doi: 10.4149/gpb_2024036

Anisodine hydrobromide injection promotes neural remodeling and recovery after ischemic stroke in mice

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Abstract. Anisodine hydrobromide injection has shown promising therapeutic effects in treating patients with cerebral infarction, improving recovery of neurological function during the post-cerebral infarction period. However, the effects of anisodine hydrobromide on brain recovery and neuroplasticity are unclear. This study explores the therapeutic effects and underlying mechanisms of anisodine hydrobromide in mice experiencing the chronic phase of an ischemia stroke. The electrocautery method established a distal middle cerebral artery occlusion (MCAO) model in healthy male C57BL/6 mice. Neurological deficits were evaluated using Golgi and immunofluorescence staining to measure the effects of anisodine hydrobromide on neural proliferation, migration and remodeling. DAPT (dipeptidic γ-secretase-specific inhibitor) was employed to explore the involvement of the Notch signaling pathway post-anisodine hydrobromide treatment. Compared to the control and MCAO groups, mice treated with anisodine hydrobromide showed improved post-stroke neurological function, increased neurite intersections, and dendritic spine density in the peri-infarct cortex. Anisodine hydrobromide also promoted neural cell regeneration which is dendritic and axonal structures and synaptic vesicle protein restructuring. Gap43, NGF, Notch1, and Hes1 protein level increased significantly in the ANI group provided inhibitor DAPT was absent. Anisodine hydrobromide can promote neurological function, neurotrophic factors, and neuroplasticity. Notch signaling pathways also impact the effects of anisodine hydrobromide on neural plasticity in ischemia stroke.

Key words: Ischemia stroke in mice — Anisodine hydrobromide — Notch signaling pathway — Neural remodeling

Abbreviations: CCA, common carotid artery; DAPT, dipeptidic γ-secretase-specific inhibitor; DG, dentate gyrus; dMCAO, distal middle cerebral artery occlusion; Map2, microtubule-associated protein 2; MCA, middle cerebral artery; mNSS, modified neurological severity score; NF, neurofilament; NGF, nerve growth factor; NICD, Notch internal cellular domain; SGZ, subgranular zone; SVZ, subventricular zone; Syp, synaptophysin; TTC, 2,3,5-triphenyltetrazolium chloride.

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Introduction

During a cerebral infarction, narrowed or blocked blood vessels limit oxygen and nutrients in brain tissue. The resulting neurological impairment can be temporary and reversible if thrombolytic therapy is applied within 6 hours of ischemia, a therapeutic window that can be too narrow for some patients (Lee and Ding 2020). Therefore, clinicians have sought treatments targeting the subacute and recovery phases of strokes to reduce the probability of residual effects in stroke patients (Hawe et al. 2019; Grefkes and Fink 2020). When the central nervous system suffers ischemic damage, the neurons in the affected area regenerate to meet the demands of neural function (Egawa et al. 2017). Neuroplasticity therapy for stroke patients may thus reduce permanent neurological impairments (Xing and Bai 2020).

Anisodine is a tropane alkaloid extracted from the roots of the club moss plant *Huperzia serrata*. Previous clinical observations have demonstrated that during the recovery period of ischemic stroke and neurodegenerative conditions such as traumatic optic neuropathy, the use of anisodine hydrobromide injection can stimulate neural cell growth and improve patients' neurological function (Varma and Yue 1986; Chang et al. 2006; Wan et al. 2023; Wang et al. 2023). Chen et al. (2017) have proved that a low dose of anisodine hydrobromide can revise the imbalance of the monoamine neurotransmitter, regulate cholinergic dysfunction, and attenuate neuronal cell death and apoptosis. However, further research is required to determine whether anisodine hydrobromide can promote neural remodeling during focal cerebral infarction's subacute and recovery phases.

The Notch signaling pathway is crucial in neural cell differentiation, proliferation, and apoptosis (Wu et al. 2020). Mathieu et al. (2019) have recently shown that when a cerebral infarction occurs, the Notch signaling pathway is activated and regenerates and remodels damaged neural cells. The Notch receptors are transmembrane proteins activated by Delta or Jagged ligands. The receptors need the γ-secretase enzyme complex to cleave the Notch internal cellular domain (NICD) to activate the signal pathway. Then, NICD translocates into the nucleus and activates transcription (Wang et al. 2009). According to Abedin et al. (2022), a dipeptidic γ-secretase-specific inhibitor (DAPT) is an inhibitor of the γ-secretase complex. Therefore, DAPT indirectly inhibits the Notch pathway activation. In this study, DAPT was administrated as an inhibitor of the Notch signal pathway to assess the effect of anisodine hydrobromide on neural remodeling when the Notch signal pathway has been inhibited.

This process resembles anisodine hydrobromide's actions when used to treat focal cerebral infarction. This study therefore assesses the effects and mechanisms of anisodine hydrobromide on neurological function and plasticity at the chronic phase in ischemia stroke mice.

Materials and Methods

Animals

The animal studies used young male C57BL/6 mice (age 8–12 weeks, weight 23–25 g) purchased from the Vital River Laboratory Animal Technology Co., ltd (Beijing, China). Mice had free access to water and food in a home cage with controlled temperature (22 ± 3°C) and humidity (60 ± 5%) under a 12-h light/dark cycle. The mice care, and experimental procedures were carried out according to ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines.

Surgery: middle cerebral artery occlusion

The model of distal middle cerebral artery occlusion (dMCAO) was used in this study. The mice were anestheted with Avertin (400 mg/kg, Sigma Aldrich, Cat#T48402-25G). Permanent focal cortical ischemia was induced by permanent occlusion of the right middle cerebral artery (MCA) and the common carotid artery (CCA). Mice were secured supine, and the right CCA was double-ligated with sutures. Subsequently, the right cortical branch of the MCA was dissected, followed by cauterization using an electrocoagulation pen. Muscular tissue and skin were sutured, and the mice were placed on a 37°C warming pad to recover. The mice underwent the same surgical procedure in the control group without artery occlusion.

Drug administration and experimental groups

The purity of the anisodine hydrobromide product (Chengdu First Pharmaceutical Co., Ltd., Sichuan, China) is 99.6%. The anisodine hydrobromide was dissolved in 0.9% saline. The mice received tail intravenous injections of anisodine hydrobromide (0.2 ml) for 14 days, starting within 4 hours of the dMCAO surgery.

In preliminary experiments, the mice were divided into four groups: (1) MCAO group: dMCAO surgery and equal volume of 0.9% saline; (2) control group: surgery without occlusion and 0.9% saline; (3) AniH group: dMCAO surgery and high dose (0.3 mg/kg) of anisodine hydrobromide; and (4) AniL group: dMCAO surgery and low dose (0.15 mg/kg) of anisodine hydrobromide. Behavioral assessments showed that the AniH group had improved neurological function; the associated anisodine hydrobromide dose of 0.3 mg/kg was therefore used to evaluate Notch pathways.

In the Notch pathway evaluation, a dipeptidic γ-secretase-specific inhibitor, DAPT (40 mg/kg, N-[N-(3,

5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) was dissolved with dimethylsulfoxide, and administered *via* intraperitoneal injection (2 ml) for 14 days post-dMCAO surgery. The five experimental groups included (1) MCAO group: dMCAO surgery and 0.9% saline; (2) control group: surgery without occlusion and 0.9% saline; (3) ANI group: dMCAO surgery and anisodine hydrobromide 0.3 mg/kg; (4) ANI+DAPT group: dMCAO surgery, DAPT 40 mg/kg and anisodine hydrobromide 0.3 mg/kg; and (5) MCAO+DAPT group: dMCAO surgery and DAPT 40 mg/kg.

Behavioral assessment and weight evaluation

The neurological function of mice was assessed *via* the Rotarod test, the Adhesive removal test, mNSS, and weight evaluation. Mice were tested in each task 5 days pre-surgery to establish a baseline, and post-surgery on Days 1, 3, 7, 14, 21, and 28. Evaluation was blind, as tests were scored without experimenter knowledge of group membership. Behavioral data were collected over time and averaged for statistical analysis.

Rotarod test

The Rotarod test assesses coordination, balance, and motor endurance in mice. Mice capable of remaining on a rotating rod for at least 60 seconds were selected for grouping. At different time points, mice were placed on a gradually accelerated rotating rod (4–40 rpm in 4 min). Time measurement began when a mouse was positioned on the rod, and ended when that mouse fell off. Each mouse underwent the test three times, with at least a 15-min interval between trials.

mNSS behavioral assessment

The modified Neurological severity score (mNSS) is used to assess sensory function, motor abilities, balance, and reflexes in mice. Scores range from 0 to 18: 13–18 indicates severe injury, 7–12 moderate injury, and 1–6 mild injury.

