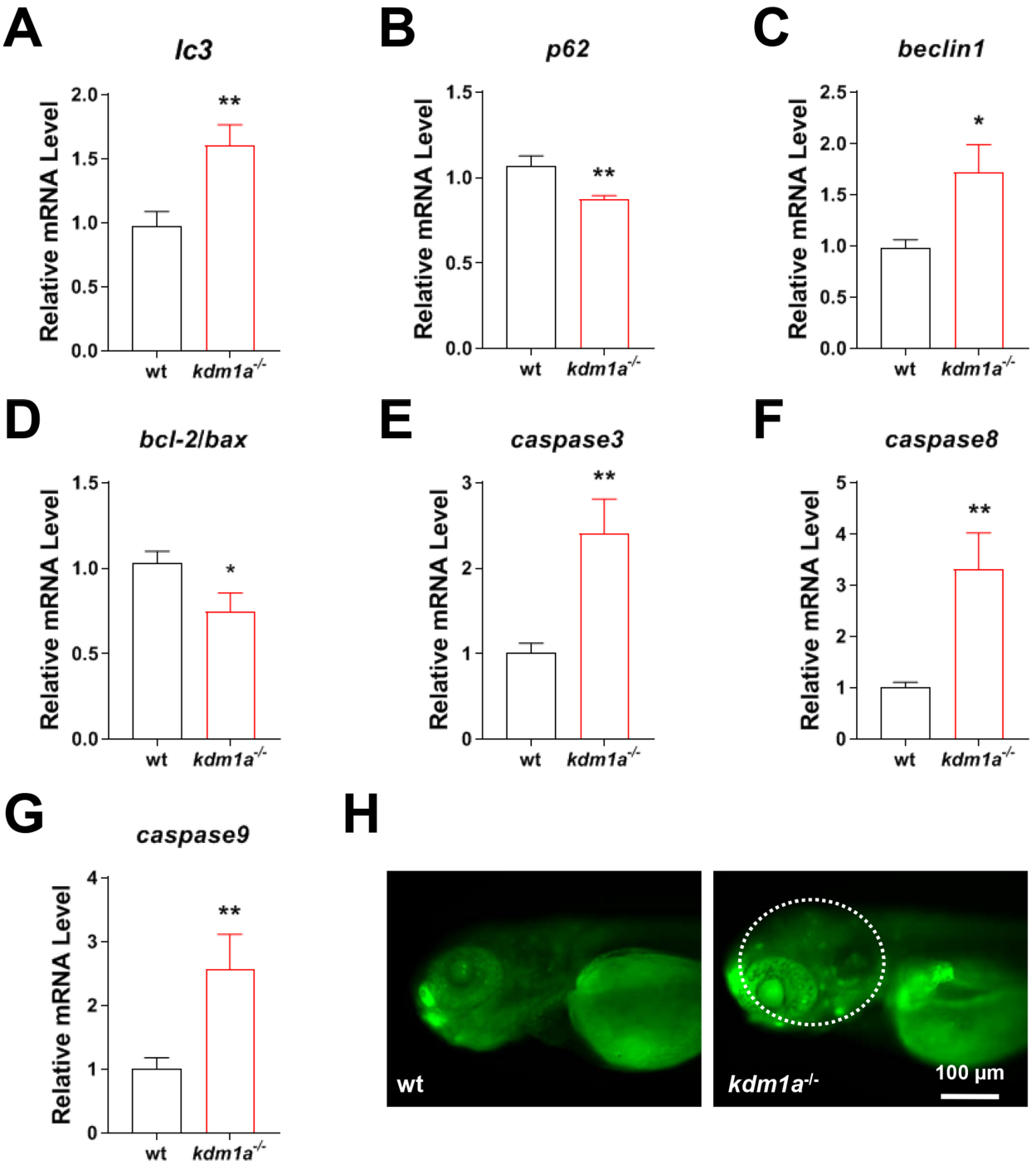

Fig. 5. *Kdm1a* deficiency induced neuronal autophagy and apoptosis in zebrafish larvae. Relative transcription activity of (A–C) autophagy (*lc3*, *p62* and *beclin1*) and (D–G) apoptosis (*bcl-2*/*bax*, *caspase 3*, *caspase 8* and *caspase 9*). (H) Apoptotic cells in the brain. Scale bar: 100 μm. Data were presented as mean ± SD of at least three independent experiments (n = 20 in each group). $^{*}p < 0.05$, $^{**}p < 0.01$, compared to wt group. lc3, microtubule-associated protein 1A/1B-light chain 3; p62, Sequestosome-1; bcl-2/bax, B-cell lymphoma 2/Bcl-2-associated X protein.

