Original Research *Highlight article*

N-acetylcysteine improves autism-like behavior by recovering autophagic deficiency and decreasing Notch-1/Hes-1 pathway activity

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Impact statement

N-acetylcysteine (NAC) has been reported to improve social interaction behavior, self-injury, and anxiety-like behavior in autism. The Notch-1/Hes-1 pathway and autophagy have been shown to participate in the pathogenesis of autism. Reactive oxygen species (ROS) have been reported to induce autophagy and play a vital role in regulating the Notch pathway and upregulating the Notch receptor Notch-1. However, whether the molecular mechanism by which NAC ameliorates autism-like behavioral abnormalities is related to the Notch-1/Hes-1 pathway and autophagy remains unclear. The current results indicated that NAC improves autism-like behavioral abnormalities and shows protective roles by inactivating Notch-1/Hes-1 signaling pathway and recovering autophagic deficiency in valproic acid (VPA)-induced autism model rats and SH-SY5Y neural cells exposed to VPA. This evidence helps to elucidate a novel molecular mechanism that underlies the therapeutic actions of NAC in autism and suggests its potential to ameliorate behavioral abnormalities in neurodevelopmental disorders.

Abstract

N-acetylcysteine (NAC) has been reported to improve social interaction behavior, irritability, self-injury, and anxiety-like behavior in autism. However, the molecular mechanism underlying the therapeutic roles of NAC in autism remains unknown. This study mainly aimed to investigate the therapeutic effect of NAC on valproic acid (VPA)-induced autism model and the underlying mechanisms. Our results showed that NAC ameliorated the deficits in sociability and the anxiety- and repetitive-like behaviors displayed by VPA-exposed rats. In addition, VPA exposure induced autophagic deficiency and enhanced Notch-1/Hes-1 pathway activity based on lowered Beclin-1 and LC3B levels, while increased expression of p62, Notch-1, and Hes-1 expression at the protein level. However, NAC recovered VPA-induced autophagic deficiency and reduced Notch-1/Hes-1 pathway activity in a VPA-exposed autism rat model and SH-SY5Y neural cells. The present results demonstrated that NAC improves autism-like behavioral abnormalities by inactivating Notch-1/Hes-1 signaling pathway and recovering autophagic deficiency. Taken together, this study helps to elucidate a novel molecular mechanism that underlies the therapeutic actions of NAC in autism and suggests its potential to ameliorate behavioral abnormalities in neurodevelopmental disorders.

Keywords: N-acetylcysteine, autophagic deficiency, Notch-1 pathway, Hes-1, autism, neuroscience

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Introduction

The core clinical manifestations of autism include social dysfunction and repetitive/rigid behavior.[1,2] A growing body of evidence is available about the relationship between oxidative stress and the development of autism.[3,4] N-acetylcysteine (NAC), a glutathione precursor with antioxidant and anti-inflammatory activities, has been reported to improve part of the clinical symptoms of autistic patients. NAC can reduce irritable behavior and hyperactivity in patients with autism.[5,6] Moreover, NAC also improves core symptoms of autism, such as social dysfunction,[6] as well as autism-related behaviors, such as self-injury behaviors.[7] In our previous studies, we also demonstrated that oxidative stress occurs in a valproic acid (VPA)-induced model of autism,[8] whereas NAC ameliorated repetitive/stereotypic behaviors in rats with autism.[9] However, the mechanism by which NAC improves autism remains unclear.

The Notch-1/Hes-1 pathway affects cell differentiation, proliferation, apoptosis, and other processes during

the course of neural development. When Notch ligands bind to receptors between adjacent cells, Notch-mediated signal transduction can receive and transmit signals to the nucleus to activate transcription factors, thereby initiating the transcription of target genes, such as Hes-1 and Hes-5. The Notch pathway is abnormally expressed in autism or autism spectrum disorders (ASDs). Compared with healthy subjects, patients with ASD have different expression profiles of downstream target gene Hey-1.[10] Our recent research results showed that the expression levels of Notch-1 receptor, Jagged-1 ligand, Notch intracellular domain (NICD) and Hes-1 were increased in autism model rats, while a specific inhibitor of the Notch pathway, DAPT can improve social interaction disorders and repetitive/stereotypic behavior,[11] suggesting the Notch signaling pathway may play a key role in the pathogenesis of autism.

The Notch pathway is associated with autophagy. The activation of autophagy in mouse T-regulatory cells (Tregs) depends on the activity of NICD, which regulates mitochondrial remodeling and the survival of activated Tregs, suggesting that Notch-1 regulates autophagy and controls the differentiation and fate of Tregs.[12,13] When γ-secretase inhibitor was used to inhibit Notch activity in malignant glioma, the autophagy marker LC3B-II/LC3B-I was significantly increased.[14] The downregulation of Notch pathway activity in human blood stem cells was negatively correlated with the increase of autophagy-related genes.[15] These findings suggest that Notch pathway may negatively regulate autophagy.

Several studies have found that enhancing autophagy during development can promote the formation of synapses, and inhibiting autophagy causes disorders of synaptic formation,[16] leading to social behavior defects and, as a result, autism and other mental diseases.[17,18] Therefore, autophagy is closely related to the onset of autism, and autophagic activation during development is conducive to clearing excessive and unnecessary cells, promoting synapse development, and improving autism symptoms.

VPA, which inhibits the activity of γ-aminobutyric transaminase and enhances the neurotransmission of γ-aminobutyric acid, is a conventional drug used in the treatment of epilepsy, bipolar disorder, and migraine. However, epidemiological studies in recent years have found that children exposed to VPA in the first trimester of gestation have a significantly increased risk for autism. Administration of VPA on day 12.5 of gestation revealed reduced social activities, and increased frequency and duration of repetitive-like behaviors, the main symptoms displayed by patients with autism.[19–21] Therefore, VPA model of autism has been widely used to study the pathogenesis of autism.

This study mainly aimed to investigate the therapeutic effects of NAC on VPA-induced autism model, and whether this therapeutic effect is related to Notch signaling pathway and autophagy *in vitro* and *in vivo*.

Materials and methods

Animals and experimental groups

All animal experiments were performed according to the Health Guidelines for the Care and Use of Laboratory Animals and the Regulations of Xinxiang Medical University (Xinxiang, China). The animals and the experimental groups were the same as those described in our previous study,[8] with several modifications. Adult female Wistar rats (Charles River Laboratories, Inc.) were bred overnight. If the female rats were found to have vaginal plugs or if vaginal smears were positive for sperm as determined by microscopy, the female rats were housed alone, and that day was recorded as the first day of pregnancy (E1). At day E12.5, the pregnant rats were randomly divided into two groups. One group was treated with a single intraperitoneal injection of 600 mg/kg VPA (CAS no. 1069665; Sigma-Aldrich; Merck KGaA). Another group of pregnant rats was intraperitoneally injected with the same volume of normal saline. After 23 days of weaning, the male offspring of the VPA-treated rats were randomly divided into two groups: VPA and VPA + NAC (Sigma-Aldrich; Merck KGaA) groups. The male offspring of the saline-treated rats were divided into control and NAC groups. The NAC or VPA + NAC groups were intraperitoneally injected with 150 mg/kg NAC once daily for 4 weeks as previously described.[9] VPA or NAC was dissolved in 0.9% saline solution. The offspring were observed for repetitive and social behavior and then sacrificed for further assay.

Behavioral testing

Open-field test

The open-field test (90 cm × 90 cm × 40 cm) was conducted on an infrared detection system to measure the repetitive behavior of the experimental rats. The open space was divided into central and surrounding areas. The central area was composed of nine squares (30 cm × 30 cm), and the surrounding area was composed of squares close to the wall. The rats were placed in the central area of the space and moved freely for 10 min. SuperMaze software (Xinruan Information Technology Co., Ltd, Shanghai, China) was used to record the data. The frequency of crossing the center and the time spent engaged in self-grooming were analyzed.

