

Neuronal ABHD6 Exacerbates Ischemic Brain Injury Through 2-AG Depletion and DDIT3/BNIP3-Dependent Autophagy Dysregulation

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Abstract

The endocannabinoid 2-arachidonoylglycerol (2-AG) confers critical neuroprotection against ischemic stroke (IS) by restricting neuronal apoptosis. While monoacylglycerol lipase (MAGL) mediates 85% of central nervous system 2-AG hydrolysis, its chronic inhibition induces severe cannabinoid receptor desensitization. In contrast, postsynaptic alpha/beta hydrolase domain 6 (ABHD6) governs a highly localized 4% pool of 2-AG degradation, uniquely positioning it as a precise, on-demand therapeutic target devoid of off-target effects. However, its exact pathological contribution to ischemic neuronal death remains elusive. This study investigates the molecular mechanisms by which ABHD6 regulates apoptosis and autophagic crosstalk following IS. Utilizing a middle cerebral artery occlusion/reperfusion mouse model and an in vitro oxygen-glucose deprivation followed by 24-hour reoxygenation (OGD/R) model in primary neurons, assays demonstrated that ABHD6 expression and enzymatic activity were significantly upregulated post-ischemia. This elevation accelerated postsynaptic 2-AG hydrolysis, restricted neuroprotective endocannabinoid activation, and aggravated apoptosis. Pharmacological inhibition of ABHD6 with WWL70 and CRISPR/Cas9-mediated genetic ablation effectively suppressed enzymatic activity, preserved endogenous 2-AG levels, and attenuated OGD/R-induced programmed cell death. Mechanistically, ABHD6 impaired neuroprotective autophagy by modulating the DDIT3/BNIP3 pathway; the consequent suppression of autophagic flux directly exacerbated neuronal apoptosis and subcellular structural disintegration. In conclusion, ABHD6 exerts a dual detrimental role in IS by depleting neuroprotective 2-AG and suppressing DDIT3/BNIP3-dependent autophagy. Targeting postsynaptic ABHD6 to restore endocannabinoid homeostasis and autophagic flux represents a highly specific molecular strategy for attenuating ischemia-induced neuronal apoptosis.

Introduction

Globally, an estimated 7.4 million incident cases of ischemic stroke (IS) occur annually, imposing a substantial medical and economic burden on individuals and society^[1]. Because current therapeutic strategies are unable to completely cure IS, it remains the second leading cause of death and a primary cause of long-term disability worldwide^[2]. Consequently, there is an urgent need to identify effective intervention targets to mitigate IS-induced neurological damage.

Recently, researchers have focused on mobilizing endogenous neuroprotective mechanisms to alleviate IS-induced injury^[3]. The endocannabinoid system (ECS)—comprising endocannabinoids, cannabinoid receptors (CBRs), and their metabolic enzymes—is a complex, ubiquitous lipid signaling network that plays a crucial role in maintaining central nervous system (CNS) homeostasis^[4, 5]. A growing body of evidence indicates that ECS activation can attenuate IS injury and promote neurological recovery^[6–8]. Thus, targeting the ECS via its primary ligands, anandamide and 2-arachidonoylglycerol (2-AG), along with their degrading enzymes, has emerged as a promising therapeutic strategy for IS.

As one of the two primary endocannabinoids, 2-AG is a monoacylglycerol that serves as a crucial endogenous ligand for CBRs (CB1R and CB2R). It is the most abundant and potent endocannabinoid in

the brain, where it modulates neurotransmitter release by functioning as a retrograde messenger^[9]. Specifically, 2-AG is synthesized in postsynaptic neurons, released into the synaptic cleft, and retrogradely diffuses to activate presynaptic CB1Rs. In response to neuronal injury, 2-AG reduces excitotoxicity, limits neuroinflammation, and modulates cerebral blood flow^[10]. Recent studies have demonstrated that elevating 2-AG levels or inhibiting its degradation can reduce ischemic infarct size and improve both early and long-term neurological outcomes following IS^[10–12]. Therefore, enhancing 2-AG signaling within the ECS represents a novel therapeutic avenue for IS.

To ensure transient and precise signaling, 2-AG is rapidly degraded after fulfilling its biological function. The enzymes primarily responsible for its hydrolysis are monoacylglycerol lipase (MAGL), alpha/beta hydrolase domain 6 (ABHD6), and ABHD12. In the CNS, MAGL mediates approximately 85% of 2-AG degradation, whereas ABHD6 and ABHD12 account for roughly 4% and 9%, respectively.^[13] Although recent studies have highlighted the therapeutic potential of targeting MAGL in neurological diseases^[10], chronic pharmacological inhibition of MAGL causes 2-AG overload, partial CB1R desensitization, and a localized loss of cannabinoid-mediated efficacy, culminating in adverse side effects such as hypoactivity and hyperreflexia^[14]. Notably, these three hydrolytic enzymes exhibit distinct subcellular localizations: MAGL is predominantly distributed in the presynaptic cytoplasm and astrocytes; ABHD12 is primarily located on the synaptic cleft face of the presynaptic membrane and in microglia; and ABHD6, alongside 2-AG synthesizing enzymes, is localized primarily within postsynaptic structures^[15, 16]. Consequently, ABHD6 is uniquely positioned to precisely and locally regulate the "on-demand" availability of 2-AG under pathological conditions. These findings suggest that ABHD6 is a novel, highly specific target for modulating 2-AG-mediated neuroprotection after IS.