Adhesive removal test

The Adhesive removal test evaluates sensory deficits and motor impairment in mice before and after cerebral ischemia. During the experiment, two adhesive-backed small pieces of paper (0.3 × 0.4 cm) are placed with equal pressure on both sides of the forepaws of the mouse. The timing starts when the mouse is returned to its cage, assessing the time taken for the mouse to make contact and remove the adhesive strips from both forepaws. Self-induced sensory deficits were measured *via* the following ratio: (time taken to remove affected forelimb paper – time taken to remove unaffected forelimb paper)/(time taken to remove affected forelimb paper + time taken to remove unaffected forelimb paper).

Weight measurement

The body weight of mice reflects their recovery of feeding ability and was measured *via* a weight gain rate (WGR) calculated using the formula: (final weight – initial weight)/initial weight.

Measurement of cerebral infarction volume

The cerebral infarction area was determined using the 2,3,5-triphenyl tetrazolium chloride (TTC) staining method at the 3rd and 7th days post-modeling. Brain tissue was sliced into six coronal sections (2 mm). These sections were then immersed in a 2% TTC staining solution and incubated in a light-protected 37°C incubator for 30 min until uniform staining occurred and fixed in 4% paraformaldehyde solution for 24 hours before being photographed by digital camera. Hemisphere lesion volume ratio (%) = [total infarct volume – (volume of intact ipsilateral hemisphere – volume of intact contralateral hemisphere)]/contralateral hemisphere volume × 100.

Golgi staining method

The Golgi staining method can distinguish subtle morphological changes in dendrites and dendritic spines. The location for Golgi staining is the peri-infarct cortex of the ipsilateral hemisphere in the experimental mice. This study used a rapid Golgi staining kit (FD NeuroTechnologies, Baltimore, MD, USA) to perform staining on brain tissue obtained from mice in each experimental group after being euthanized (intraperitoneal administration of pentobarbital sodium 150–200 mg/kg) 28 days after modeling. All experimental steps following the manufacturer's protocols. The number of dendritic bifurcations and spine density were counted and branch points were analyzed by NeuronJ plugin, ImageJ software and Sholl analysis.

Immunofluorescence staining

Immunofluorescence staining was performed to evaluate the remodeling of neuronal dendrites, axons, and synapses using microtubule-associated protein 2 (Map2), synaptophysin (Syp), neurofilament (NF), NeuN, and DCX staining (Abcam Co., UK). In immunofluorescence detection, Map2, NF, and Syp were analyzed in the peri-infarct cortex of the ipsilateral hemisphere, while DCX staining was performed in the ipsilateral subventricular zone (SVZ). After 14-day treatment, mice were anesthetized and perfused transcardially with saline to clear blood, followed by 4% paraformaldehyde

(PFA). Brain tissue was then dehydrated in 30% sucrose for 48 hours, embedded, and stored in a freezer at −80°C. Frozen brain tissue was sectioned in 20 μm slices using a cryotome (Thermo Scientific, USA), permeabilized using 0.5% Triton X-100 for 15 min, blocked with 10% donkey serum at 37°C for 1 hour, and incubated with different primary antibodies at 4°C overnight. The following day, slices were washed with PBS and treated with secondary antibodies (Alexa Fluor 488 or 594, Jackson Immuno Research, USA) at 37°C for 1 hour. Finally, following another PBS wash, the slices were exposed to droplets of an anti-fluorescence quenching agent subjected to coverslipping, after which the sections were observed and photographed with laser scanning confocal microscopy (Zeiss LSM880, German). This study performed immunofluorescence staining on mouse tissues on the 28th day, but the results were not ideal. This may be due to scar formation and tissue atrophy, leading to abnormal tissue sections (immunofluorescence typically requires 15–20 μm thick sections). Therefore, we ultimately chose to perform immunofluorescence staining on mouse tissues on the 14th day of the experiment.

Western blot detection

After the 14-day treatment, the brains were harvested for further experiments. We used the Western blot method to conduct experimental analysis on the protein levels of GAP43 (axonal growth marker), NGF, Notch internal cellular domain (NICD), and Hes1. Proteins from the cortex surrounding the ischemic infarct area were obtained using Radio Immunoprecipitation Assay (RIPA) lysis buffer (Solarbio, China). The protein concentration was detected by a bicinchoninic acid protein assay reagent kit (Thermo Fisher Scientific, USA). An equivalent amount (50 μg) of protein samples were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Roche, USA). These membranes were blocked with 5% nonfat milk for 1 hour at room temperature, followed by overnight incubation with primary antibodies at 4°C. GAPDH or β-actin was used as an internal reference for total protein to calculate the relative levels of the target protein. The next day, membranes were washed with TBST three times and incubated with either anti-rabbit IgG secondary antibody (Rockland, USA) or anti-mouse IgG secondary antibody (Rockland, USA) for 1 hour at room temperature. Finally, the membrane was scanned by an infrared scanner (LICOR Bioscience, USA) and analyzed using ImageJ software. Primer antibodies are presented in Table 1.

Table 1. Basic information of the Western blot rabbit antibodies

Antibodies		Concentration
Monoclonal		
	GAP43	1 : 800
	NGF	1 : 800
	NICD	1 : 800
	Hes1	1 : 800
Polyclonal		
	GAPDH	1 : 10000
	β-actin	1 : 10000

Source: Abcam.

Statistical analysis

All statistical analyses of the data in this study were conducted using SPSS 26.0 software (IBM, USA). Normally distributed data were presented as mean ± SEM. Paired comparisons were conducted using t-tests, while multiple groups were analyzed using one-way analysis of variance (ANOVA). For continuous data that did not meet the criteria for normality, non-parametric tests were employed for comparative analysis. A significance level of $p < 0.05$ was considered statistically significant.

Results

Anisodine hydrobromide promotes neurological function of mice after ischemia stroke

In order to investigate whether Anisodine hydrobromide promotes neurological function recovery, we measured the Rotarod test, Adhesive removal test, mNSS and weight at different time points over 4 weeks post-surgery. Compared to the MCAO group, mice in the AniH group showed significantly longer average times on the Rotarod test at 3, 7, 14, 21, and 28 days post-surgery (Day 3: 42.23 ± 3.70 *vs.* 68.33 ± 3.32, $p < 0.01$; Day 7: 50.42 ± 3.39 *vs.* 79.54 ± 8.44, $p < 0.01$; Day 14: 60.54 ± 3.01 *vs.* 82.96 ± 3.14, $p < 0.01$; Day 21: 62.66 ± 4.70 *vs.* 80.88 ± 6.93, $p < 0.01$; Day 28: 67.79 ± 3.40 *vs.* 83.60 ± 5.40, $p < 0.01$) (Fig. 1A). Additionally, compared to the AniL group, mice in the AniH group had longer average times on days 3, 7, and 14 (Day 3: 55.09 ± 3.63 *vs.* 68.33 ± 3.32, $p < 0.01$; Day 7:61.30 ± 5.28 *vs.* 79.54 ± 8.44, $p < 0.01$; Day 14: 79.92 ± 4.56 *vs.* 82.96 ± 3.14, $p < 0.01$) (Fig. 1A). Compared to the MCAO group, the mNSS scores consistently decreased in the AniH group on Days 3, 7, 14, 21, and 28 (Day 3: 5.50 ± 1.07 *vs.* 4.25 ± 0.89, $p < 0.05$; Day 7: 4.63 ± 1.30 *vs.* 3.13 ± 0.83, $p < 0.05$; Day 14: 4.13 ± 1.13 *vs.* 2.75 ± 0.71, $p < 0.05$; Day 21: 2.75 ± 0.89 *vs.* 1.63 ± 0.74, $p < 0.05$; Day 28: 2.25 ± 0.89 *vs.* 1.25 ± 0.46, $p < 0.05$) (Fig. 1B). Compared to the AniL group, the mNSS scores were lower in the AniH group on Day 14, 21 and 28 ($p < 0.05$) (Day 14: 3.63 ± 0.74 *vs.* 2.75 ± 0.71, $p < 0.05$; Day 21: 2.75 ± 0.71 *vs.* 1.63 ± 0.74, $p < 0.05$; Day 28: 2.38 ± 0.92 *vs.*