Three-chamber test

The three-chamber test was utilized to analyze the sociability of the subject rats. The subject rat was placed in the center area for a 5 min habituation period, then, a rat (stranger 1) was introduced into the chamber on the left, and the chamber on the right was empty (Object). The subject rat in the center was allowed to explore the three chambers for 10 min after the removal of the gate of each chamber. Subsequently, the subject rat was introduced into the center area, and another rat was introduced into the chamber on the right (stranger 2) on the same day. The subject rat was allowed to explore the three chambers for another 10 min. The time spent in each chamber was recorded using the SuperMaze software (Xinruan).

Western blot analysis

The offspring rats were decapitated and the prefrontal cortex (PFC), hippocampus (HC), and cerebellum (CB) were removed quickly from ice trays and collected and stored at –80°C for further use. The brain tissues or cells were

homogenized with protein lysis buffer. After heat denaturation, equal amounts of protein were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore). Membranes were blocked with 5% non-fat milk in TBS-Tween-20 buffer at room temperature for 2 h. Subsequently, membranes were washed and secondary antibodies were added and incubated for 2 h. Protein bands were detected by enhanced chemiluminescence (cat. no. P0018-1; Beyotime Institute of Biotechnology). ImageJ software (1.44 P; National Institutes of Health) was used for grayscale measurement. The relative expression of the target proteins was normalized to that of the internal reference GAPDH (1:5000; Kangen Biotechnology Co.). Then, the primary antibodies were incubated at 4°C overnight. Horseradish peroxidase-labeled primary antibodies used were as follows: Rabbit anti-Notch-1 (1:1000; Cat. No. Bioss-1335R; Boster Biological Technology), anti-NICD (1:500; Cat. No. ab83232; Abcam), rabbit anti-Jagged-1 (1:1000; Cat. No. Bioss-1448R; Boster Biological Technology), rabbit anti-Hes-1 (1:1000; Cat. No. sc-25392; Santa Cruz Biotechnology, Inc.), rabbit anti-Hes-5 (1:1000; Cat. No. sc-293445; Santa Cruz Biotechnology, Inc.), rabbit anti-Beclin-1 (1:1000; Cat. No. cst-3738s; Cell Signaling Technology, Inc.), rabbit anti-p62 (1:1000; Cat. No. cst-13121s; Cell Signaling Technology, Inc.), and rabbit anti-LC3B (1:1000; Cat. No. AF5402; Affinity Biosciences, Inc.).

SH-SY5Y neural cell culture and experimental treatments

SH-SY5Y neural cells were obtained from American Type Culture Collection. SH-SY5Y neural cells were cultured with DMEM/F12 medium containing 10% fetal bovine serum, 25 U/mL penicillin and 25 μg/mL streptomycin. The cells were cultured and maintained at 37°C under 5% CO_2. SH-SY5Y neural cells were treated with 0, 2, 4, 8, 10, and 12 mM VPA for 24 h or 2 mM NAC for 2 h. Phosphate-buffered saline was used to dissolve VPA or NAC.

Cell viability assay and cell morphology observation

Cell viability was determined using an MTT assay. Briefly, SH-SY5Y neural cells were cultured in 96-well plates and treated with VPA or NAC. Subsequently, 10 μL MTT solution was added and incubated for 4 h at 37°C. The MTT solvent was then added, and the spectrophotometric absorbance of the samples was analyzed with a microplate reader. In addition, morphological changes in SH-SY5Y neural cells following VPA exposure were observed under a light microscope.

Measurement of intracellular reactive oxygen species levels

The levels of intracellular reactive oxygen species (ROS) were measured with 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes; Thermo Fisher Scientific, Inc.). DCFH-DA has no fluorescence and freely crosses the cell membrane and can enter the cell and be hydrolyzed by intracellular esterase to form DCFH. DCFH is unable to permeate the cell membrane, thus facilitating the labeling of the cell with the probe. Intracellular ROS can oxidize non-fluorescent DCFH to generate fluorescent DCF. Detection of the DCF fluorescence can determine the levels of intracellular ROS. The cells were collected and suspended in 10 μM DCFH-DA at 37°C for 20 min. Subsequently, the cells were washed with serum-free culture medium three times to completely remove DCFH-DA in order for it not to enter the cells. Fluorescence-activated cell sorting with a fluorescence spectrometer (LSRFortessa X-20; BD Biosciences) was used to analyze the levels of intracellular ROS. The excitation and emission wavelengths were 485 and 530 nm, respectively.

Statistical analysis

All data are expressed as the mean ± SD and analyzed with GraphPad Prism version 8.0 (GraphPad Software, CA). Independent sample *t*-tests were used to compare differences between two groups, and four-group comparisons were analyzed by one-way analysis of variance (ANOVA) with the Tukey method for pairwise comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

NAC ameliorates autistic-like behavioral abnormalities

Previous studies have shown that NAC improved several clinical symptoms of autistic patients. Therefore, this study investigated the effects of NAC on autism-like behavioral abnormalities in a VPA-induced autism model, such as repetitive and social interaction behaviors.

The anxiety and repetitive/stereotypic behavior in rats were assessed in an open field. The results of the open-field test showed that compared with control rats, VPA rats showed a decreased frequency of crossing the center ($P < 0.01$; see Figure 1(A) and (B)) and spent more time in self-grooming behavior ($P < 0.01$; see Figure 1(C)), while VPA + NAC rats exhibited an increased number of crossing the center ($P < 0.01$; see Figure 1(A) and (B)) and spent less time performing in self-grooming behavior ($P < 0.05$; see Figure 1(C)) in comparison with the VPA group. There was no significant difference in the distance traveled among four groups (see Figure 1(D)). It is worth mentioning that NAC alone did not change the frequency of crossing the center and self-grooming behavior. These results suggested that NAC improved anxiety, repetitive/stereotypic behaviors in a VPA-induced autism model.

The three-chamber test showed that the control group was more interested in novel things, as well as the NAC group. The VPA group showed no preference in each chamber, while the VPA + NAC group showed more interest in novel things (see Figure 2(A)). The statistical results showed that the time for the control group to enter the chamber of stranger rat 1 was higher than the time for the empty chamber, and the difference was statistically significant ($P < 0.01$; see Figure 2(B)). In the VPA group, there was no statistical difference in the time spent in the empty chamber and the chamber of stranger rat 1 (see Figure 2(B)). In the VPA + NAC group, the time spent in the chamber of stranger rat 1 was higher than the time spent in the empty chamber, and the difference was statistically significant ($P < 0.05$; see Figure 2(B)). In addition, in the