In this study, we generated the first *kdm1a*$^{-/-}$ zebrafish using the CRISPR/Cas9 system and documented its morphological, behavioral and neurological characteristics. Potential off-target effects of the CRISPR/Cas9 system were mitigated by the use of a high-specificity sgRNA, designed to minimize sequence homology elsewhere in the genome, and by the genetic outcrossing of founders, which dilutes any random, off-target mutations. The consistent phenotypes observed across multiple independent mutant lines further support that they are the result of *kdm1a* loss-

of-function specifically in the *kdm1a*$^{-/-}$ zebrafish. Previous studies focused on functional changes in *kdm1a* in adult mice, and *kdm1a* deficiency-induced developmental abnormalities have been less studied in embryos. In our study, *kdm1a* deficiency caused lower hatching and survival rates, and a higher malformation rate during early development. *Kdm1a* deficiency resulted in partial embryonic lethality and multiple morphological abnormalities during early development of zebrafish larvae. Interestingly, these observations, such as skeletal and cardiac malformations coincided with the clinical features reported in human *kdm1a* mutated patients [12,13].

A series of behavioral tests have been applied in zebrafish, including assessments of locomotor activity, fear and anxious behavior, social interaction, novelty seeking, aggression and learning and memory [41]. In this study, we used some of these behavioral assays to analyze *kdm1a* deficiency in zebrafish and found some abnormal behaviors. The *kdm1a*-deficient zebrafish developed spontaneous locomotor deficit, suggesting that *kdm1a* deficiency decreases the ability to move, which coincides with the severe paralysis of *kdm1a*-deficient adult mice. In the open field test, *kdm1a*$^{-/-}$ zebrafish displayed significantly more exploratory behavior toward the center of the field, suggesting that *kdm1a* deficiency decreased the ability to perceive danger in a new environment, which somehow represents low intelligence [24]. The mirror attack test is typically used to study social behavior and the response to novelty in zebrafish. Interestingly, *kdm1a*$^{-/-}$ zebrafish interacted very little with familiar zebrafish (itself in the mirror). Zebrafish are interested in familiar fish, so they usually interact with the familiar opponent in the mirror. One of the possible reasons behind the abnormal behavior of *kdm1a*$^{-/-}$ zebrafish might be dysfunction in cognitive ability, particularly learning and memory deficits. More specifically, the zebrafish expressing exploratory activity in the open field test and interacting very little in the mirror test are thought to be related to cognitive deficits [42]. Therefore, we hypothesized that loss of *kdm1a* impairs the learning and memory ability in zebrafish and thus impacts cognition.

The Y-maze test was used to assess learning and memory, and the response to novelty in zebrafish was similar to that of rodents. The time and the distance traveled in the novel arm are the behavioral parameters in this test [43]. Zebrafish usually prefer the unexplored arm (novel arm). In this study, the *kdm1a*$^{-/-}$ zebrafish traveled a greater distance in the open arm and less in the novel arm, indicating memory deficits. These observations further suggest that loss of *kdm1a* induces learning and memory impairment. Taken together, *kdm1a* KO zebrafish exhibited motor deficits and intellectual disabilities, which were consistent with Christopher *et al.*'s findings [14] that *kdm1a* deficiency causes paralysis and learning and memory deficits in adult mice. Few studies have investigated the role of *kdm1a* during early neurodevelopment. These behavioral phenotypes provide deeper insight into *kdm1a*-KO, indicating the important role of *kdm1a* in neurodevelopmental behaviors in zebrafish larvae.

Neurobehavioral deficits are closely associated with neurogenic disruption. Interestingly, the HE and Nissl-stained sections revealed that *kdm1a*$^{-/-}$ zebrafish had significantly fewer neuronal cells than the control group, suggesting that loss of *kdm1a* causes neurogenic impairment, which may lead to morphological and behavioral abnormalities. The results of this study follow previous findings demonstrating that neural cell death assessed by Nissl staining has a detrimental effect on animal behavior [44].

The neurobehavioral changes in zebrafish larvae are closely related to their neurogenetic or axonogenetic disorders [20]. To further validate this assumption, the effects of *kdm1a* on CNS and motor neuron development were evaluated using *HuC:egfp* and *hb9:egfp* transgenic zebrafish. In *HuC:egfp* transgenic zebrafish, GFP was integrated into the promoter sequence of the *elavl3* gene, which encodes the neuron-specific RNA-binding protein HuC. HuC is one of the earliest neuronal markers in zebrafish and is expressed in the CNS [45]. In the *hb9:egfp* zebrafish, GFP is specifically localized to motor neurons under the regulatory control of the *hb9* gene, a key regulator essential for motor neuron development [46]. Consistent with the neurobehavioral changes, *kdm1a* deficiency significantly reduced GFP intensity in the brain of *HuC:egfp* transgenic zebrafish at 72 hpf and inhibited motor neuron axon growth in *hb9:egfp* zebrafish.