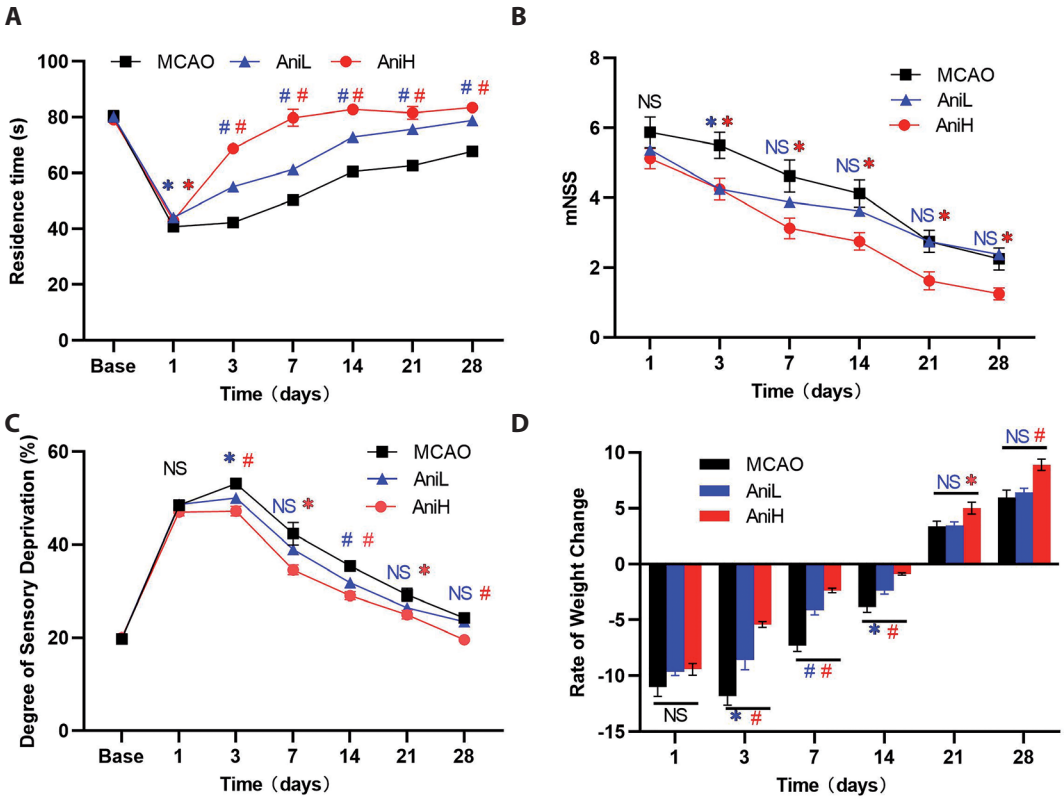

Figure 1. Anisodine hydrobromide promotes neurological recovery. Rotarod test (**A**), mNSS evaluation (**B**), Adhesive removal test (**C**) and the growth rate of body weight (**D**) were performed to assess neurological function on Days 1, 3, 7, 14, 21, and 28 after stroke. $n = 8$, each group; * $p < 0.05$, # $p < 0.01$; NS, no statistical difference. (For color figure see online version.)

1.25 ± 0.46, $p < 0.05$) (Fig. 1B). In Adhesive removal test, the most severe self-sensory deficits and motor impairments in all three groups of mice occurred on the 3rd day after surgery. Compared to the MCAO group, mice in the AniH group displayed milder self-sensory deficits and motor impairments on the 3rd, 7th, 14th, 21th and 28th days (Day 3: 53.10 ± 2.11 *vs.* 47.25 ± 2.82, $p < 0.01$; Day 7: 42.35 ± 6.91 *vs.* 34.59 ± 2.88, $p < 0.05$; Day 14: 35.41 ± 2.09 *vs.* 29.05 ± 2.40, $p < 0.01$; Day 21: 29.24 ± 3.94 *vs.* 24.94 ± 2.62, $p < 0.05$; Day 28: 24.22 ± 2.26 *vs.* 19.58 ± 0.68, $p < 0.01$) (Fig. 1C). Compared to the AniL group, the AniH group showed milder self-sensory deficits and motor impairments on Day 7, 14, and 28 (Day 7: 38.95 ± 1.21 *vs.* 34.59 ± 2.88, $p < 0.05$; Day 14: 31.80 ± 2.42 *vs.* 29.05 ± 2.40, $p < 0.05$; Day 28: 23.45 ± 1.74 *vs.* 19.58 ± 0.68, $p < 0.01$) (Fig. 1C). In addition, AniH group showed higher weight gain rate than MCAO group on Days 3, 7, 14, 21 and 28 after modeling (Day 3: −11.83 ± 2.31 *vs.* −5.42 ± 0.72, $p < 0.01$; Day 7: −7.31 ± 1.45 *vs.* −2.37 ± 0.60, $p < 0.01$; Day 14: −3.84 ± 1.34 *vs.* −0.86 ± 0.28, $p < 0.01$; Day 21: 3.39 ± 1.28 *vs.* 5.00 ± 1.48, $p < 0.05$; Day 28: 5.97 ± 1.84 *vs.* 8.89 ± 1.44, $p < 0.01$) (Fig. 1D). Compared to the AniL group, mice in the AniH group showed better recovery in average weight gain rate on Days 3, 7, 14, 21 and 28 (Day 3: −8.62 ± 2.37 *vs.* −5.42 ± 0.72, $p < 0.01$; Day 7: −4.16 ± 1.10 *vs.* −2.37 ± 0.60, $p < 0.01$; Day 14: −2.38 ± 0.90 *vs.* −0.86 ± 0.28, $p < 0.01$; Day 21: 3.46 ± 0.91 *vs.* 5.00 ± 1.48, $p < 0.05$; Day 28: 6.41 ± 1.12 *vs.* 8.89 ± 1.44, $p < 0.01$). After 28 days of modeling, the weight gain rates were 5.98% for the MCAO group, 6.41% for the AniL group, and 8.89% for the AniH group (Fig. 1D).

Anisodine hydrobromide promotes neurological function of mice after ischemia stroke through Notch signal pathway

In order to investigate the effect of the DAPT inhibitor and the effect of Ani with DAPT, we also measured the behavioral tests at different time points.

Compared to the MCAO+DAPT group, mice in the MCAO group showed significantly longer average times on the Rotarod test at 7, 14, 21, and 28 days post-surgery (Day 7: 50.84 ± 2.73 *vs.* 46.73 ± 2.25, $p < 0.01$; Day 14: 61.14 ± 2.19 *vs.* 57.13 ± 3.00, $p < 0.01$; Day 21: 63.2 ± 2.85 *vs.* 58.00 ± 2.83, $p < 0.01$; Day 28: 67.45 ± 2.57 *vs.* 61.5 ± 1.85, $p < 0.01$) (Fig. 2A). Compared to the ANI+DAPT group,