Although its role in 2-AG hydrolysis is the most extensively studied and clinically relevant function, ABHD6 is a versatile serine hydrolase with several other regulatory capacities. ABHD6 can hydrolyze alternative monoacylglycerols (e.g., 1-monoacylglycerols) and various other lipid esters, indicating its broader involvement in cellular lipid metabolism beyond endocannabinoid degradation^[17, 18]. Furthermore, ABHD6 is involved in the regulation of glucose-stimulated insulin secretion (GSIS), with studies demonstrating that its pharmacological inhibition or genetic knockdown in mice enhances GSIS^[19]. In microglia, ABHD6 may also influence the production of other lipid mediators and intracellular signaling cascades that drive inflammatory responses, extending beyond the mere modulation of 2-AG-mediated anti-inflammatory effects^[20]. Despite these diverse functions, the precise role of postsynaptic ABHD6 following IS remains unclear.

In the present study, we demonstrate that neuronal ABHD6 expression is upregulated following IS, leading to accelerated 2-AG hydrolysis and the restriction of ECS activation, which exacerbates neuronal damage. Furthermore, we reveal that ABHD6 impairs neuronal autophagy by modulating the DDIT3/Bnip3 signaling pathway, thereby further aggravating ischemic neuronal injury.

Methods

Animals

An *Abhd6* knockout (ABHD6-KO) mouse model was generated by Shanghai Model Organisms (Shanghai, China) utilizing CRISPR/Cas9 technology. The genotyping protocol for the ABHD6-KO mice is detailed in Figure S1. Male mice, aged 10–12 weeks with an initial body weight of approximately 25 g, were utilized for all experiments. Prior to the commencement of the study, the animals were housed in a temperature-controlled facility maintained at approximately 27°C on a 12-h light/dark cycle for a minimum of 7 d. All animal procedures were conducted in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of the Fourth Military Medical University.

Cell culture

The methodology for culturing primary neurons was performed as previously described^[21]. Briefly, cerebral cortices were extracted from 16–18-day-old embryos. Tissue dissociation was achieved by gentle trituration in 0.25% trypsin at 37°C for 15 min. The dissociated neuronal cells were resuspended in Neurobasal medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.5 mM L-glutamine and 2% B27 supplement (Thermo Fisher Scientific). Cells were then plated at a density of 3×10^5 cells/cm². Prior to seeding, the culture vessels were pre-coated with poly-L-lysine (50 µg/mL) overnight at room temperature. The cultured neurons were maintained in a humidified incubator at 37°C with 5% CO₂, and the culture medium was replaced every other day. Neurons exhibiting a viability of greater than 95% were utilized for subsequent experiments. Additionally, the HT22 neuronal cell line was obtained from the American Type Culture Collection. These cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

Brain ischemia models

An in vitro oxygen-glucose deprivation/reoxygenation (OGD/R) model was employed to simulate brain ischemia. To induce OGD, the culture medium was removed, and the cells were washed three times with phosphate-buffered saline (PBS). Subsequently, the cells were incubated in a hypoxic chamber (94% N₂, 1% O₂, and 5% CO₂) for 1, 2, 4, or 6 h. Following hypoxic exposure, fresh culture medium was added, and the cells were returned to a standard normoxic incubator at 37°C for 24 h to simulate reoxygenation.

To simulate brain ischemia in vivo, a middle cerebral artery occlusion/reperfusion (MCAO/R) model was utilized. The MCAO/R procedure was performed following a standard protocol^[22]. The occlusion was maintained for 1 h prior to reperfusion. Following ischemia, neurological deficits were evaluated by a blinded observer utilizing the 5-point Longa scoring system (0 = no deficit; 1 = failure to extend contralateral forepaw; 2 = circling to the contralateral side; 3 = falling to the contralateral side; 4 = depressed level of consciousness or lack of spontaneous movement). To ensure model consistency,

only animals achieving a Longa score of 1 to 3 were deemed successful MCAO/R models and retained for downstream experiments.

Measurement of cerebral blood flow

To confirm successful brain ischemia and reperfusion, real-time cerebral blood flow (CBF) was monitored utilizing a laser speckle imaging system (RWD Life Science, Shenzhen, China). Under 4% isoflurane anesthesia and normothermic conditions (maintained at 37°C), the mouse skull was exposed via a midline scalp incision. The imaging probe was positioned directly above the skull, and CBF was subsequently recorded.

qRT-PCR

Total RNA was extracted utilizing TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For mRNA quantification, cDNA was reverse-transcribed from 2 µg of total RNA utilizing the PrimeScript RT Reagent Kit (TaKaRa Biotechnology, Dalian, China). Quantitative real-time PCR (qRT-PCR) was performed on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Amplification reactions were conducted utilizing SYBR Premix Ex Taq (TaKaRa Biotechnology), with β-actin serving as the endogenous normalization control. All primer sequences are detailed in Table S1.

Lentiviral Transduction and Stable Cell Line Generation

Lentiviral particles targeting ABHD6 for overexpression, shRNA-mediated knockdown, and their respective negative controls were designed and synthesized by Genechem (Shanghai, China). Target cells were seeded into 6-well plates at a density of 2×10^5 cells per well and cultured until 50%–60% confluence was achieved. Subsequently, the cells were transduced with the respective lentiviral particles at a multiplicity of infection of 20. To enhance transduction efficiency, polybrene (8 µg/mL; Genechem) was added to the culture medium. Following a 24-h incubation period, the virus-containing medium was replaced with fresh complete culture medium. To establish stable cell lines, the cells were subjected to selection utilizing puromycin (2 µg/mL) for 7 d, commencing 48 h post-transduction. Prior to downstream experiments, the overexpression and knockdown efficiencies of ABHD6 in the surviving stable cells were validated via qRT-PCR and Western blot analysis.