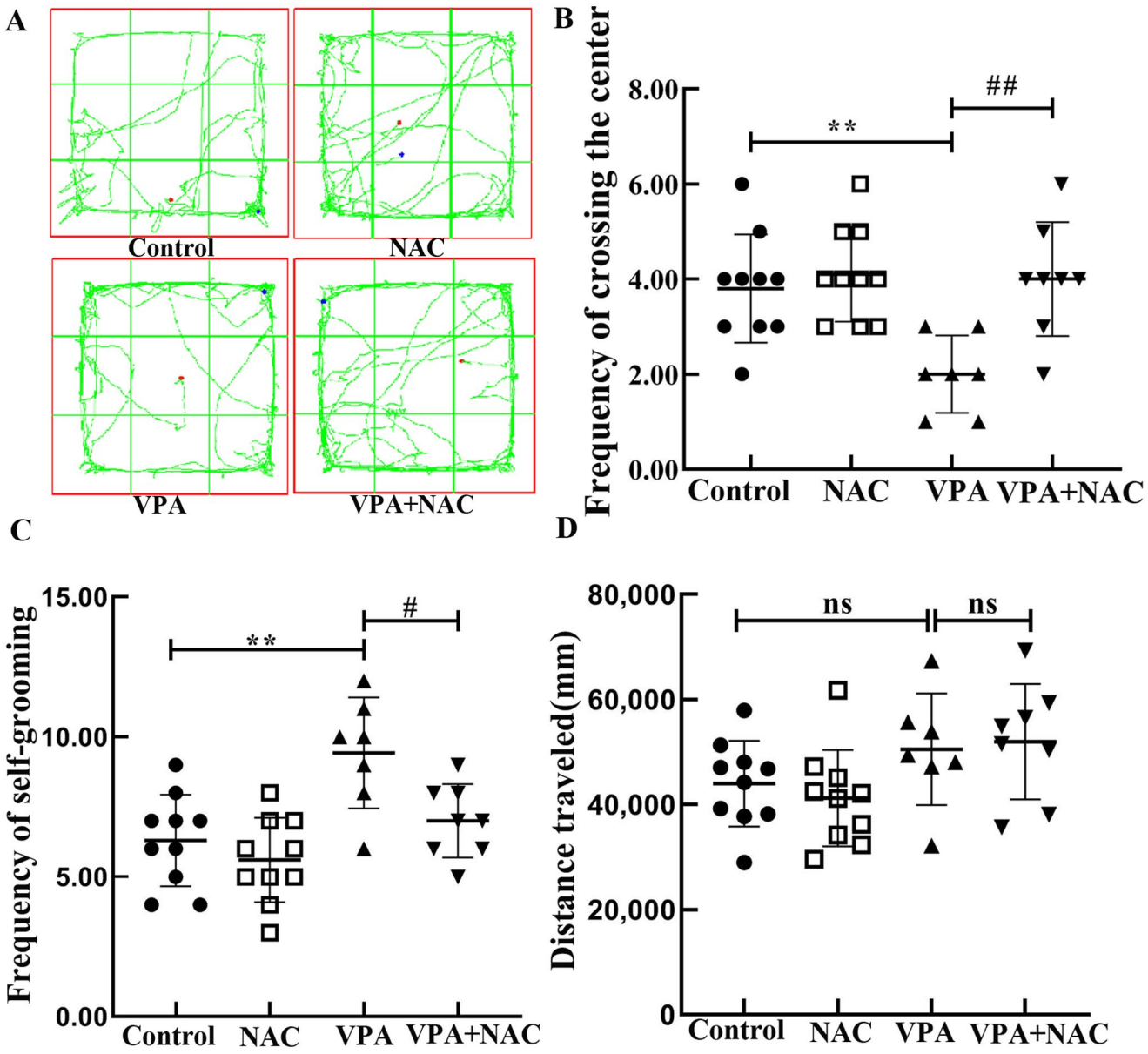

Figure 1. NAC ameliorated anxiety and repetitive behavior in VPA-exposed rats. The (A) motion trials, (B) frequency of crossing the center, (C) time spent in self-grooming, and (D) distance traveled were measured for 10 min in autotracking cages.
Data are expressed as the mean ± SD. and $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus VPA group. Control group, $n = 10$; NAC group, $n = 10$; VPA group, $n = 7$; VPA + NAC group, $n = 8$. NAC: N-acetylcysteine; VPA: valproic acid.

VPA + NAC group, the time spent in the chamber of stranger rat 2 was higher than that of stranger rat 1, and the difference was statistically significant ($P < 0.01$; see Figure 2(C)). In the VPA group, there was no statistical difference between the time spent in the chamber of stranger rat 2 and the time spent in the chamber of stranger rat 1 (see Figure 2(C)). The time for the control group to enter the chamber of stranger rat 2 was higher than that of stranger rat 1, and the difference was statistically significant ($P < 0.05$; see Figure 2(C)).

VPA induces, while NAC reverses autophagic deficiency

As we have demonstrated above, NAC ameliorates autism-like behavioral abnormalities, but whether the therapeutic effect of NAC is related to autophagy remains unclear. Moreover, numerous studies have demonstrated that abnormal autophagy may be associated with autism.[22,23] Therefore, autophagy was first detected in a VPA-induced autism model. The expression levels of autophagy-related proteins LC3B, p62, and Beclin-1 were detected by western blotting (also see Supplemental material). Our present results showed that VPA decreased the expression level of LC-3B ($P < 0.001$ for PFC, $P < 0.01$ for HC, $P < 0.01$ for CB; see Figure 3(A) to (D)) and Beclin-1 ($P < 0.001$ for PFC, $P < 0.001$ for HC, $P < 0.001$ for CB; see Figure 3(A) to (D)), while increased the level of p62 in the PFC, HC, and CB ($P < 0.01$ for PFC, $P < 0.01$ for HC, $P < 0.05$ for CB; see Figure 3(A) to (D)), suggesting that VPA induced autophagic deficiency. Then, this study determined whether NAC ameliorates VPA-induced autism rats via autophagy. The expression levels of LC3B ($P < 0.01$ for PFC, $P < 0.001$ for HC, $P < 0.01$