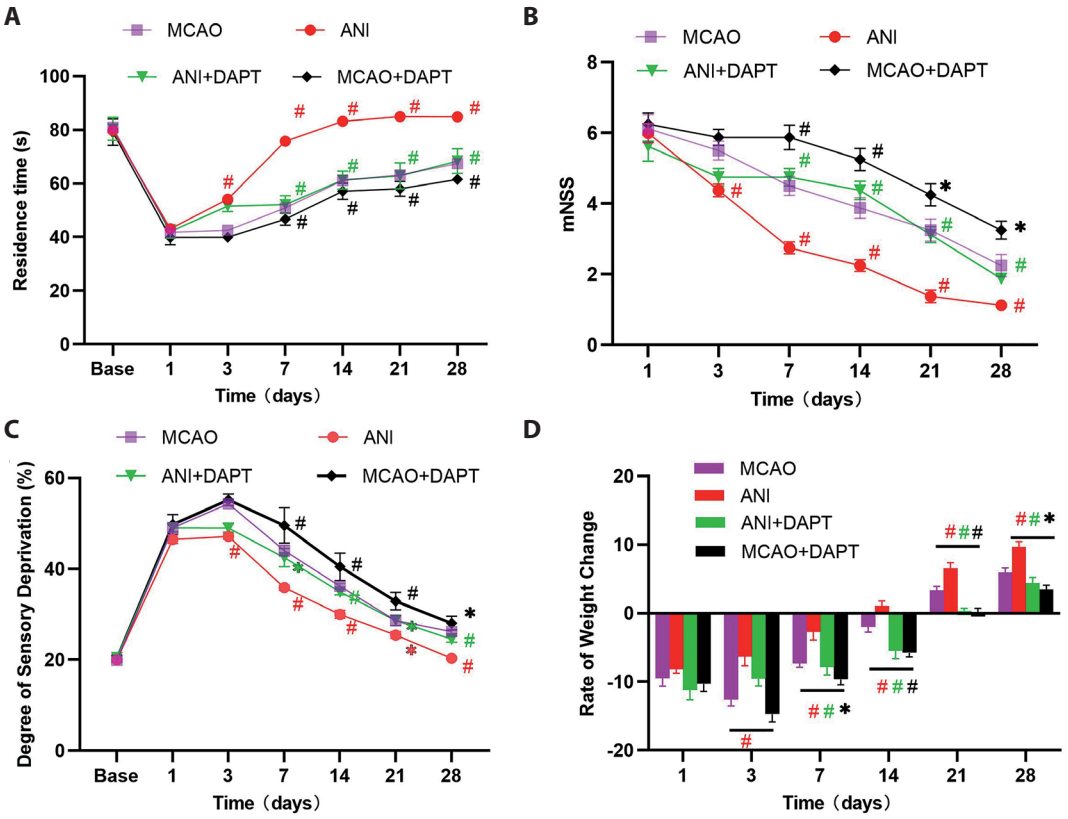

Figure 2. The effect of anisodine hydrobromide promotes neurological recovery inhibited by DAPT. Rotarod test (**A**), mNSS evaluation (**B**), Adhesive removal test (**C**) and the growth rate of body weight (**D**) were performed to assess neurological function with the administration of anisodine hydrobromide and DAPT on Days 1, 3, 7, 14, 21, and 28 after stroke. $n = 8$, each group; * $p < 0.05$, $^{\#}$ $p < 0.01$. (For color figure see online version.)

mice in the ANI group showed significantly longer average times on the Rotarod test at 7, 14, 21, and 28 days post-surgery (Day 7: 52.15 ± 3.35 *vs.* 75.79 ± 2.66, $p < 0.01$; Day 14: 61.33 ± 3.36 *vs.* 83.18 ± 3.47, $p < 0.01$; Day 21: 62.88 ± 4.81 *vs.* 84.93 ± 2.69, $p < 0.01$; Day 28: 68.38 ± 4.55 *vs.* 84.86 ± 3.21, $p < 0.01$) (Fig. 2A). The mNSS scores decreased in the MCAO group compared to the MCAO+DAPT group on Days 7, 14, 21, and 28 (Day 7: 4.50 ± 0.76 *vs.* 5.88 ± 0.99, $p < 0.01$; Day 14: 3.88 ± 0.83 *vs.* 5.25 ± 0.89, $p < 0.01$; Day 21: 3.25 ± 0.89 *vs.* 4.25 ± 0.89, $p < 0.05$; Day 28: 2.25 ± 0.89 *vs.* 3.25 ± 0.71, $p < 0.05$) (Fig. 2B). Compared to the ANI+DAPT group, the mNSS scores were lower in the ANI group on Day 7, 14, 21 and 28 (Day 7: 4.75 ± 0.71 *vs.* 2.75 ± 0.46, $p < 0.01$; Day 14: 4.38 ± 0.74 *vs.* 2.25 ± 0.46, $p < 0.01$; Day 21: 3.13 ± 0.64 *vs.* 1.38 ± 0.52, $p < 0.01$; Day 28: 1.88 ± 0.35 *vs.* 1.13 ± 0.35, $p < 0.01$) (Fig. 2B). In Adhesive removal test, compared to the MCAO+DAPT group, mice in the MCAO group displayed milder self-sensory deficits and motor impairments on the 7th, 14th, 21th, and 28th days (Day 7: 44.01 ± 3.45 *vs.* 49.54 ± 3.88, $p < 0.01$; Day 14: 36.18 ± 1.84 *vs.* 40.46 ± 3.03, $p < 0.01$; Day 21: 28.61 ± 3.11 *vs.* 32.9 ± 1.93, $p < 0.01$; Day 28: 26.20 ± 1.35 *vs.* 28.04 ± 1.54, $p < 0.05$) (Fig. 2C). Compared to the ANI+DAPT group, mice in the ANI group displayed milder self-sensory deficits and motor impairments on the 7th, 14th, 21th, and 28th days (Day 7: 35.85 ± 1.57 *vs.* 42.47 ± 5.69, $p < 0.05$; Day 14: 30.00 ± 2.45 *vs.* 34.92 ± 1.80, $p < 0.01$; Day 21: 25.43 ± 2.05 *vs.* 28.63 ± 3.33, $p < 0.05$; Day 28: 20.34 ± 0.94 *vs.* 24.52 ± 1.81, $p < 0.01$) (Fig. 2C). In addition, MCAO group showed higher weight gain rate than MCAO+DAPT group on days 7, 14, 21 and 28 after modeling (Day 7: −7.34 ± 1.64 *vs.* −9.66 ± 2.21, $p < 0.05$; Day 14: −2.05 ± 2.10 *vs.* −5.79 ± 1.66, $p < 0.01$; Day 21: 3.33 ± 1.68 *vs.* 0.10 ± 1.77, $p < 0.01$; Day 28: 5.94 ± 1.80 *vs.* 3.44 ± 1.83, $p < 0.05$) (Fig. 2D). Compared to the ANI+DAPT group, mice in the ANI group showed better recovery in average weight gain rate on Days 7, 14, 21 and 28 (Day 7: −2.77 ± 3.25 *vs.* −7.91 ± 3.15, $p < 0.01$; Day 14: 1.06 ± 2.01 *vs.* −5.51 ± 3.24, $p < 0.01$; Day 21: 6.50 ± 2.42 *vs.* 0.36 ± 1.04, $p < 0.01$; Day 28: 9.68 ± 2.09 *vs.* 4.41 ± 2.30, $p < 0.01$) (Fig. 2D). In this part of the behavioral experiment, the ANI group performed better than the MCAO group in all four behavior tests.

Anisodine hydrobromide promotes histological recovery of mice after ischemia stroke

To investigate whether anisodine hydrobromide could promote histological recovery after stroke, we evaluated infarct volume on Days 3 and 7 and Golgi staining on Day 28. Infarct volume was significantly reduced in both the ANI groups relative to the MCAO group on 3 and 7 days after stroke (Day 3: 16.07 ± 0.88% *vs.* 9.90 ± 1.67%, $p < 0.01$; Day 7: 12.45 ± 1.81% *vs.* 6.33 ± 1.20%, $p < 0.01$) (Fig. 3A,B). Compared to Day 3, the cerebral infarction volume on Day 7 in the MCAO and ANI groups decreased by 22.51% and 25.47%, respectively; notably, the reduction in cerebral infarction volume was more significant in the ANI group.

The behavioral experiment results show that the inhibitor DAPT has an effect at the recovery stage. Therefore, in order to investigate the effect of inhibitor DAPT and the effect of Ani with DAPT, we evaluated the Golgi staining at Day 28. Using Sholl statistical analysis to evaluate neuronal recovery by assessing the number of neuronal dendritic intersections in the brains of mice on Day 28 after successful modeling of focal cerebral ischemia, we found that the radial distance from cell soma ranged from 20μm to 250 μm, with a morelimited range of 50 to 150 μm in the MCAO and ANI groups. Compared to the MCAO group, mice in the ANI group exhibited more neuronal dendritic intersections ranging from 40 to 130 μm around the infarct area ($p < 0.01$) (Fig. 4A,B). Compared to the MCAO+DAPT group, mice in the MCAO group exhibited more neuronal dendritic intersections ranging from 20 to 90 μm around the infarct area ($p < 0.01$) (Fig. 4A,B). Compared to the ANI+DAPT group, mice in the ANI group exhibited more neuronal dendritic intersections ranging from 10 to 120 μm around the infarct area ($p < 0.01$) (Fig. 4A,B).

The Sholl plot in ANI group reflects increased dendritic growth. Consistently, results from staining for dendritic spine density at 28 days after successful modeling of focal cerebral ischemia in mice showed an increased density of neuronal dendritic spines in the ANI group compared to the MCAO group (6.92 ± 0.60 *vs.* 5.13 ± 1.67%, $p < 0.01$) (Fig. 4C,D). The mice also showed an increased density of neuronal dendritic spines in the MCAO group compared to the MCAO+DAPT group (4.09 ± 0.54 *vs.* 5.13 ± 1.67%, $p < 0.01$) (Fig. 4C,D). The mice in the ANI group showed an increased density of neuronal dendritic spines compared to the ANI+DAPT group (6.92 ± 0.60 *vs.* 5.59 ± 0.68%, $p < 0.01$) (Fig. 4C,D).