Western blot

Total protein was extracted from brain tissues and cultured cells. Following extraction, the protein samples were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were subsequently blocked in phosphate-buffered saline containing Tween 20 for 1 h. Following this, the membranes were incubated overnight at 4°C with primary antibodies specific for ABHD6 (Cell Signaling Technology, Danvers, MA, USA), Ddit3

(Proteintech, Chicago, IL, USA), Bnip3 (ImmunoWay, Plano, TX, USA), LC3 (Proteintech), β -Tubulin (Proteintech), and p62 (ImmunoWay) at a dilution of 1:1000. Finally, appropriate secondary antibodies were applied, and the membranes were incubated for 1 h at room temperature.

Cell viability assay

Cell viability was evaluated utilizing a Cell Counting Kit-8 (CCK-8) assay (Beyotime, Shanghai, China). Briefly, cells were seeded into 96-well plates at a density of 5×10^3 cells/well and subjected to the designated treatments. Subsequently, 10 μ L of CCK-8 solution was added to each well. Following a 2-h incubation at 37°C, the absorbance at 450 nm was recorded utilizing a microplate reader. Cell viability was expressed as a percentage relative to the untreated control group, following the subtraction of the background absorbance from the blank wells.

ABHD6 activity assay

The assessment of ABHD6 activity was performed as previously described^[23]. ABHD6 hydrolase activity was determined by measuring the hydrolysis of the fluorogenic substrate 4-methylumbelliferyl-heptanoate (4-MUH; Sigma-Aldrich, St. Louis, MO, USA). Cell lysates were prepared in Tris buffer (pH 7.4), and total protein concentrations were normalized. The lysates (20 μ g/well) were pre-incubated with the ABHD6-specific inhibitor WWL70 (10 μ M) or a vehicle control for 30 min at 37°C in black 96-well plates. Subsequently, 4-MUH (50 μ M) was added, and the generated fluorescence was kinetically measured at 37°C (excitation/emission = 355/460 nm) utilizing a microplate reader (BioTek, Bad Friedrichshall, Germany). ABHD6-specific activity was defined as the difference in reaction velocity between the vehicle-treated and WWL70-treated samples, and was normalized to the total protein concentration (pmol/min/mg).

Enzyme-Linked Immunosorbent Assay (ELISA)

Blood chemistry analysis was conducted utilizing a biochemical analyzer (Randox RX Daytona U98a; Randox Laboratories, Crumlin, UK). For the quantification of 2-AG levels in the culture supernatants and tissue homogenates, specific ELISA kits (Meimian, Yancheng, China) were utilized in strict accordance with the manufacturer's instructions.

Histology and immunohistochemistry

Following the designated treatments, the mice were euthanized, and their brains were rapidly extracted. The collected brain tissues were fixed overnight in 4% paraformaldehyde (PFA). The tissues were subsequently embedded in paraffin, sectioned at a thickness of 5 μ m, and stained with hematoxylin and eosin (H&E). For immunohistochemical analysis, primary antibodies targeting NeuN (1:200; Abcam, Cambridge, MA, USA), LC3 (1:200; Proteintech, Chicago, IL, USA), and p62 (1:200; ImmunoWay, Plano, TX, USA) were utilized. Briefly, the brain sections were blocked for 1 h at room temperature in 4% bovine serum albumin (BSA) containing 0.3% Triton X-100. The sections were then incubated with the primary

antibody solutions (diluted in the blocking buffer) on a shaker overnight at 4°C. Subsequently, the sections were incubated with secondary antibodies conjugated with S-vision 488, 555, and 647 (Servicebio, Wuhan, China) for 1 h at room temperature.

For immunocytochemical staining, the cultured cells were fixed in 4% PFA, washed three times with PBS, and permeabilized with 0.2% Triton X-100 for 10 min. Following blocking with 1% BSA for 30 min, the cells were incubated overnight at 4°C with a primary anti-LC3 antibody (1:200; Proteintech). Subsequently, the cells were incubated with a DyLight 594-conjugated secondary antibody (1:500; GeneTex, Alton, CA, USA) for 1 h. Finally, the cell nuclei were counterstained with DAPI for 5 min.

TUNEL staining

Apoptosis was assessed utilizing a TUNEL assay kit (Beyotime, Shanghai, China) in strict accordance with the manufacturer's protocol. Cultured cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and incubated with the TUNEL reaction mixture for 1 h at 37°C in the dark. Cell nuclei were subsequently counterstained with DAPI. Finally, images were captured utilizing a fluorescence microscope.

Modified neurological severity score (mNSS)

Neurological function was assessed utilizing the mNSS, which comprehensively evaluates motor, sensory, reflex, and balance indicators in injured mice. As previously detailed^[24], this scoring system ranges from 0 (normal function) to 18 points (maximal deficit), with higher scores corresponding to more severe brain injury.

Rotarod test

Motor coordination and balance were evaluated utilizing a rotarod apparatus (Shanghai Xin Ruan Information Technology, Shanghai, China). Each mouse was placed on the rotating rod for an acclimation period of 10 s at an initial speed of 5 rpm. Subsequently, the rotation speed was accelerated from 5 to 40 rpm over a 5-min period. The latency to fall from the rod was recorded for each mouse, and the average value from three independent trials was calculated for statistical analysis.