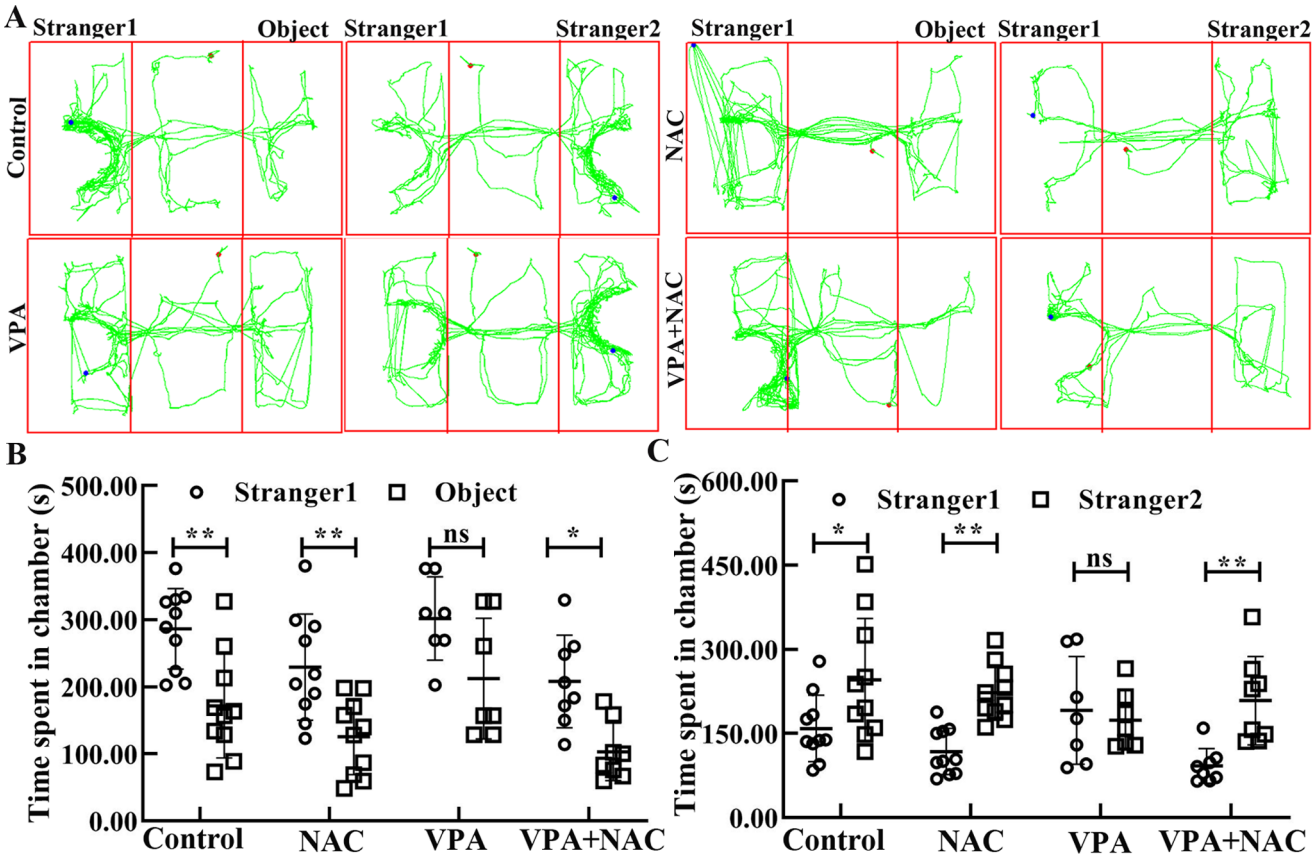

Figure 2. NAC ameliorated social interaction behavior in VPA-exposed rats. The (A) motion track, (B) time spent in chamber of stranger 1 and the empty chamber, (C) time spent in chamber of stranger 1 and stranger 2 were measured for two 10 min time series in three-chamber test.
Data are expressed as the mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus Stranger1. Control group, $n = 10$; NAC group, $n = 10$; VPA group, $n = 7$; VPA + NAC group, $n = 8$.
NAC: N-acetylcysteine; VPA: valproic acid.

for CB; see Figure 4(A) to (D)) and Beclin-1 ($P < 0.05$ for PFC, $P < 0.001$ for HC, $P < 0.05$ for CB; see Figure 4(A) to (D)) were significantly higher, whereas the levels of p62 were lower in the PFC, HC, and CB of VPA + NAC rats compared with VPA rats ($P < 0.05$ for PFC, $P < 0.01$ for HC, $P < 0.05$ for CB; see Figure 4(A) to (D)). These data demonstrated that NAC reversed autophagic deficiency in VPA-exposed autism rats, suggesting that NAC ameliorated autism-like behavior by recovering autophagic dysfunction.

VPA upregulates, while NAC downregulates the Notch-1/Hes-1 pathway

Notch-1 is involved in the regulation of autophagy,[12,13] and several studies have shown that the expression of regulatory molecules related to the Notch-1/Hes-1 pathway or other related molecules related to the pathway is abnormal in patients with autism.[24,25] Thus, this study determined whether the Notch-1/Hes-1 signaling pathway is involved in the improvement of autism-like behavioral abnormalities by NAC. First, this study examined the activity of the Notch-1/Hes-1 pathway in VPA-induced autism rats. Western blotting results showed that compared with the control group, the levels of Notch-1 (($P < 0.01$ for PFC, $P < 0.05$ for HC, $P < 0.01$ for CB; see Figure 5(A) to (D)) and Hes-1 (($P < 0.001$ for PFC, $P < 0.01$ for HC, $P < 0.05$ for CB; see Figure 5(A) to (D)) were significantly increased in the PFC, HC, and CB of VPA-exposed group, suggesting that the Notch-1/Hes-1 pathway activity was increased in the VPA-induced autism model, and activation of the Notch-1/Hes-1 pathway may lead to increased susceptibility to autism. Then, the effects of NAC on the Notch signaling pathway were measured in rats with VPA-induced autism. Western blot analysis demonstrated that, compared with VPA group, the expression of Notch-1 (($P < 0.001$ for PFC, $P < 0.01$ for HC, $P < 0.01$ for CB; see Figure 6(A) to (D)) and Hes-1 (($P < 0.01$ for PFC, $P < 0.001$ for HC, $P < 0.01$ for CB; see Figure 6(A) to (D)) was decreased in the PFC, HC, and CB of the VPA + NAC rats. However, rats treated with NAC alone did not alter expression levels of key molecules of the Notch-1/Hes-1 pathway. The results showed that NAC inhibited abnormal activation of the Notch-1/Hes-1 pathway in VPA-induced autism models, suggesting that NAC ameliorated autism-like behavior by inactivating the Notch-1/Hes-1 pathway.

VPA decreases cell viability, induces autophagic deficiency and ROS generation, while NAC reverses autophagic deficiency in SH-SY5Y neural cells

To further confirm the protection mechanism of the effects of NAC on VPA, SH-SY5Y neural cells were cultured. To investigate the effects of VPA on the survival and morphology of SH-SY5Y neural cells, the cells were exposed to 0, 2, 4, 8, 10,

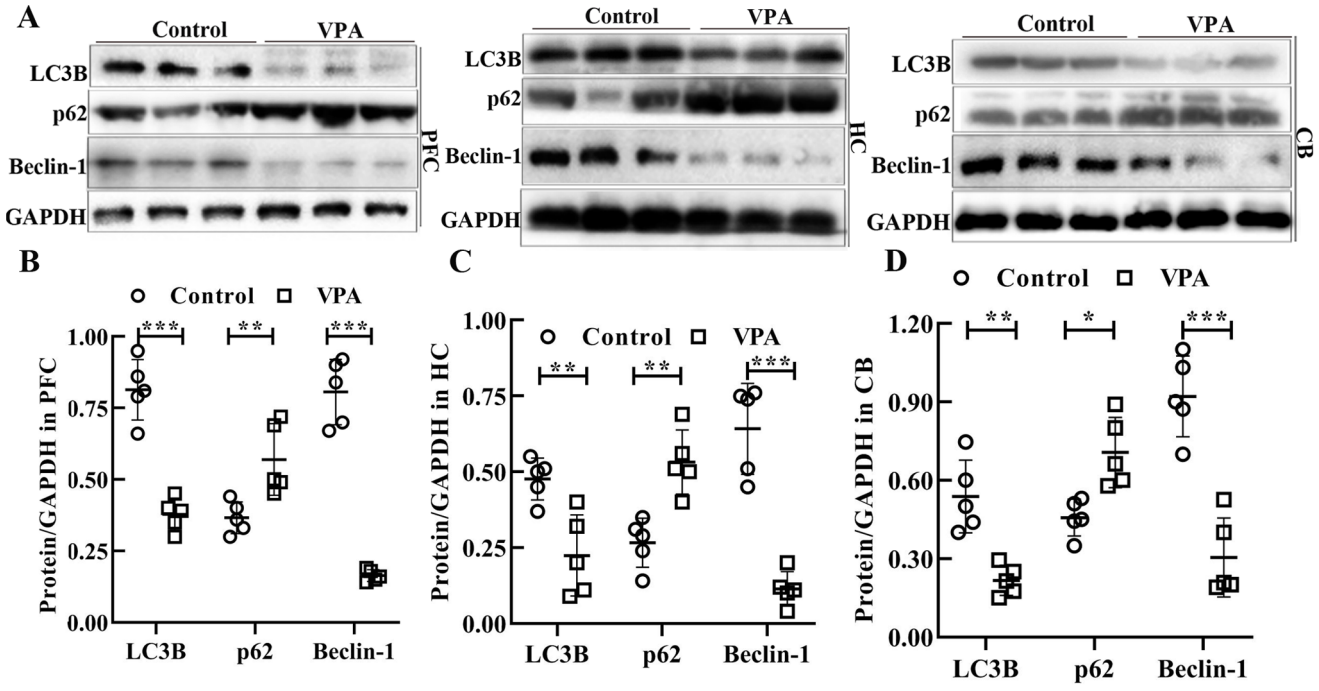

Figure 3. VPA induced autophagic deficiency in rats. The protein levels of (A to D) LC3B, (A to D) p62, and (A to D) Beclin-1 were determined using western blotting. Data are expressed as the mean ± SD. $n = 5$ for each group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus Control group. VPA: valproic acid; PFC: prefrontal cortex; HC: hippocampus; CB: cerebellum.