However, no increase was observed in any specific type of dendritic spine (Fig. 4C,D). In conclusion, the data collected from infarct volume, dendritic intersections, and dendritic spine density suggest that anisodine hydrobromide significantly promotes the histological recovery of mice after ischemia strokes. DAPT can inhibit the promotion effect.

Anisodine hydrobromide facilitated neural plasticity of mice after ischemia stroke

To further explore the effect of anisodine hydrobromide on neuronal cell structure plasticity, we performed immunostaining for Map2, NF, and Syp on the 14th day post-stroke to assess the remodeling of neuronal cell structure. Map2 is enriched in dendrites, determining and stabilizing dendritic shape during neuron development. NF comprises the cytoskeleton and functionally maintains neuronal caliber. Syp is characteristic of a type of small neurosecretory vesicles and is involved in the regulation of short-term and long-term synaptic plasticity. In the immunostaining, compared with the control group at the same time points, the MCAO and ANI groups showed a reduction in Map2, NF, and Syp density at 14th day post-stroke. Compared with the MCAO group, the ANI group exhibited a significant increase in Map2 (1.3 ± 0.15 *vs.* 2.08 ± 0.34, $p < 0.001$, Fig. 5A,B), NF (5.42 ± 0.63 *vs.* 7.64 ± 1.02, $p = 0.001$, Fig. 5A,C), and Syp (4.77 ± 0.65 *vs.* 7.83 ± 0.59, $p < 0.001$, Fig. 6A,B). With the inhibitor, compared with the MCAO+DAPT group, the MCAO group exhibited a significant increase in Map2 (1.3 ± 0.15 *vs.* 1.03 ± 0.12, $p < 0.01$, Fig. 5A,B), NF (5.42 ± 0.63 *vs.* 4.31 ± 0.70, $p < 0.05$, Fig. 5A,C), and Syp (4.77 ± 0.65 *vs.* 2.69 ± 0.27, $p < 0.001$, Fig. 6A,B). Compared with the ANI+DAPT group, the ANI group also exhibited a significant increase

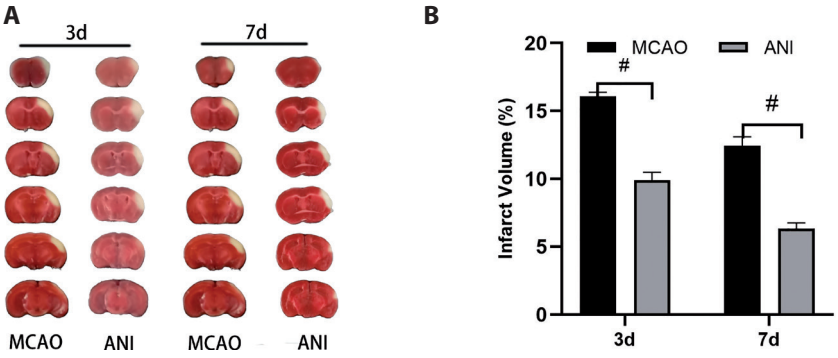

Figure 3. Anisodine hydrobromide reduces infarct volume. **A.** TTC-stained sections in MCAO and ANI groups of the Days 3 and 7 after stroke. **B.** The infarct volume was significantly reduced in ANI group. $n = 8$, each group; * $p < 0.05$, $^{\#}$ $p < 0.01$.

in Map2 (1.49 ± 0.18 *vs.* 2.08 ± 0.34, $p < 0.01$, Fig. 5A,B), NF (6.21 ± 0.52 *vs.* 7.64 ± 1.02, $p < 0.05$, Fig. 5A,C), and Syp (5.09 ± 0.72 *vs.* 7.83 ± 0.59, $p < 0.001$, Fig. 6A,B).

In addition, DCX staining was utilized to observe the migration of newly formed neurons toward the ischemic lesion. Compared to the control group, both the MCAO and ANI groups showed increased expression of DCX in the SVZ area adjacent to the lesion on the 14th day post-stroke. Moreover, the ANI group exhibited significantly higher DCX expression than the MCAO group (3.91 ± 0.47 *vs.* 6.77 ± 0.83, $p < 0.001$, Fig. 6A,C). With the inhibitor, the MCAO group exhibited significantly higher DCX expression than the MCAO+DAPT group (3.91 ± 0.47 *vs.* 2.92 ± 0.66, $p < 0.05$, Fig. 6A,C). The ANI group also exhibited significantly higher DCX expression than the ANI+DAPT group (4.59 ± 0.67 *vs.* 6.77 ± 0.83, $p < 0.01$,

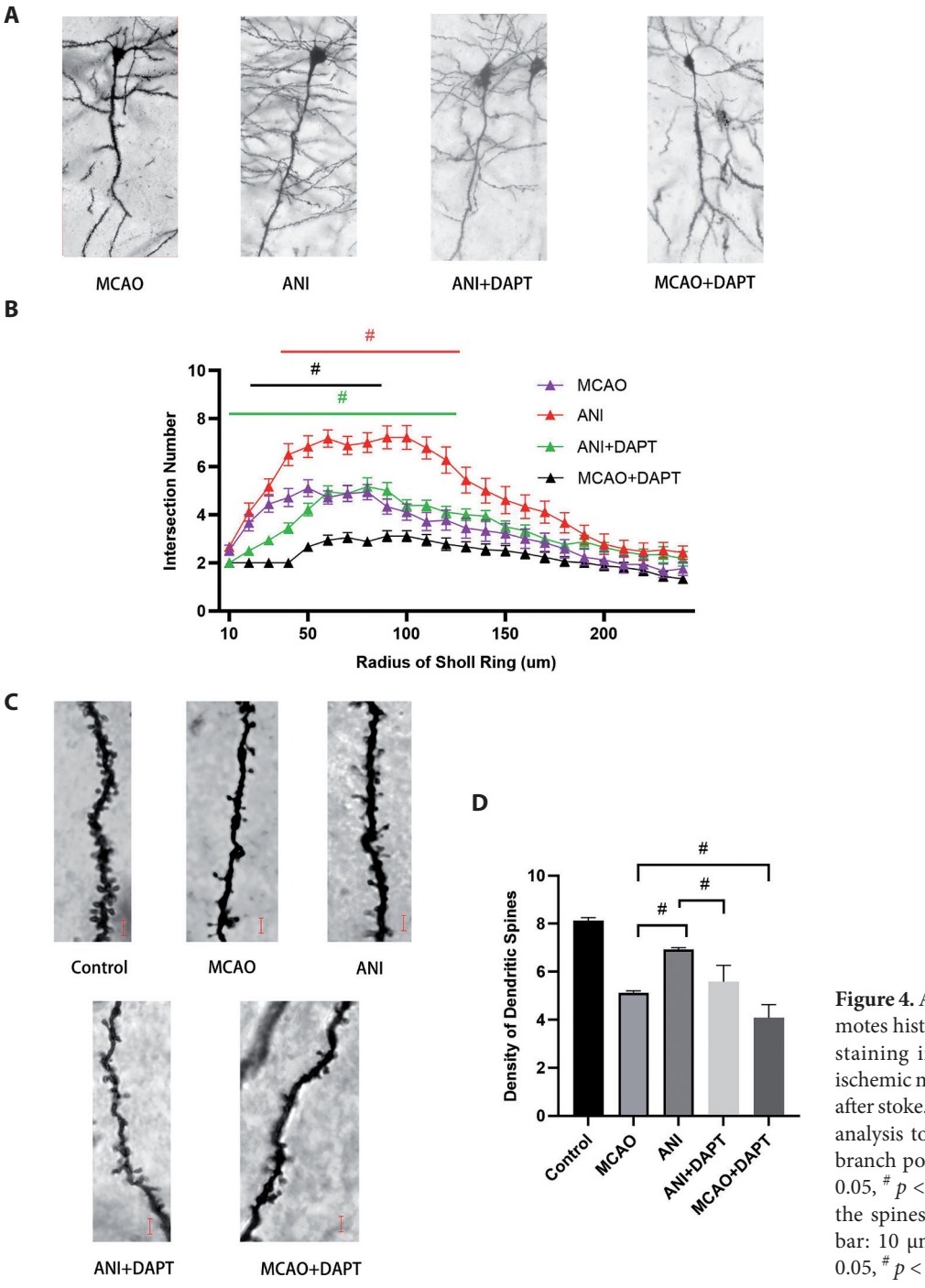

Figure 4. Anisodine hydrobromide promotes histological recovery. **A, B.** Golgi staining in the peri-infarct cortex of ischemic mice were performed at Day 28 after stoke. Using Sholl and spine density analysis to evaluate dendritic complex, branch points. $n = 6$, each group; * $p < 0.05$, $^{\#}$ $p < 0.01$. **C, D.** Golgi staining of the spines at Day 28 after stoke (scale bar: 10 μm). $n = 6$, each group; * $p < 0.05$, $^{\#}$ $p < 0.01$.