Forelimb use asymmetry (FUA) test

Forelimb use asymmetry was assessed by placing each mouse in a transparent cylinder and allowing it to perform a minimum of 15 exploratory rearing movements within a 10-min observation period. Forelimb usage during weight-bearing contact with the cylinder wall was documented. Contacts made exclusively with the forelimb on the side of the brain injury were recorded as ipsilateral, whereas contacts made with the forelimb on the side opposite to the injury were recorded as contralateral. The simultaneous use of both forelimbs was recorded as a bilateral measurement. The FUA score was subsequently calculated utilizing the following formula: $[(\text{Ipsilateral} - \text{Contralateral}) / (\text{Ipsilateral} + \text{Contralateral} + \text{Bilateral})] \times 100$.

Elevated plus maze (EPM) test

The EPM test was conducted in a sound-attenuated room under low-light conditions to minimize external stress. To eliminate olfactory cues, the maze apparatus was thoroughly cleaned with 75% ethanol prior to each trial. To initiate the 5-min trial, each mouse was placed on the central platform facing an open arm, directed away from the experimenter. Strict silence was maintained throughout the testing period to ensure behavioral consistency. Animal movements were automatically recorded and tracked utilizing a video tracking system (Smart 3.0; Panlab, Barcelona, Spain). Key indicators, including the duration of time spent in the open arms and the total distance traveled, were subsequently analyzed to assess anxiety-like behaviors.

RNA sequencing (RNA-seq)

Total RNA was extracted from the tissue samples utilizing TRIzol reagent in accordance with the manufacturer's instructions. Comprehensive RNA-seq library construction and subsequent bioinformatic analyses were performed by Wuhan MetWare Biotechnology Co., Ltd. (Wuhan, China). Briefly, following mRNA enrichment and fragmentation, first-strand cDNA was synthesized utilizing random hexamer primers, followed by second-strand cDNA synthesis. The resulting cDNA fragments were purified, subjected to end-repair and A-tailing, and ligated to sequencing adapters utilizing a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). To generate strand-specific sequencing libraries, the dUTP-labeled second-strand cDNA was selectively digested utilizing uracil-N-glycosylase (UNG). The resulting products were size-selected via agarose gel electrophoresis and amplified via PCR. Finally, the constructed libraries were sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) to generate paired-end reads.

Statistics.

Statistical analyses were performed utilizing GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA). An unpaired Student's t-test was utilized to evaluate the statistical significance of differences between two independent groups. For comparisons involving more than two groups, a one-way analysis of variance was employed, followed by an appropriate post hoc test. All data are presented as the mean $\pm$ standard error of the mean (SEM). A *P*-value of < 0.05 was considered to indicate statistical significance.

Results

The expression level and enzymatic activity of ABHD6 increased after IS

Accumulating evidence indicates that ABHD6 plays a significant role in the pathogenesis of neurological diseases^[25–27]. However, the specific role of ABHD6 following IS remains unclear. To elucidate the role of ABHD6 in the pathological progression of IS, a MCAO/R mouse model was established to simulate IS in vivo. Laser speckle contrast imaging revealed a significant reduction in cerebral blood flow in MCAO/R mice compared with healthy mice (Figs. 1A, B). Neurological function was evaluated utilizing the Longa

scoring system, which demonstrated an average score greater than 2 in the MCAO/R group (Fig. 1C). Subsequent triphenyltetrazolium chloride (TTC) staining of the extracted brain tissues revealed extensive ischemic lesions in the MCAO/R mice (Figure S2). These findings confirm the successful establishment of the in vivo cerebral ischemia model.

To evaluate ABHD6 expression in the brains of MCAO/R mice, qPCR and Western blot analyses were conducted. The results demonstrated that ABHD6 expression was significantly upregulated in the brain tissues of MCAO/R mice compared with healthy controls (Figs. 1D–F). In vitro, mouse primary neurons were cultured and subjected to OGD/R treatment. Microscopic examination revealed distinct morphological alterations in the primary neurons following OGD/R, characterized by cellular shrinkage, cytoplasmic reduction, and disintegration of the neural network (Fig. 1G). Furthermore, TUNEL staining indicated a significant increase in apoptosis among primary neurons following OGD/R (Figs. 1H, I). Collectively, these observations confirm the successful establishment of the in vitro OGD/R model in primary neurons. Subsequently, ABHD6 expression in primary neurons subjected to OGD for varying durations was quantified via qPCR and Western blot analyses. The data revealed that ABHD6 expression levels increased significantly at 1 h post-injury, remained elevated up to 4 h post-injury, and peaked at 2 h post-injury (Figs. 1J–L). Consequently, a 2-h OGD duration was utilized for all subsequent in vitro experiments. These findings indicate that ABHD6 expression is upregulated following ischemic injury. Furthermore, the enzymatic activity of ABHD6 in the brain tissues of MCAO/R mice was assessed, revealing significantly higher activity in the MCAO/R group compared with the sham-operated group (Figs. 1M, N). Consistently, ABHD6 enzymatic activity in the primary neurons of the OGD/R group was significantly elevated relative to the control group (Figs. 1O, P). Together, these results demonstrate that both the expression level and the enzymatic activity of ABHD6 are significantly elevated following IS.