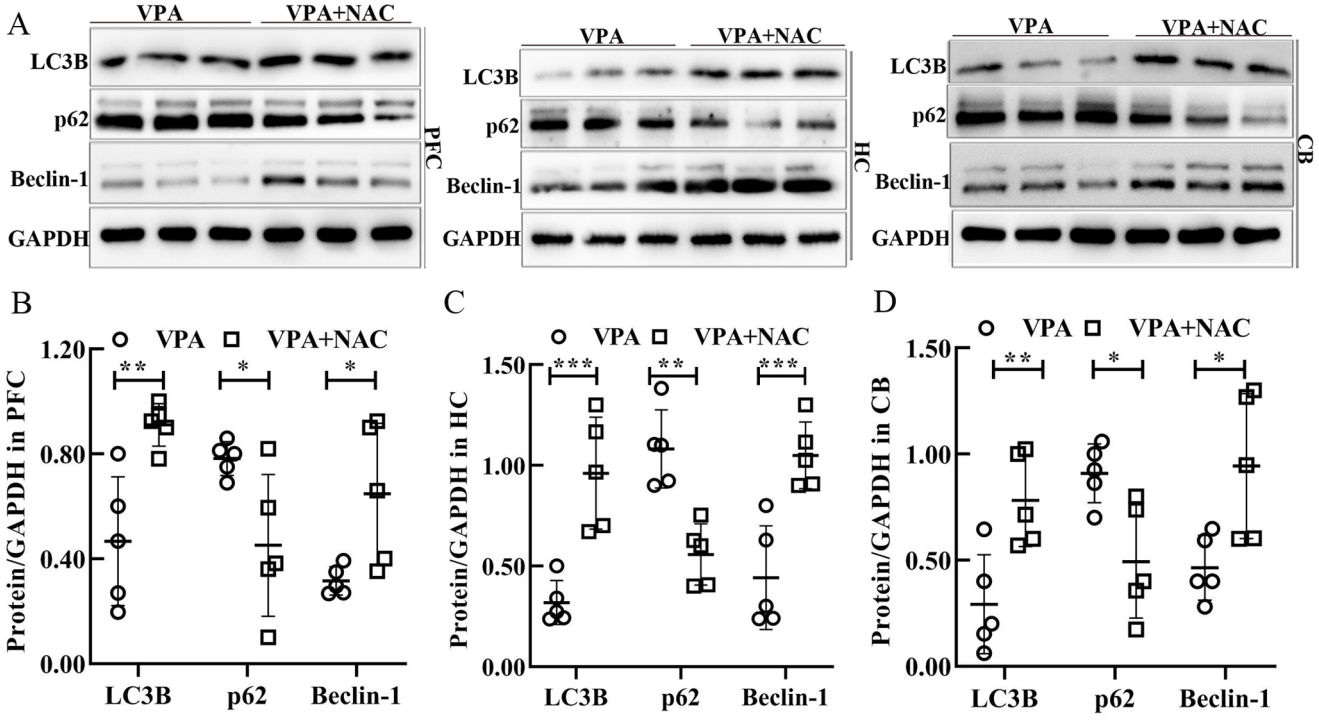

Figure 4. NAC treatment recovered autophagic deficiency in VPA-exposed rats. The protein levels of (A to D) LC3B, (A to D) p62, and (A to D) Beclin-1 were determined using western blotting.
Data are expressed as the mean ± SD. $n = 5$ for each group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus VPA group. VPA: valproic acid; NAC: N-acetylcysteine; PFC: prefrontal cortex; HC: hippocampus; CB: cerebellum.

or 12 mM VPA for 24 h, and an MTT assay was performed to determine cell viability. VPA decreased SH-SY5Y survival rate in a dose-dependent manner ($P < 0.001$, see Figure 7(A)). The survival rate of cells treated with 4 or 8 mM VPA was ~50% (see Figure 7(A)). In addition, compared with the control group, the group exposed to 4 or 8 mM VPA exhibited irregular morphology, decreased cell density, and increased cell space (see Figure 7(B)). Autophagy plays different roles

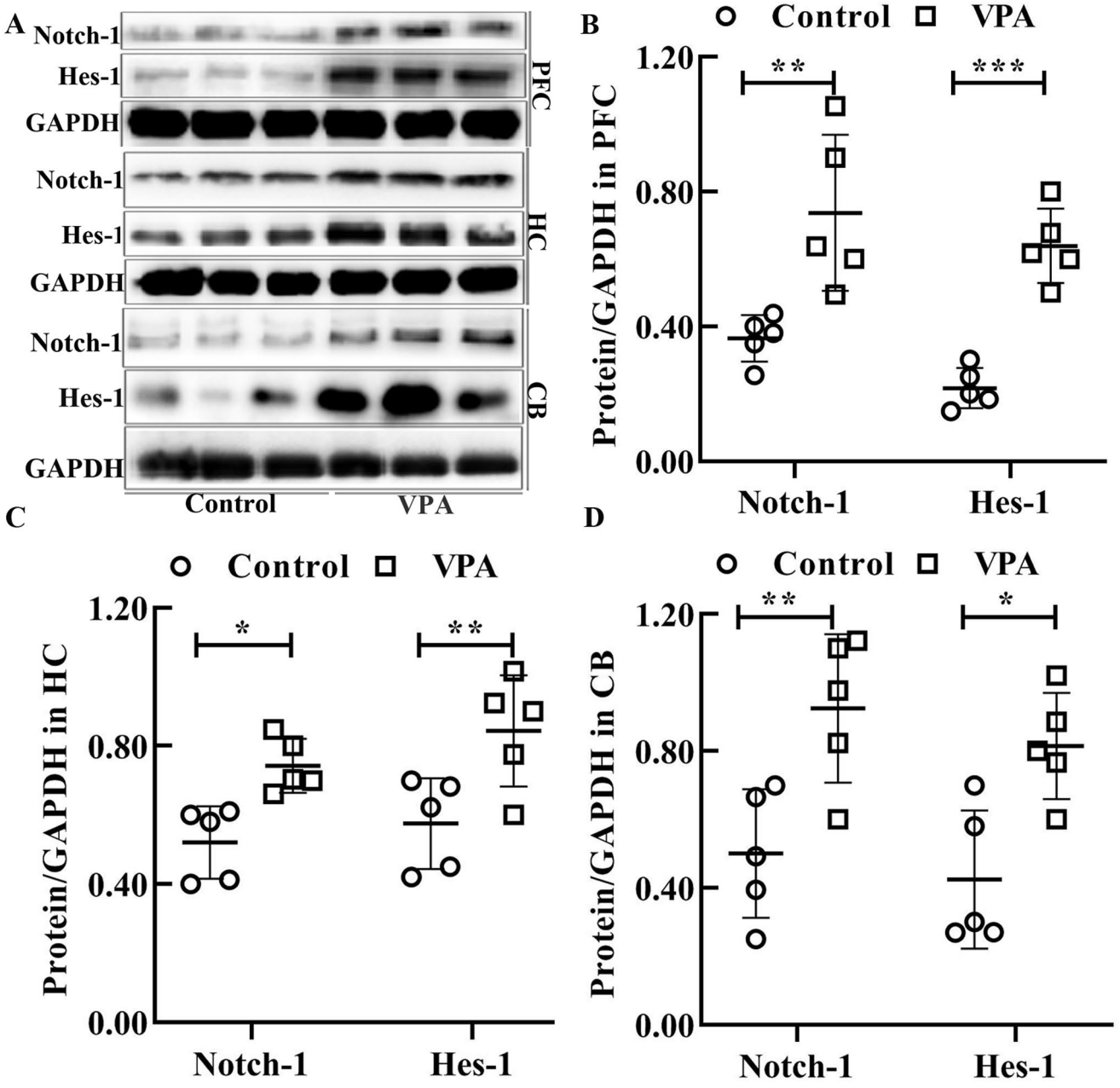

Figure 5. VPA activated Notch-1/Hes-1 signaling pathway in the PFC, HC, and CB. (A–D) The protein levels of Notch-1 and Hes-1 were determined by western blotting.
Data are expressed as the mean ± SD. $n = 5$ for each group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control. VPA: valproic acid; PFC: prefrontal cortex; HC: hippocampus; CB: cerebellum.

under different conditions, resulting in either cell death or cell survival. To assess whether VPA decreased cell survival was associated with autophagy, the cells were exposed to 4 mM VPA for 24 h and autophagy markers were measured. Western blotting analysis demonstrated that VPA decreased the expression levels of LC-3B and Beclin-1 ($P < 0.01$ for LC-3B, $P < 0.05$ for Beclin-1; see Figure 7(C)), but enhanced the levels of p62 in SH-SY5Y neural cells ($P < 0.01$, see Figure 7(C)). These results suggested that VPA inhibited autophagic flux and reduced cell viability.