Neuronal nuclei (NeuN) and microtubule-associated protein 2 (MAP2) are two neuron-specific proteins. Due to conservation among species and their stable expression during specific developmental stages, NeuN and MAP2 are reliable, conserved markers of mature neurons [47]. MAP2 and NeuN expression levels have been thought to indicate neuronal death or loss [48]. In this study, the brightness of the fluorescent NeuN and MAP2 staining decreased, indicating neuronal damage. In addition, we measured the mRNA expression of neurodevelopmental genes (*elavl3*, *neurog1*, *neurod1*, *tuba1*, *gap43*, *gfap* and *syn2a*) to verify the neurotoxic effect of *kdm1a*. *Elavl3*, *neurog1* and *neurod1* serve as biomarkers for early neurogenesis in zebrafish [49], whereas *gap43*, *gfap* and *syn2a* are linked to neural maturation, axonal growth and neurotransmitter secretion, particularly synaptic functions [20]. In our study, loss of *kdm1a* downregulated these genes, which further demonstrated that loss of *kdm1a* exerted direct effects on neurogenetic and motor neuron axonogenetic injury, thereby changing the neurobehaviors of zebrafish larvae.

The mechanisms of *kdm1a*-induced damage on motor neuron axonogenesis and neurogenesis are largely unexplored. Recent studies have demonstrated that Autophagy has been recently reported to participate in the development process of NDDs [50,51]. Autophagy is a conserved self-destructive process used to remove damaged organelles

and proteins via lysosomal degradation. Autophagy plays a crucial role in the organogenesis in zebrafish, including neurogenesis. Multiple lines of evidence point to *kdm1a* as an essential regulator of autophagy [52]. Some studies have shown that *kdm1a* affects autophagy by epigenetically modifying the expression of some proteins [53]. Moreover, *kdm1a* may also directly affect proteins involved in autophagy, such as P62 [54]. In this study, we observed the changes in autophagy-related molecules (P62, Beclin1 and LC3) and found that a deficiency of *kdm1a* increased the expression of *beclin1*, *lc3* and decreased the expression of *p62*. Our data demonstrated that a lack of *kdm1a* induced excessive autophagy in zebrafish larvae.

More importantly, excessive autophagy is a potential pathway to induce neuronal apoptosis [55]. For example, myclobutanil exposure causes excessive autophagy and neuronal apoptosis, leading to developmental neurotoxicity in zebrafish [56]. Therefore, autophagy is an inducer of apoptosis by activating the mitochondrial apoptosis pathway in zebrafish [57]. In the present study, we further investigated the changes in mitochondrial apoptosis-related molecules (*bcl-2*, *bax*, *caspase3*, *caspase9* and *caspase8*) and found that *kdm1a* deficiency increased the expression of *caspase3*, *caspase9* and *caspase 8*, and decreased the expression of *bcl2*/*bax*, resulting in the accumulation of apoptotic cells in the brain of zebrafish larvae. Collectively, our data demonstrated that a lack of *kdm1a* induced hyperactive autophagy and neuronal apoptosis, coinciding with aberrant behaviors and neurodevelopment in zebrafish larvae. This suggests that the dysregulation of these cellular processes may be a significant contributor to the observed neurotoxicity.

Nevertheless, this study has limitations as we cannot directly confirm the loss of KDM1A protein at the biochemical level due to the lack of a validated antibody against zebrafish KDM1A, future efforts will focus on obtaining or generating a specific antibody to definitively confirm protein ablation. Due to the absence of *kdm1a* overexpression in both wild-type and *kdm1a-deficient* zebrafish models, conducting gain-of-function and rescue experiments in future work will be essential to further clarify the precise functional contributions of *kdm1a*, its influence on neural development, and the molecular mechanisms involved. While our findings link *kdm1a* loss to autophagy/apoptosis activation and neuronal defects, the exact mechanism requires further investigation. Future studies should use autophagy and apoptosis inhibitors in zebrafish to determine whether suppressing these pathways rescues the neurodevelopmental phenotypes.

5. Conclusions

In summary, the present study demonstrates that *kdm1a* deficiency leads to excessive autophagy and neuronal apoptosis, which are likely responsible for the impairments in neurogenesis, motor axon outgrowth, and learning and memory in zebrafish larvae. These findings establish a critical link between *kdm1a* dysfunction and behavioral abnormalities relevant to NDDs, advancing our mechanistic understanding of neurodevelopmental and axonal pathogenesis mediated by *kdm1a* dysregulation. Our study provides new insight into developing potential therapeutic strategies for autophagy and apoptosis to limit the pathogenesis of NDDs.

Availability of Data and Materials

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

Author Contributions

LZ, Conceptualization, Data curation, Visualization, Original draft, Writing-Reviewing and Editing. JW, Conceptualization, Data curation, Methodology, Software. MY, Methodology, data analysis. QX, Methodology, data analysis. QH, Methodology, data analysis. JZ, Methodology, Data curation, Writing-Reviewing and Editing, Validation, Funding acquisition. XC, Conceptualization, Supervision, Writing-Reviewing and Editing, Funding acquisition. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experiments complies with American Veterinary Medical Association (AVMA) guidelines for euthanasia of zebrafish and adhered of the Animal Care Committee of Nanjing Medical University (IACUC-2205028).

Acknowledgment

Not applicable.

Funding

This work was supported by the National Natural Science Foundation of China [82103877], the Natural Science Foundation of Jiangsu Province [BK20211017].

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JIN44394.

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