ABHD6 exacerbated brain injury following IS by hydrolyzing 2-AG

To explore the in vitro biological effects of ABHD6 following cerebral ischemia, primary neurons were treated with 20 nmol of WWL70 (an ABHD6 inhibitor) following OGD/R. The enzymatic activity of ABHD6 in the primary neurons was measured, and the results demonstrated that WWL70 effectively inhibited ABHD6 enzymatic activity following OGD/R (Figs. 2A, B). Subsequent quantification of 2-AG levels revealed that OGD/R treatment decreased 2-AG levels, whereas WWL70 administration elevated 2-AG levels by inhibiting ABHD6 enzymatic activity post-OGD/R (Fig. 2C). To further evaluate the biological effects of ABHD6 in primary neurons, cell viability was quantified. The data indicated that WWL70 treatment increased the viability of primary neurons following OGD/R, an effect that was abolished by the introduction of AM251 (a competitive inhibitor of 2-AG) (Fig. 2D). These in vitro experiments indicate that ABHD6 exacerbates cellular damage by hydrolyzing 2-AG following OGD/R.

Subsequently, 2-AG levels in the brain tissues of mice subjected to MCAO/R and treated with WWL70 were quantified. At 7 days post-MCAO/R, 2-AG levels were significantly lower in the MCAO/R group than in the sham group; however, these levels were significantly higher in the WWL70-treated group compared

with the control group (Fig. 2E). To evaluate the *in vivo* biological effects of ABHD6, H&E staining was performed on the brain sections. Histological analysis revealed that WWL70 treatment reduced the cerebral ischemic volume following MCAO/R, suggesting that ABHD6 exacerbates neuronal damage in the MCAO/R mouse model via 2-AG hydrolysis (Figs. 2G, H). Neurological function was subsequently evaluated at 7 days post-MCAO/R utilizing the mNSS. The mNSS scores were significantly lower in the WWL70 group than in the control group at 7 days post-MCAO/R (Fig. 2I). Furthermore, the rotarod and FUA tests were conducted to evaluate the biological effects of ABHD6 on motor function in the MCAO/R mice. As illustrated in Fig. 2J, the WWL70 group exhibited improved rotarod performance at 7 days post-MCAO/R compared with the control group. Additionally, forelimb usage by mice in the WWL70 group was greater than that observed in the control group (Fig. 2K). These results suggest that ABHD6 exacerbates motor dysfunction in the MCAO/R mouse model by hydrolyzing 2-AG. To comprehensively assess neurological impairment following MCAO/R, anxiety-like behaviors were evaluated utilizing the EPM test. Mice in the WWL70 group exhibited a significantly higher percentage of distance traveled and time spent in the open arms compared with the control group, with no significant difference observed in the total distance traveled (Figs. 2L–O). These findings suggest that ABHD6 aggravates anxiety-like behaviors in mice following MCAO/R by hydrolyzing 2-AG. Taken together, these results demonstrate that ABHD6 exacerbates brain injury following IS via 2-AG hydrolysis.

Knockdown of ABHD6 improved the cell viability of HT22 cells after OGD/R

To further investigate the *in vitro* biological effects of ABHD6 on neuronal cells following cerebral ischemia, lentiviral vectors designed for the overexpression (OE-ABHD6) and knockdown (sh-ABHD6) of ABHD6 were synthesized and utilized to transduce HT22 cells (a representative neuronal cell line). The mRNA expression levels of ABHD6 in the HT22 cells were initially quantified via qPCR. As illustrated in Fig. 3A, ABHD6 mRNA expression in the OE-ABHD6 group was significantly elevated compared with the control group (> 1.8-fold), whereas expression in the sh-ABHD6 group was significantly reduced (> 70% reduction). Additionally, the protein levels of ABHD6 in the HT22 cells were evaluated via Western blot analysis (Figs. 3B, C). Consistently, ABHD6 protein levels were significantly upregulated in the OE-ABHD6 group (> 1.8-fold) and significantly downregulated in the sh-ABHD6 group (> 70% reduction) relative to the control group.

Subsequently, the transduced HT22 cells were subjected to OGD/R, and both ABHD6 enzymatic activity and 2-AG levels were measured. The analysis revealed that ABHD6 enzymatic activity was significantly enhanced in the OE-ABHD6 group and significantly suppressed in the sh-ABHD6 group compared with the control group (Fig. 3D, E). Consistent with the hydrolytic activity of ABHD6 on 2-AG, the 2-AG levels in the OE-ABHD6 group were significantly lower than those in the control group, whereas the levels in the sh-ABHD6 group were significantly higher (Fig. 3F). Finally, the viability of the HT22 cells following OGD/R was assessed utilizing a CCK-8 assay. The data demonstrated that cell viability was significantly reduced in the OE-ABHD6 group and significantly increased in the sh-ABHD6 group relative to the control

group (Fig. 3G). Collectively, these findings indicate that the knockdown of ABHD6 improves HT22 cell viability following OGD/R by attenuating 2-AG hydrolysis.

Knockdown of ABHD6 improved neurological function after MCAO/R in mice

To investigate the in vivo biological effects of ABHD6 following cerebral ischemia, an *Abhd6* knockout (KO) mouse line was generated (Figure S1). Both heterozygous and homozygous KO mice were viable and fertile, remaining phenotypically indistinguishable from their wild-type (WT) littermates regarding gross appearance and general behavior. Following the successful establishment of the ABHD6-KO mouse line, ABHD6 protein levels in the brain tissues were assessed via Western blot analysis, which confirmed the absence of ABHD6 expression in the KO mice (Figs. 4A, B).