VPA can enhance ROS and lipid peroxidation generation.[26,27] ROS are a natural byproduct in the process of normal oxygen metabolism and play vital roles in cell signal transduction and homeostasis. To determine whether VPA-induced autophagic deficiency is associated with ROS, SH-SY5Y cells were exposed to 2 mM NAC for 2 h following VPA treatment. As shown in Figure 7(D), VPA increased ROS generation in SH-SY5Y neural cells, and this effect was blocked by NAC. ROS have been reported to induce autophagy.[28,29] To determine whether NAC can improve VPA-induced autophagic deficiency, the effects of NAC on autophagy were measured in SH-SY5Y neural cells. The results showed that NAC increased the expression levels of LC-3B and Beclin-1 ($P < 0.01$ for LC-3B, $P < 0.05$ for Beclin-1; see Figure 7(C)), but decreased the levels of p62 in SH-SY5Y neural cells treated with VPA ($P < 0.001$, see Figure 7(C)),

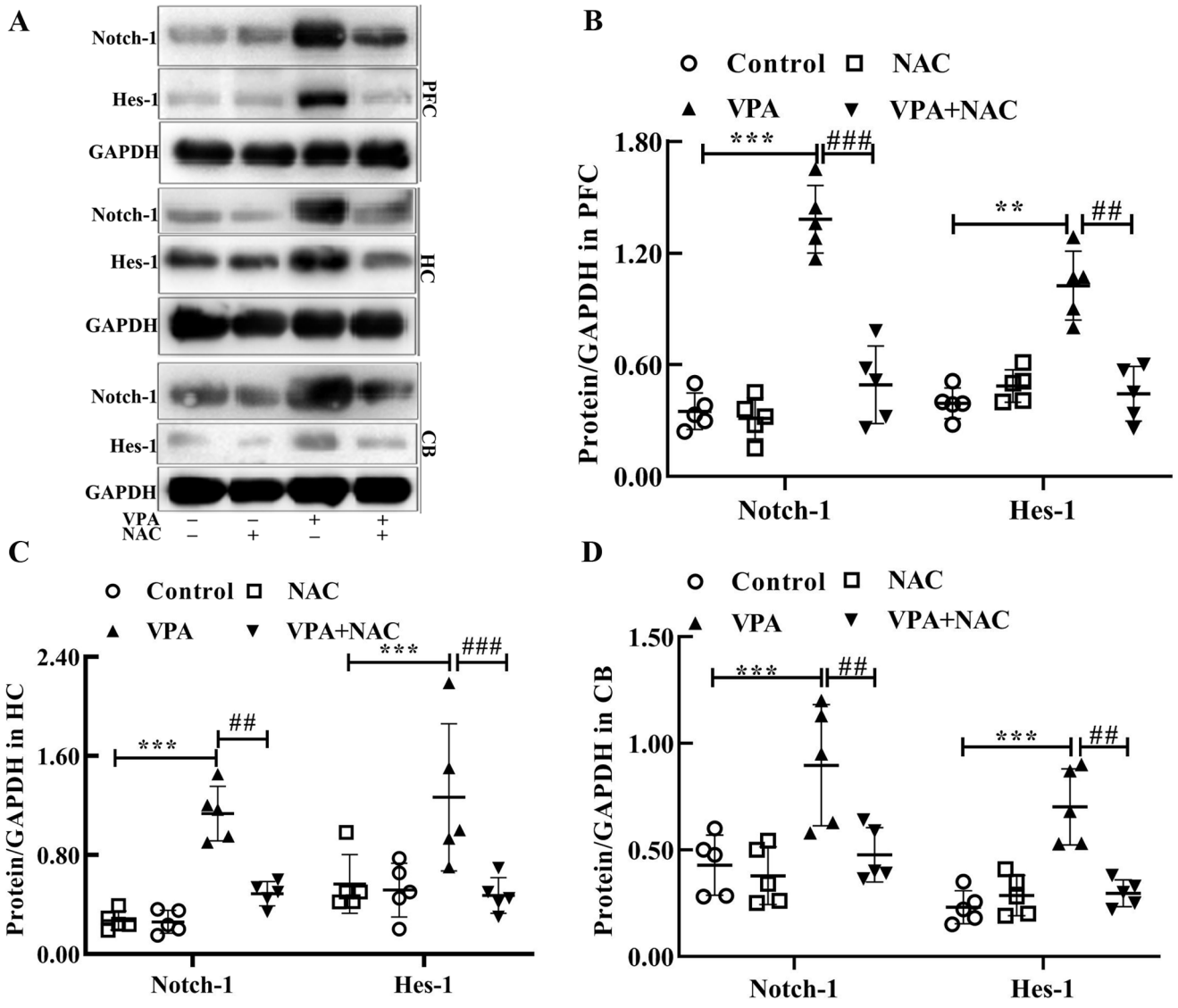

Figure 6. NAC downregulated Notch-1/Hes-1 signaling pathway activation in the PFC, HC, and CB. (A–D) The protein levels of Notch-1 and Hes-1 were determined by western blotting.
Data are expressed as the mean ± SD. $n = 5$ for each group. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus VPA group. NAC: N-acetylcysteine; VPA: valproic acid; PFC: prefrontal cortex; HC: hippocampus; CB: cerebellum.

suggesting that VPA-induced autophagic dysfunction is attributed to ROS generation.

VPA upregulates, while NAC downregulates the Notch-1/Hes-1 pathway in SH-SY5Y neural cells

It was reported that the Notch signaling pathway is highly important in the regulation of autophagy.[12,30] Therefore, this study investigated whether Notch signaling pathway is involved in NAC-ameliorated autophagy defects in VPA-exposed SH-SY5Y neural cells. The activity of the Notch pathway in VPA-treated cells was detected by western blotting. Western blotting analysis showed that VPA increased Notch-1, NICD, and Jagged-1 levels in SH-SY5Y neural cells ($P < 0.05$ for Notch-1, $P < 0.05$ for NICD, $P < 0.01$ for Jagged-1, see Figure 8(A) to (D)). Similarly, VPA increased the protein synthesis of downstream target genes, such as *Hes-1* and *Hes-5* in SH-SY5Y neural cells ($P < 0.001$ for Hes-1, $P < 0.01$ for Hes-5; see Figure 8(E) and (F)). But, VPA and NAC decreased Notch-1, NICD, Jagged-1, Hes-1, and Hes-5 expression at the protein level ($P < 0.05$ for Notch-1, $P < 0.05$ for NICD, $P < 0.05$ for Jagged-1, $P < 0.001$ for Hes-1, $P < 0.01$ for Hes-5; see Figure 8(A) to (F)). The results suggested that VPA activates, but NAC inactivates the Notch-1/Hes-1 pathway activity in SH-SY5Y neural cells.