Fig. 6A,C). The foregoing data all suggest that anisodine hydrobromide improves neural plasticity. And, the effect can be inhibited by DAPT.

Anisodine hydrobromide augmented expression of neurotrophic factors and regulation of factors involved in the Notch signaling pathway

To further validate the specific processes through which anisodine hydrobromide operates during the growth and remodeling of neural tissues in the focal cerebral ischemia mouse model. We observed changes in neurotrophic factors Gap43 and NGF after 14 days of continuous intravenous administration. Results from Western blot analysis revealed that compared to the MCAO group, the ANI group exhibited an upward trend in protein expression of Gap43 (1.45 ± 0.25 *vs.* 0.98 ± 0.19, $p < 0.01$) and NGF at Day 14 (1.31 ± 0.34 *vs.* 0.84 ± 0.08, $p < 0.05$) (Fig. 7A,C). To further verify the specific mechanisms through which anisodine hydrobromide operates in the growth and regeneration of neural cells, we evaluated the Notch signaling pathway, including Notch1 (NICD) and its downstream target gene Hes1 in the infarcted cortical brain tissue of mice at Day 14, finding that the expression of NICD and its downstream target gene Hes1 significantly increased in the ANI group relative to the MCAO group (NICD: 1.22 ± 0.23 *vs.* 0.86 ± 0.19, $p < 0.05$; Hes1: 0.91 ± 0.05 *vs.* 0.75 ± 0.10, $p < 0.05$) (Fig. 7A,D,E).

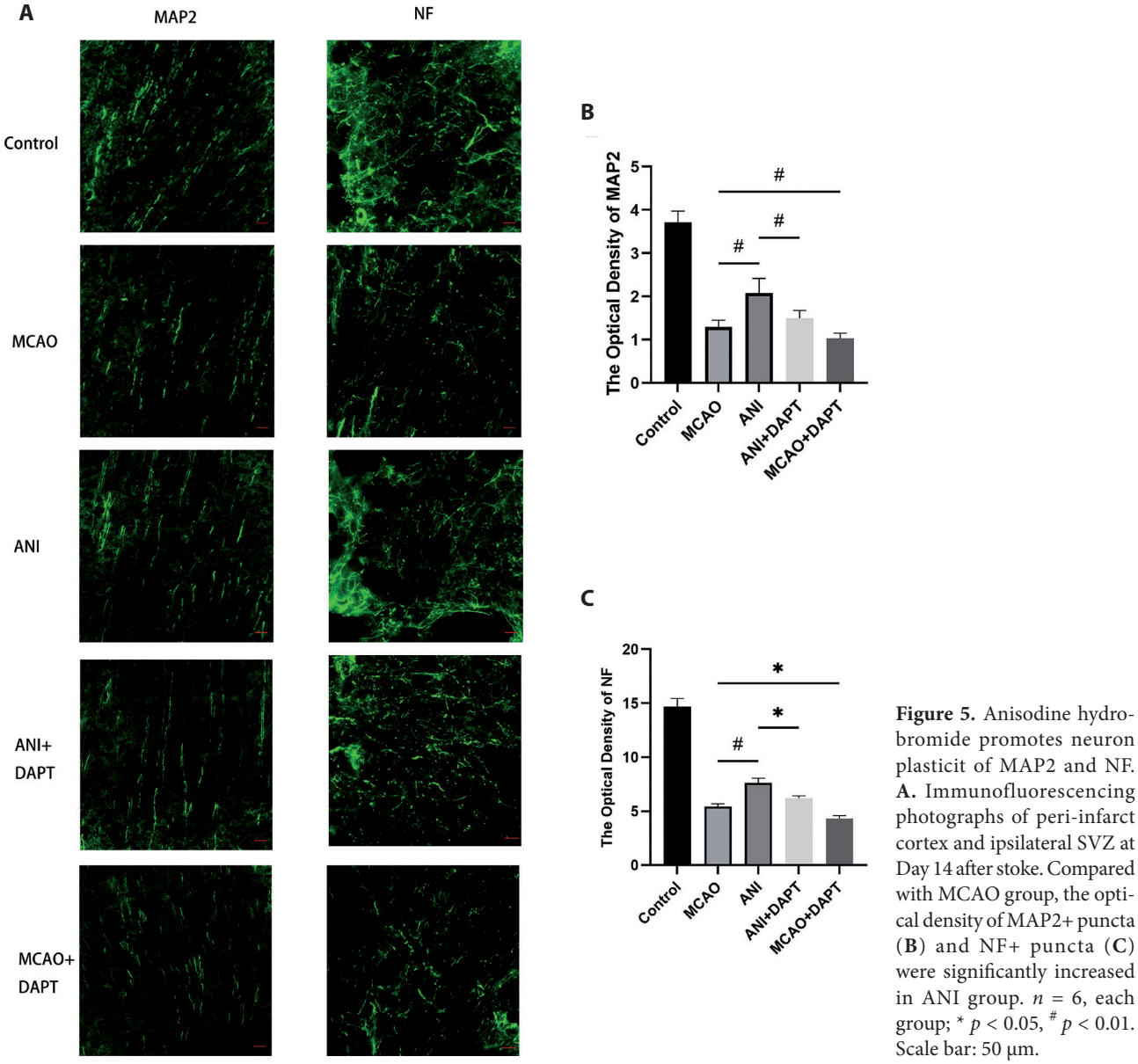

Figure 5. Anisodine hydrobromide promotes neuron plasticit of MAP2 and NF. **A.** Immunofluorescencing photographs of peri-infarct cortex and ipsilateral SVZ at Day 14 after stoke. Compared with MCAO group, the optical density of MAP2+ puncta (**B**) and NF+ puncta (**C**) were significantly increased in ANI group. $n = 6$, each group; * $p < 0.05$, $^{\#}$ $p < 0.01$. Scale bar: 50 μm.

Impact of anisodine hydrobromide on neurotrophic factors following inhibition of the Notch pathway with DAPT

To further explore Anisodine hydrobromide's involvement in the Notch signaling pathway during stroke treatment, we leveraged the Notch pathway inhibitor DAPT. Initially, we tested the impact of continuous DAPT administration. After 14 days of treatment, we examined NICD and its downstream gene, Hes1, in the infarcted cortical brain tissue. Compared to the MCAO group, the expression of NICD and Hes 1 in the MCAO+DAPT group decreased (NICD: 0.50 ± 0.11 *vs.* 0.86 ± 0.19, $p < 0.01$; Hes1: 0.49 ± 0.09 *vs.* 0.75 ± 0.10, $p < 0.01$) (Fig. 7A,D,E). At same time, the expression of neurotrophic factors – Gap43 and NGF – also decreased. (GAP43: 0.77 ± 0.12 *vs.* 0.98 ± 0.19, $p < 0.05$; NGF: 0.57 ± 0.07 *vs.* 0.84 ± 0.08, $p < 0.01$) (Fig. 7A,C). It therefore appears that DAPT can interfere with the Notch1 pathway and decrease the expression of neurotrophic factors.