Subsequently, 2-AG levels in the brain tissues of WT and KO mice were quantified following MCAO/R. The analysis revealed that 2-AG levels in the brain tissues of KO mice were significantly higher than those in WT mice post-MCAO/R (Fig. 4C). To evaluate the neuroprotective efficacy of 2-AG following MCAO/R, AM251 was administered to block 2-AG signaling. Histological evaluation via H&E staining at 7 days post-MCAO/R revealed that the cerebral infarct area in the KO group was significantly reduced compared with the WT group. However, treatment with AM251 abolished this neuroprotective effect, resulting in a significantly larger cerebral infarct area in the AM251-treated KO group compared with the untreated KO group (Figs. 4E, F). Neurological function was evaluated within 7 days following MCAO/R utilizing the mNSS. The mNSS scores were significantly lower in the KO group than in the WT group; conversely, these scores were significantly elevated in the AM251-treated KO group relative to the untreated KO group (Fig. 4G).

Subsequently, rotarod and FUA tests were conducted to evaluate the effects of ABHD6 on motor function recovery in the MCAO/R mice. As illustrated in Fig. 4H, the KO group exhibited improved rotarod performance compared with the WT group. However, this functional improvement was attenuated in the AM251-treated KO group. Similarly, forelimb usage was significantly greater in the KO group than in the WT group, whereas this metric was significantly reduced in the AM251-treated KO group (Fig. 4I). To comprehensively assess neurological impairment following MCAO/R, anxiety-like behaviors were evaluated utilizing the EPM test. Mice in the KO group exhibited a significantly higher percentage of distance traveled and time spent in the open arms compared with the WT group, with no significant difference observed in the total distance traveled. Conversely, the AM251-treated KO group exhibited a significantly lower percentage of distance traveled and time spent in the open arms relative to the untreated KO group (Figs. 4J–L, Figure S3). Collectively, these observations indicate that the knockout of ABHD6 improves neurological function following MCAO/R in mice by inhibiting 2-AG hydrolysis.

Mechanism of ABHD6 aggravating neuronal injury after IS.

Emerging evidence indicates that beyond its canonical role in 2-AG hydrolysis, ABHD6 is broadly implicated in diverse physiological processes, including glucose and lipid metabolism, as well as the regulation of inflammatory responses^[17–20]. Consequently, it was investigated whether ABHD6 regulates the biological activities of neurons following IS via its noncanonical functions. To explore the effects of ABHD6 on neurons post-IS, the transcriptomes of primary neurons transduced with OE-ABHD6 or sh-ABHD6 were compared with those of their respective negative controls (NC) utilizing RNA-seq. Principal component analysis (PCA) revealed that both the overexpression and knockdown of ABHD6 induced dramatic transcriptional changes in the primary neurons (Figures S4, S5). Specifically, a total of 13 upregulated and 199 downregulated genes were identified in primary neurons following ABHD6 overexpression (Figs. 5A–C). Conversely, a total of 218 upregulated and 95 downregulated genes were identified in primary neurons following ABHD6 knockdown (Figs. 5D–F).

To elucidate the biological implications of these differentially expressed genes (DEGs), Gene Ontology (GO) enrichment analysis—encompassing three sub-ontologies: biological process (BP), cellular component (CC), and molecular function (MF)—was performed. The GO analysis primarily indicated significant enrichment in terms associated with extracellular matrix organization, chemotaxis, and synaptic structures (Figs. 5G, H and Figures S6, S7). To gain further functional insights, the DEGs were subsequently subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. This analysis revealed that the DEGs were predominantly clustered in inflammatory and immune-related signaling cascades, including the TNF, NF- κ B, and IL-17 pathways (Figs. 5I, J). Furthermore, significant enrichment was observed in the FoxO and NOD-like receptor signaling pathways. Given that both FoxO and NOD-like receptors are canonical upstream regulators of the autophagic machinery^[28, 29], these results collectively suggest that ABHD6 is involved in the regulation of inflammatory signaling and autophagy in primary neurons following IS.

ABHD6 aggravate OGD/R injury by inhibiting autophagy through negatively regulating Ddit3/Bnip3 in HT22 cells

To elucidate the downstream molecular mechanisms by which ABHD6 exacerbates ischemic injury in vitro, the DEGs in HT22 cells were analyzed. Venn diagram analysis identified an intersection of 8 specific genes that were concomitantly downregulated following ABHD6 overexpression (OE-ABHD6) and upregulated following ABHD6 knockdown (sh-ABHD6) (Fig. 6A). Subsequent protein-protein interaction (PPI) network analysis identified Ddit3 as a critical hub gene within this network (Fig. 6B). Given the established function of Ddit3 (also known as CHOP) in the regulation of autophagy^[30] alongside the aforementioned KEGG pathway enrichment results, a potential role for ABHD6 in regulating neuronal autophagy following IS was hypothesized. As Ddit3 and Bnip3 are well-characterized inducers of autophagy^[31], their expression profiles were subsequently evaluated. RT-qPCR and Western blot analyses demonstrated that ABHD6 overexpression significantly suppressed both the mRNA and protein levels of Ddit3 and Bnip3 in OGD/R-treated HT22 cells (Figs. 6C–G). Conversely, ABHD6 knockdown significantly elevated the expression of both Ddit3 and Bnip3 compared with the control

group (Figs. 6C–G). These findings indicate that ABHD6 acts as a negative regulator of the Ddit3/Bnip3 signaling axis under OGD/R conditions.