Discussion

This study demonstrated that the activity of Notch-1/Hes-1 pathway was upregulated in rats with VPA-induced autism, while pharmacological inhibition with NAC ameliorated autism-like abnormal behavior, reduced abnormal Notch-1/Hes-1 signaling pathway activation, and recovered autophagic deficiency. NAC also protected SH-SY5Y neural cells against VPA-induced autophagic deficiency and Notch-1/Hes-1 pathway activation. The aforementioned

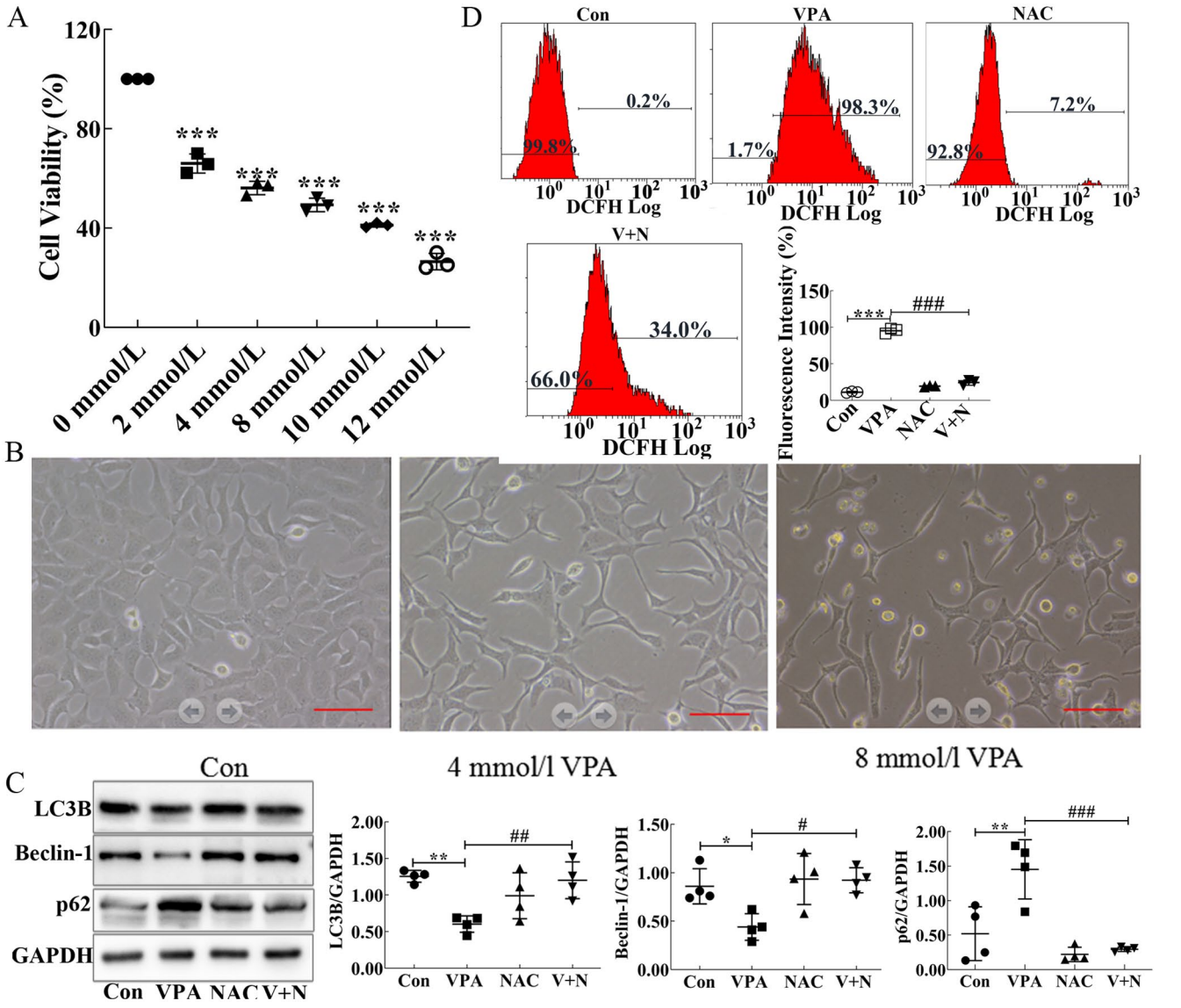

Figure 7. VPA altered (A) cell viability and (B) cell morphology of SH-SY5Y neural cells, but NAC recovered (C) autophagic deficiency and (D) ROS generation of VPA-exposed SH-SY5Y neural cells. SH-SY5Y neural cells were treated with 4 mM VPA for 24 h in the absence or presence of 2 mM NAC for 2 h.
Scale bar: 100 μm. Data are expressed as the mean ± SD. Cell viability, morphology, and ROS generation: $n = 3$ in each group; Western blotting: $n = 4$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus Con group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus VPA group. NAC: N-acetylcysteine; VPA: valproic acid; ROS: reactive oxygen species.

in vivo and *in vitro* results suggested that NAC ameliorates autism-like behavioral abnormalities by recovering autophagic deficiency and decreasing the Notch-1/Hes-1 pathway activation.

VPA can activate numerous signaling pathways, such as the canonical Wnt signaling pathway[9] and the ERK/Akt pathway.[31] This study found that the Notch-1/Hes-1 signaling pathway was activated in SH-SY5Y neural cells treated with VPA or in rats exposed to VPA, consistent with studies conducted using Clara cells[32] and human endometrial stromal cell lines.[33] However, the mechanism by which VPA activates the Notch-1/Hes-1 pathway remains unknown. The mechanism may be that VPA facilitates Hes-1 promoter activity and accelerates Notch translocation,[34] thus activating the Notch-1/Hes-1 pathway. The Notch-1/Hes-1 signaling pathway can influence cell communication and cell fate in various cell types. However, the function of Notch signaling in VPA-treated cells or animals remain controversial. Several observations demonstrated that VPA can inhibit Notch signaling in hepatocellular carcinoma cells.[35,36] Thus, the function of Notch signaling has not been fully determined and the precise Notch-mediated mechanisms remain unclear. These conflicting results are possibly associated with the fact that the effects of VPA on Notch-1/Hes-1 signaling pathway is highly cell-specific. Moreover, VPA is a pleiotropic molecule, as it can activate or suppress numerous pathways.[37] It is also possible that Notch activity induced by VPA is regulated by upstream-signaling pathways or upstream-related factors. More mechanism regarding the upstream-signaling pathways or upstream-related factors and Notch pathway functions in autism remains to be elucidated. A deeper insight into the mechanism how upstream-signaling pathways or upstream-related factors affect neuron and glial cell development, especially induced pluripotent stem cells from