Compare to the ANI groups, results showed that on 14th day, the expression of NICD and Hes1 decreased significantly in the ANI +DAPT group (NICD: 1.22 ± 0.23 *vs.* 0.85 ± 0.27, $p < 0.05$; Hes1: 0.91 ± 0.05 *vs.* 0.71 ± 0.09, $p < 0.01$). The inhibitory effects on Notch1 and Hes1 thus persisted with continuous DAPT administration in ANI+DAPT group (Fig. 7A,D,E). When the Notch signaling pathway was inhibited, we assessed the expression of related neurotrophic

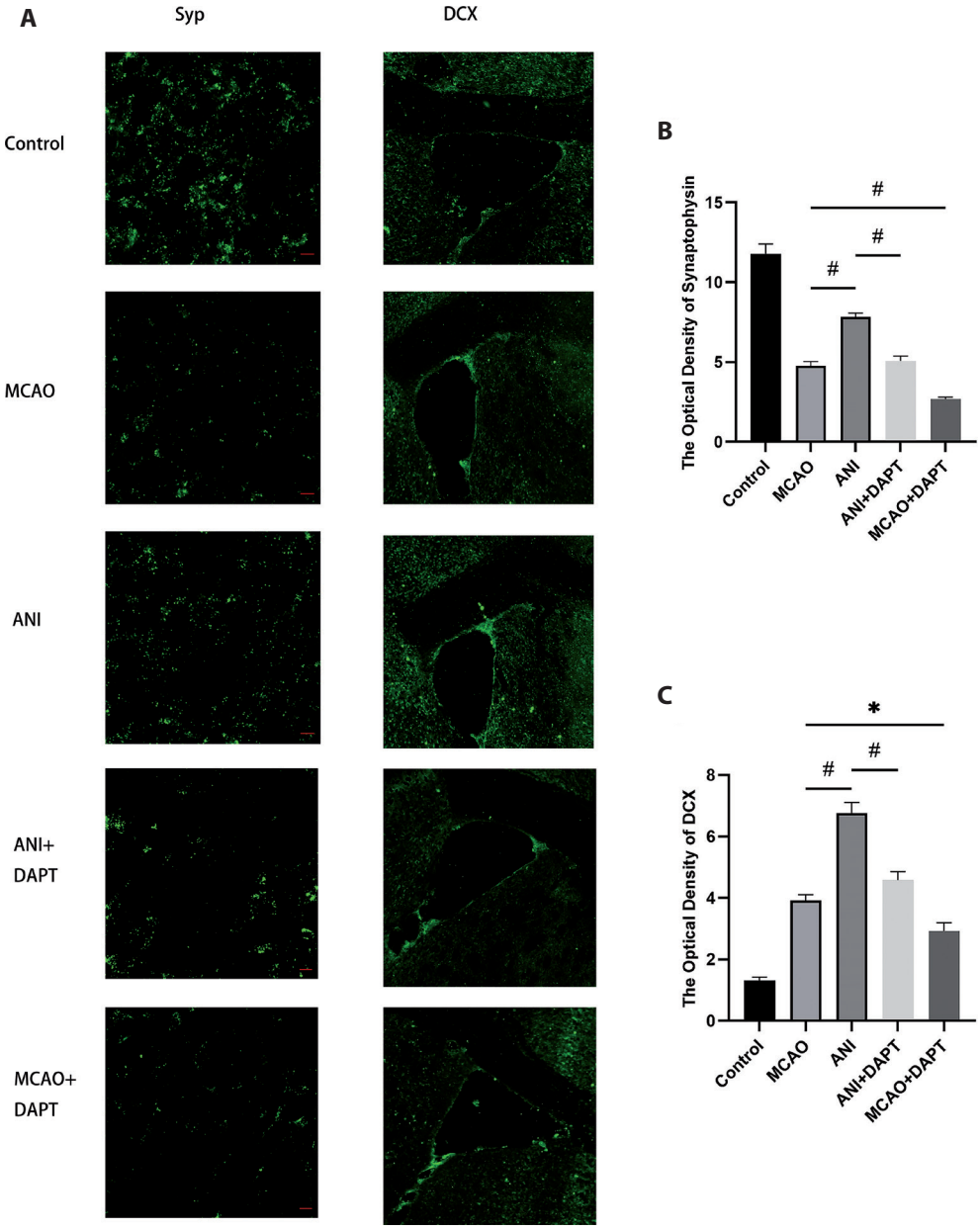

Figure 6. Anisodine hydrobromide promotes neuron plasticit of synaptophysin (Syp) and DCX. **A.** Immunofluorescencing photographs of peri-infarct cortex and ipsilateral SVZ at Day 14 after stoke. Compared with MCAO group, the optical density of Synaptophysin+ puncta (**B**) and DCX+ cells (**C**) were significantly increased in ANI group. $n = 6$, each group; * $p < 0.05$, $^{\#}$ $p < 0.01$. Scale bar: 50 μm.

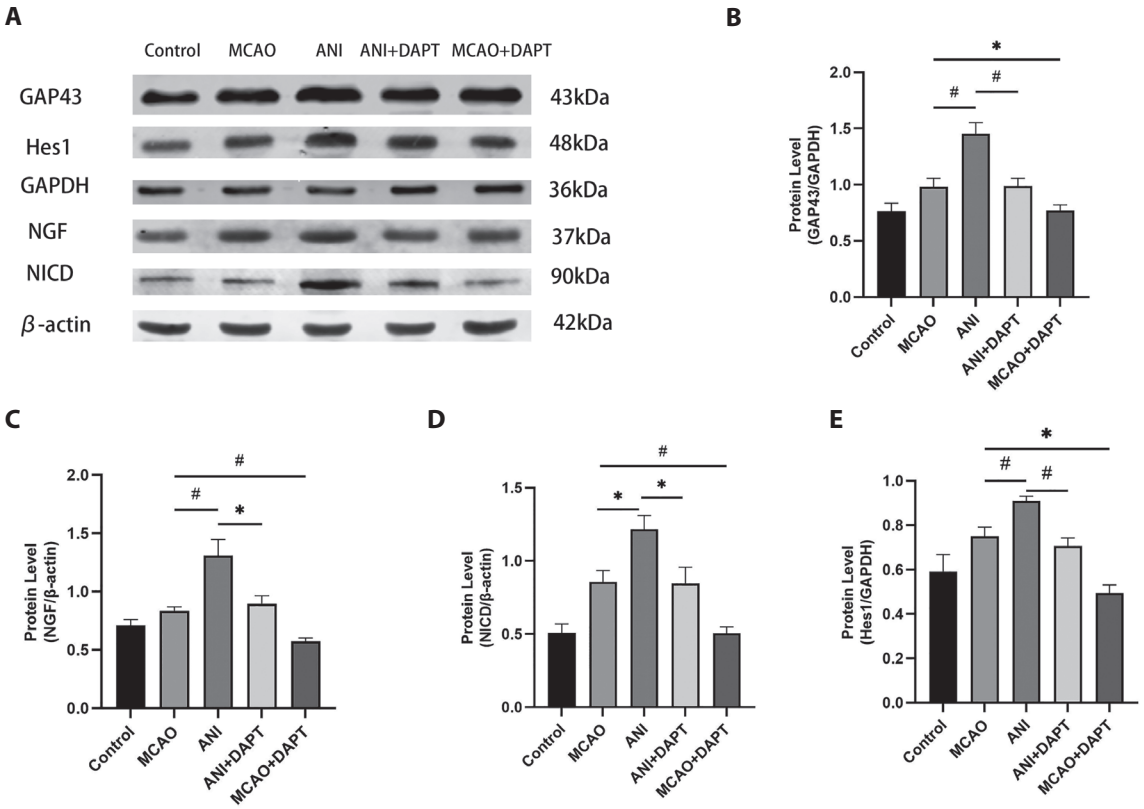

Figure 7. Anisodine hydrobromide promotes the expression of neurotrophic factors and Notch signal pathway factors. **A.** Western blotting. The protein levels of neurotrophic factors levels GAP43 (**B**) and NGF (**C**). Notch signal pathway factors NICD (**D**) and Hes1 (**E**). $n = 6$, each group; * $p < 0.05$, $^{\#}$ $p < 0.01$.

factors-Gap43 and NGF-following anisodine hydrobromide treatment for stroke, finding that both decreased in the ANI+DAPT group on Day 14 relative to the ANI group (GAP43: 1.45 ± 0.25 *vs.* 0.99 ± 0.17, $p < 0.01$; NGF: 1.31 ± 0.34 *vs.* 0.90 ± 0.17, $p < 0.05$) (Fig. 7A–C).

Discussion

As a non-selective muscarinic acetylcholine receptor antagonist, anisodine hydrobromide has been applied in the clinical treatment of conditions including migraine, retinal vascular spasm, ischemic stroke, and organophosphate poisoning (Chang et al. 2006; Chen et al. 2017; Mathieu et al. 2019; Chen and Yeong 2020). Several studies have confirmed its role in relieving vascular spasms, improving circulation, stabilizing cell membranes, and prolonging the survival time of hypoxic cells (Wu et al. 2016; Wang et al. 2017; Chen et al. 2019). Notably, Chen et al. (2017) observed anisodine hydrobromide's potential to improve cognitive deficits by reducing neuron necrosis and apoptosis. At the same time, clinical observations also indicate that anisodine hydrobromide treatment is associated with favorable outcomes for patients with ischemia stroke in the recovery phase (Chang et al. 2006; Wan et al. 2023; Wang et al. 2023). Building on this research, we explored the effects of anisodine hydrobromide on neurological function recovery, neuron structure remodeling, and axonal growth markers, while evaluating the mechanisms of Notch signal pathways during neural remodeling.