To determine whether the ABHD6-mediated downregulation of Ddit3/Bnip3 is associated with impaired autophagic activity, the expression of classical autophagy markers was evaluated via Western blot analysis. As illustrated in Figs. 6H–J, ABHD6 overexpression significantly decreased the LC3-II/LC3-I ratio and induced a marked accumulation of the autophagic substrate p62, indicating a blockade in autophagic flux. Conversely, ABHD6 knockdown promoted autophagic turnover, as evidenced by an increased LC3-II/LC3-I ratio and the accelerated degradation of p62. To further evaluate these biochemical alterations, immunofluorescence staining for LC3 was conducted. Consistent with the Western blot data, the fluorescence intensity of LC3 were significantly reduced in the OE-ABHD6 group and significantly increased in the sh-ABHD6 group (Figs. 6K, L). Finally, transmission electron microscopy (TEM) was utilized to assess ultrastructural changes. TEM analysis revealed a significant reduction in the formation of double-membrane autophagosomes in ABHD6-overexpressing cells. Conversely, ABHD6 knockdown resulted in a significant accumulation of autophagic vacuoles per field (Figs. 6M, N). Collectively, these findings demonstrate that ABHD6 suppresses Ddit3/Bnip3-mediated autophagy in HT22 cells during OGD/R injury.

ABHD6 exacerbated MCAO/R injury by inhibiting neuronal autophagy through negatively regulating Ddit3/Bnip3

To elucidate the downstream molecular mechanisms by which ABHD6 exacerbates ischemic injury in vivo, the mRNA levels of Ddit3 and Bnip3 were analyzed in ABHD6-KO mice following MCAO/R. The analysis revealed that the mRNA levels of Ddit3 and Bnip3 were significantly higher in ABHD6-KO mice than those in WT mice (Figs. 7A, B). Consistent with the RT-qPCR data, the protein levels of Ddit3 and Bnip3 were significantly elevated in ABHD6-KO mice compared with WT mice (Figs. 7C–E). These findings indicate that ABHD6 acts as a negative regulator of the Ddit3/Bnip3 signaling axis under MCAO/R conditions.

To determine whether the ABHD6-mediated downregulation of Ddit3/Bnip3 is associated with impaired autophagic activity in vivo, Western blot analysis was conducted to evaluate the expression of LC3 and p62. As illustrated in Figs. 7F–H, the LC3-II/LC3-I ratio was significantly higher in the ABHD6-KO group than in the WT group, whereas the p62 level was significantly lower in the ABHD6-KO group compared with the WT group. Subsequently, immunofluorescence staining was utilized to assess the expression levels of LC3 and p62 in the brain sections of WT and ABHD6-KO mice. These observations were consistent with the Western blot findings (Figs. 7I–K). Finally, TEM was utilized to evaluate neuronal autophagosomes. TEM analysis revealed a significant accumulation of double-membrane autophagosomes in the ABHD6-KO mice (Figs. 7L, M). Collectively, these findings indicate that ABHD6 exacerbates MCAO/R injury by inhibiting neuronal autophagy via the negative regulation of Ddit3/Bnip3.

Discussion

IS affects approximately 7.4 million people worldwide annually and represents the second leading cause of death and a primary cause of long-term disability^[1]. Current therapeutic strategies often fail to achieve complete functional recovery, highlighting the clinical need to identify novel interventional targets capable of mobilizing the brain's endogenous neuroprotective mechanisms. The ECS is essential for maintaining central nervous system homeostasis^[4, 5]. Its primary endogenous ligand, 2-AG, functions as a retrograde messenger. Recent literature indicates that 2-AG, synthesized in postsynaptic neurons, diffuses retrogradely to activate presynaptic CB1R, thereby limiting excitotoxicity, reducing neuroinflammation, and modulating cerebral blood flow following IS^[6–8]. To maintain precise spatial and temporal signaling, 2-AG is rapidly degraded by hydrolytic enzymes such as ABHD6. However, the dynamic alterations of ABHD6 following IS and its potential impact on IS prognosis via 2-AG modulation have remained poorly understood.

In the present study, the potential pathogenic role of ABHD6 in IS was explored. Utilizing an in vitro OGD/R primary neuron model and an in vivo murine MCAO/R model, it was observed that ischemic stress is associated with a rapid and sustained upregulation of ABHD6 expression and enzymatic activity. The data suggest that this upregulation may exacerbate ischemic neural damage by excessively hydrolyzing 2-AG, thereby compromising the endogenous neuroprotective capacity of the ECS. Conversely, the pharmacological inhibition of ABHD6 with WWL70 restored 2-AG levels, attenuated neuronal apoptosis, and reduced cerebral infarct volume. Importantly, this molecular modulation was accompanied by observable functional benefits, including improved mNSS, enhanced sensorimotor coordination (as evidenced by rotarod and FUA tests), and the amelioration of post-stroke anxiety-like behaviors in the EPM test.

The neuroprotective effects observed following ABHD6 inhibition appear to be mediated by a dual mechanism involving intercellular lipid signaling and intracellular quality control. Under physiological conditions, 2-AG serves as an "on-demand" retrograde messenger: upon postsynaptic synthesis, it traverses the synaptic cleft to activate presynaptic CB1R, suppressing the release of excitatory neurotransmitters and mitigating excitotoxicity. While MAGL is the most abundant 2-AG-degrading enzyme, it is primarily localized presynaptically^[16]. Because ABHD6 is specifically colocalized with 2-AG-synthesizing enzymes within postsynaptic structures, its pathological upregulation post-ischemia may precipitate the premature degradation of 2-AG near its site of synthesis. This localization suggests that ABHD6 overexpression potentially deprives the synapse of its innate feedback protection before the lipid mediator can exert its neuroprotective effects. This analytical framework is supported by the in vitro rescue experiments, wherein the restorative effects of the ABHD6 inhibitor WWL70 on neuronal viability were effectively attenuated by AM251, a competitive inhibitor of CB1R/2-AG signaling. These observations indicate that ABHD6 is overexpressed in neurons following IS and may exacerbate neurological dysfunction through the enhanced localized hydrolysis of 2-AG.