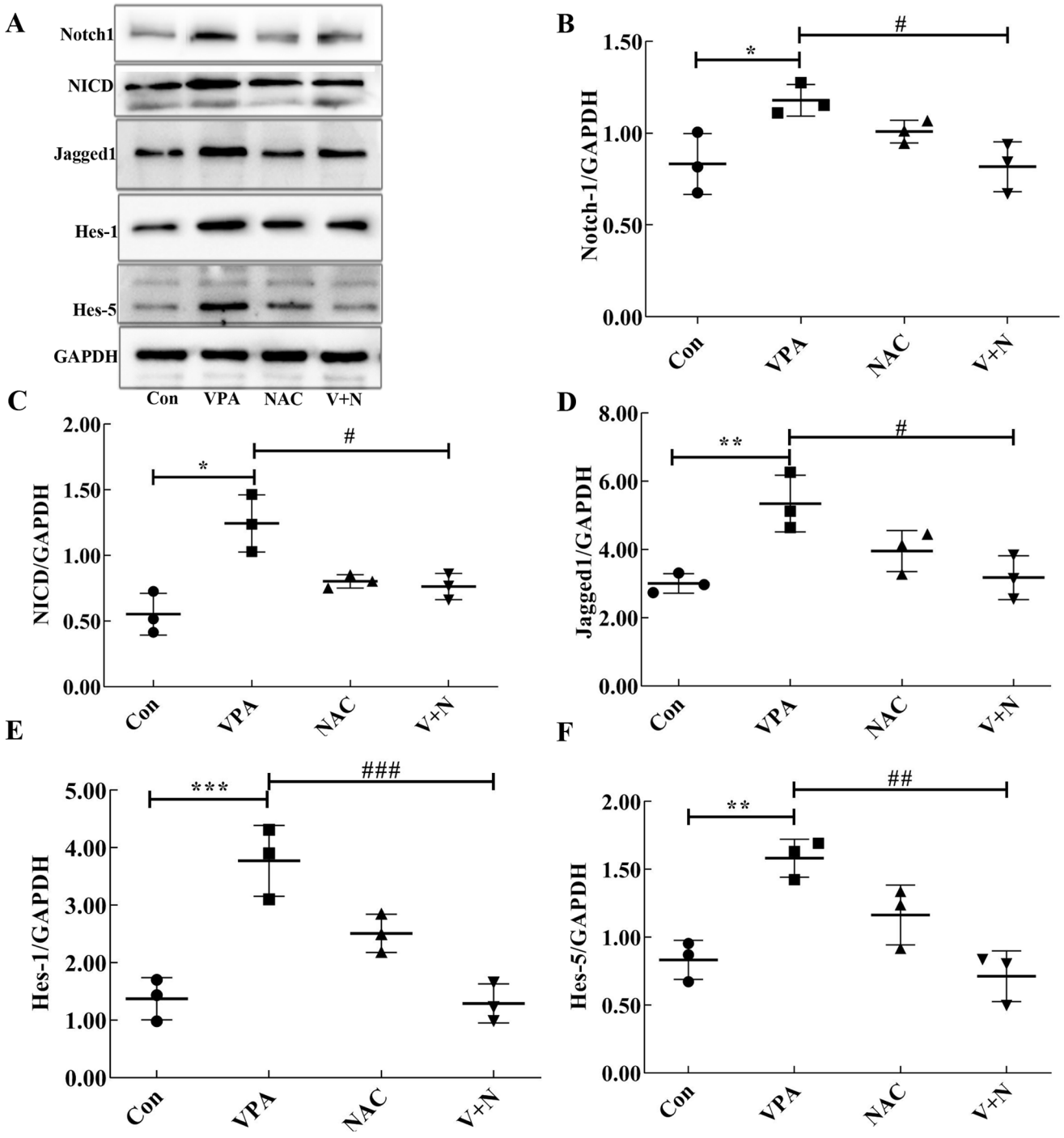

Figure 8. NAC downregulated Notch-1/Hes-1 activation in VPA-exposed SH-SY5Y neural cells. SH-SY5Y neural cells were treated with 4 mM VPA for 24 h in the absence or presence of 2 mM NAC for 2 h. The protein levels of (A and B) Notch1, (A and C) NICD, (A and D) Jagged1, (A and E) Hes-1 and (A and F) Hes-5 were determined using western blotting.
Data are expressed as the mean ± SD. $n = 3$ for each group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus Con group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus VPA group. NAC: N-acetylcysteine; VPA: valproic acid.

patients with autism, could contribute to reveal the precise pathogenesis of autism.

Oxidative stress plays an important role in the pathogenesis of autism.[38,39] Oxidative stress leads to injury in neurons and glial cells in brain areas associated with autism, thus consequent behavioral abnormalities. So, antioxidant therapy has a potential in the treatment for autism.[40] NAC is a precursor drug of cysteine, a major component of the antioxidant system, so its antioxidant effect is also considered to be one of the potential mechanisms in the treatment of autism. In our previous study, we showed that NAC (150 mg/kg), when intraperitoneally administered to VPA-induced autism model rats once daily for 4 weeks, reduces oxidative stress, based on reduced malondialdehyde and increased glutathione contents in PFC and HC of VPA-induced autism model rats after NAC treatment.[9] Although NAC has been shown to improve autism-like behavioral abnormalities, such as irritability,[5,6] social awareness, and repetitive/stereotypic

behavior,[9] the mechanism of action of NAC remains unclear. Previous evidence has shown that ROS play a vital role in regulating the Notch pathway,[41] and upregulating the Notch receptor Notch-1.[42] This study showed that NAC can ameliorate anxiety-like behaviors, repetitive/stereotypic behavior and social behavior disorder, and the mechanism may be related to its downregulation of Notch-1/Hes-1 signaling pathway activation *in vitro* and *in vivo*, suggesting that ROS may play an important role in VPA-induced activation of Notch-1/Hes-1 pathway. However, the role of ROS signaling pathway in the occurrence of autism, and how this pathway affects VPA-induced Notch-1/Hes-1 signaling pathway activation, and then participates in the occurrence of autism are still unclear, which requires further research.

ROS have also been reported to induce autophagy,[28,29] but autophagy serves as a buffer system to control the level of ROS in cells and reduce their toxic effects.[43] Autophagy plays an important role in the elimination of proteins and organelles that are damaged by oxidative stress. Our present results showed that autophagic deficiency is induced in VPA-treated SH-SY5Y neural cells or in VPA-induced autism animal models, while NAC reversed autophagic deficiency. VPA induces autophagic dysfunction in numerous diseases, such as gastric cancer,[44] myocardial dysfunction,[45] traumatic brain injury,[46] and renal fibrosis.[47] The mechanism by which VPA influences autophagy-related proteins, and by which autophagy exerts directional and specific control on the onset of different diseases, and the signaling pathway that contributes to these processes remain unknown. Autophagy is a process that relies on the lysosome pathway to degrade cytoplasmic proteins and organelles. VPA induced protein reduction of LC-3B and Beclin-1 in this study, suggesting that VPA may decrease autophagosome synthesis, or increase autophagy degradation. Autophagy protein p62 is degraded in late autophagy, and its level is negatively correlated with autophagy activity. This study showed that VPA increases protein expression of p62, indicating that the autophagy activity may be decreased or the degradation process may be hindered. On the contrary, NAC enhanced protein expression of LC-3B and Beclin-1, and lowered the p62 expression levels, indicating that the number of autophagosomes may be increased after autophagy activation in the early stage, or autophagosomes may be accumulated due to the failure of the autophagosome to fuse with the lysosome or due to lysosomal dysfunction or reduction in number in the late stage, which requires further research. Notably, Notch signaling pathway activation inhibits autophagic flux.[48] Moreover, pharmacological inhibition of the Notch pathway can induce autophagy in glioma neurospheres.[14] Interestingly, autophagy modulated Notch-1 degradation, thus regulating the Notch pathway in primary neurons.[49] Different mechanisms of the regulatory relationship between autophagy and Notch signaling have been reported, and these mechanisms may be specific to different disease types or specific cell types. Although this study did not elucidate the regulatory relationship between autophagy and the Notch signaling pathway in autism, our recent study found that the Notch pathway inhibitor DAPT reversed autophagic deficiency in a VPA-exposed autism model.[11] It is likely that the Notch pathway regulates autophagic function and may influence the occurrence of autism. Further research may be able to clarify this point.

Conclusions

This study demonstrated that VPA exposure induces Notch-1/Hes-1 activation and autophagic deficiency. NAC ameliorated VPA-induced behavioral abnormalities, inhibited VPA-induced Notch-1/Hes-1 pathway activation and recovered autophagic deficiency, suggesting that NAC may inhibit Notch-1/Hes-1 signaling pathway activity, thus recover autophagy defects, and ultimately improve autistic-like behavioral abnormalities, such as repetitive behavior and social interaction disorder. This study helps to elucidate a novel molecular mechanism that underlies the action of NAC in autism and to suggest its potential to ameliorate behavioral abnormalities in neurodevelopmental disorders.

AUTHORS' CONTRIBUTIONS

Y-HZ conceived and designed the experiments, analyzed the data, wrote the article, and contributed to the article revision. TW, Y-FL, and Y-ND performed the experiments and analyzed the data. X-LH and L-JW conducted the experiments. All authors approved the final version of the article.

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

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