The neurovascular unit maintains normal neurological function and is comprised of brain endothelial cells, pericytes or vascular smooth muscle cells, neurons, and glia. During the recovery phase after ischemia, neurovascular signaling is critically important, as repair mechanisms may involve neuron regeneration (Moskowitz et al. 2010). We thus performed our research *in vivo* to mimic the ischemia recovery phase.

To determine the dosage of anisodine hydrobromide and its effects on neurological function recovery, we established two groups with different concentrations (0.3 mg/kg, AniH group, and 0.15 mg/kg, AniL group) to observe the drug's effectiveness. Our results show that the reduction in infarction area was more significant in the AniH group, suggesting that anisodine hydrobromide can play a role in the recovery of brain tissue post-stroke. These findings align with similar results observed in the recovery phases of ischemic stroke, traumatic optic neuropathy, and other neurological disorders

(Chang et al. 2006; Wang et al. 2023). The Rotarod experiment demonstrated that mice in the AniH group showed better recovery in motor function compared to the MCAO group. While the AniL group also improved motor function compared to MCAO, their recovery was less effective than the AniH group. Therefore, 0.3 mg/kg of anisodine hydrobromide promotes better healing of motor function. This trend was further validated in the mice's adhesive experiments, mNSS behavioral score, and the recovery of their body weight. In general, anisodine hydrobromide appears to promote long-term neurological function recovery in mice post-ischemia stroke. We therefore selected 0.3 mg/kg of anisodine hydrobromide for our subsequent exploration of the neural remodeling process in mice after ischemia stroke.

Nagappan et al. (2020) has demonstrated that adult mammals possess potential for structural repair and functional recovery within the central nervous system following injury. Neuroplasticity allows brains to change neuronal structure networks to meet the need of neurological functions. This ability is influenced by various factors such as axonal growth markers, the cellular microenvironment, and genetics (Pöyhönen et al. 2019).

There are also two regions in the brain that provide new neurons in adulthood: SVZ and the dentate gyrus (DG). Xiong et al. (2019) and Beckers et al. (2019) indicate that when the central nervous system is damaged, axons, dendrites, and synapses undergo regeneration and remodeling under various cellular factors. In this process, axons grow and sprout to traverse the damaged area, reestablishing neural connections and facilitating functional restoration. During cerebral infarction, local brain tissue experiences hypoxia, leading to structural necrosis of neurons and their supporting tissues and potential neurological impairment.

Our research investigates the neuroplasticity of anisodine hydrobromide following cerebral infarction. In this experimental section, we utilized Golgi and immunofluorescence for tissue staining to observe neuronal cell structures and assess neural cell remodeling. Continuous treatment with anisodine hydrobromide post-surgery was found to promote neuronal cell structural remodeling. Golgi staining revealed that anisodine hydrobromide increases dendritic spine density, enhancing the complexity of neuronal structures during neuronal remodeling. Immunofluorescence staining of Map2, NF, Syp and DCX demonstrated that anisodine hydrobromide facilitates neuroplasticity during neural remodeling.

In order to further verify the effect of anisodine hydrobromide in promoting neuroplasticity, our research analyzes the protein levels of GAP43, and NGF. Several other researchers have found that the protein levels of GAP43 and NFG increase when neuron regeneration occurs after ischemia (Beckers et al. 2019; Caglayan et al. 2019; Russo et al. 2022). Our research showed that continuous administration of anisodine hydrobromide can also enhance the synthesis of GAP43 and NFG. Therefore, anisodine hydrobromide promotes the release of axonal growth markers and neural remodeling after ischemia.

SVZ and DG provide new neurons in adult response to ischemia. The new neurons integrate into neuronal networks. Kawai et al. (2005) have proved the role of Notch signaling in the brain's response to ischemia. The research shows that ischemia upregulates Notch pathway expression in the SVZ when neurogenesis occurs (Kawai et al. 2005; Felling et al. 2006). When Notch receptors are activated by Delta or Jaggened ligands, the receptors need the γ-secretase enzyme complex to cleave the NICD to activate the signal pathway. Then, NICD translocate into the nucleus and activates transcription (Wang et al. 2009). Hes1 is an essential downstream target gene of the Notch pathway. Wang et al. (2009) found that the expression of NICD and Hes1 was increased in the SVZ and increased neurogenesis after ischemia. Therefore, inhibiting the γ-secretase complex enzyme will lead to the inhibition of Notch pathway activation.

Dong et al. (2021) showed that DAPT works as a γ-secretase inhibitor, which can effectively inhibit the release of the Notch intracellular domain from the Notch receptor and inhibit the activation of the Notch signaling pathway. Wang et al. (2009) also show that the use of DAPT can inhibit the activation of Notch1 and thus inhibit neurogenesis after ischemia as well. In other experiments, continuous abdominal injection of DAPT was employed to achieve sustained inhibition of the Notch signaling pathway and observe the expression of neurotrophic factors (Feng et al. 2019; Dong et al. 2021). Therefore, we investigated the Notch signaling pathway's involvement during neural neuroplasticity in ischemia with anisodine hydrobromide treatment by DAPT.

In our research, we utilized DAPT to reveal the role of the Notch signaling pathway in neural neuroplasticity during the recovery phase of anisodine hydrobromide-treated ischemia. The behavioral assessment, Golgi, and immunofluorescence staining results show that continuous treatment with DAPT can inhibit neural function recovery and neuronal cell structural remodeling. Western blot measurements of the MCAO+DAPT groups also indicate that DAPT inhibits the expression of NICD, Hes1, GAP43, and NGF. Therefore, continuous administration of DAPT to inhibit the Notch signaling pathway after ischemia is a reliable approach.

The Western blot observations of the MCAO and ANI groups further revealed that the expression of GAP43 and NGF is increased by anisodine hydrobromide after ischemia. At the same time, anisodine hydrobromide enhances the presentation of NICD and Hes1, both Notch signaling pathway-related factors. This result indicates that the Notch signaling pathway has been activated in the recovery phase of anisodine hydrobromide-treated ischemia.

On the other hand, the administration of DAPT with anisodine hydrobromide can inhibit the neural remodeling

and neural function recovery compared with the ANI group. DAPT also suppressed the expression of NICD and Hes1 in the ANI+DAPT group; additionally, the expression of GAP43 and NGF decreased in ANI+DAPT relative to ANI. Therefore, DAPT with anisodine hydrobromide inhibited the activation of the Notch signaling pathway. This further inhibits the promotion of anisodine hydrobromide on neural remodeling and neural function recovery.

Ultimately, our study suggests that anisodine hydrobromide can promote neurotrophic factors and neuroplasticity following ischemic stroke, a process also impacted by Notch signaling. Chen et al. (2017) reported that anisodine hydrobromide can attenuate neuronal cell death and apoptosis by activating the Akt/GSK-3β signaling pathway. Therefore, further research requires investigating the other potential mechanisms by which anisodine hydrobromide may impact neuroplasticity.

Conclusion

Anisodine hydrobromide promotes neural plasticity and improves long term neurological recovery. These effects are mediated at least in part through axonal growth markers and the expression of the Notch signaling pathway. When the expression of the Notch signaling pathway is inhibited, anisodine hydrobromide's regulatory effect on the relevant factors of the Notch signaling pathway is suppressed, leading to a significant decrease in its promotion of neurotrophic factors, neural remodeling and neural function recovery. Whether anisodine hydrobromide may have beneficial effect through other mechanisms is a question meriting further research.

Funding. This work was generously supported by grants from the National Natural Science Foundation of China (No. 81974184 and No. 82271366), Hebei Key Laboratory of Vascular Homeostasis (20567630H), and the Key Research Project of Medical Science in Hebei Province No. 20180294.

Conflict of interest. The authors have no conflicts of interest to declare.

Data available. Data is available from the corresponding author on request.

Authors' contributions. XM: writing – original draft, validation, formal analysis, visualization, software, methodology, writing – review & editing. XZ: conceptualization, data curation, resources, funding acquisition. HJ: conceptualization, data curation. CZ: conceptualization, validation, software, methodology, investigation. RC: conceptualization, software, methodology, investigation. XZ: software, methodology, investigation. RX: software, methodology. WJ: methodology, investigation.

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Received: July 9, 2024
Final version accepted: August 20, 2024