Furthermore, ABHD6 is recognized as a 'moonlighting' protein with functions extending beyond lipid hydrolysis. Recent studies have demonstrated that ABHD6 acts as an endocytic accessory protein, contributing to the formation of long-term depression, synaptic downscaling, and reversal learning by

promoting AMPA receptor endocytosis^[32]. Additionally, ABHD6 has been shown to negatively regulate GSIS by hydrolyzing monoacylglycerols; accordingly, its inhibition in diabetic models restores GSIS and improves glucose tolerance^[19]. However, the multifarious roles of ABHD6 in the context of IS have remained largely unexplored. The present study suggests a novel non-enzymatic role for ABHD6: the suppression of cellular autophagy via the Ddit3/Bnip3 axis. Both DNA damage-inducible transcript 3 (Ddit3/CHOP) and BCL2-interacting protein 3 (Bnip3) are established positive regulators of the autophagic machinery during cellular stress^[30, 31]. In the canonical endoplasmic reticulum stress response, Ddit3 functions as a pivotal upstream transcriptional regulator of the pro-autophagic protein Bnip3^[33]. To evaluate whether ABHD6 regulates autophagy via this signaling axis following IS, a cross-model validation approach encompassing unbiased transcriptomics and bidirectional in vitro and in vivo manipulations was utilized. RNA-sequencing combined with intersectional differential expression analysis across ABHD6 gain- and loss-of-function cellular models identified Ddit3 as a central, negatively regulated downstream target. Molecular validation in vitro confirmed that ABHD6 overexpression suppresses, whereas ABHD6 silencing amplifies, the mRNA and protein levels of both Ddit3 and its downstream effector Bnip3. This transcriptional suppression correlated with an inhibition of autophagic flux, as evidenced by a reduced LC3-II/LC3-I ratio and the accumulation of the autophagic substrate p62. This blockade in autophagic activity was further substantiated by immunofluorescence imaging of LC3 puncta and TEM, which revealed a significant reduction in double-membrane autophagosomes in ABHD6-overexpressing cells and a reciprocal accumulation in sh-ABHD6 cells.

To further contextualize the physiological relevance of this pathway, the Ddit3/Bnip3-autophagy cascade was evaluated in vivo utilizing an MCAO/R stroke model in ABHD6-KO mice. Although ischemic injury intrinsically elicited a baseline upregulation of Ddit3 and Bnip3 in wild-type (WT) mice as part of the typical stress response, the genetic deletion of ABHD6 appeared to significantly amplify this activation. Compared with WT ischemic brains, ABHD6-KO mice subjected to MCAO/R exhibited significantly elevated levels of Ddit3 and Bnip3 transcripts and proteins. This in vivo activation of the Ddit3/Bnip3 axis in KO mice corresponded with an intensification of cerebral autophagic flux, demonstrated by enhanced LC3-II/LC3-I conversion, accelerated p62 degradation, and an accumulation of double-membrane autophagic vacuoles within ischemic brain tissues observed via TEM. Collectively, the data from both cellular and animal models indicate that ABHD6 may function as an endogenous negative regulator of ischemic autophagy by restraining the Ddit3/Bnip3 signaling cascade. During IS, cells endure significant energy depletion, organelle damage, and protein misfolding, making autophagy an essential cellular clearance mechanism for survival. The present findings suggest that ABHD6 overexpression obstructs autophagic flux by downregulating the Ddit3/Bnip3 pathway. This resulting accumulation of cellular debris may accelerate the progression from reversible ischemic stress to irreversible apoptosis. Consequently, it is postulated that ABHD6 exacerbates IS-induced neurological damage partly through its non-enzymatic function of inhibiting autophagy.

Despite these promising findings, several limitations of the present study should be noted. First, the in vivo MCAO/R model evaluations were restricted to the acute and subacute phases of IS (up to 7 days

post-injury). Therefore, the long-term implications of ABHD6 inhibition on chronic neurorestoration, including neurogenesis and synaptic remodeling, remain to be elucidated. Second, stroke pathology is governed by complex multicellular interactions. While the in vitro experiments were conducted in isolated primary neurons, ABHD6 is also expressed in glial cells, such as microglia and astrocytes, which critically mediate the post-stroke neuroinflammatory response. The specific contributions of glial ABHD6 to the observed ischemic outcomes were not delineated in the current experimental design and warrant future investigation.

These findings contribute to the existing literature by highlighting the regulatory potential of ABHD6 in IS. While MAGL is responsible for approximately 85% of 2-AG degradation^[13], chronic pharmacological inhibition of MAGL can induce 2-AG overload, partial desensitization of CB1R, and a subsequent loss of cannabinoid-mediated efficacy, occasionally leading to adverse effects such as hypoactivity and hyperreflexia^[14]. Although ABHD6 accounts for a smaller overall fraction of 2-AG hydrolysis, it is uniquely positioned at the postsynaptic membrane, unlike MAGL and the microglia-associated ABHD12. The present data suggest that inhibiting ABHD6 may afford precise, localized control over "on-demand" 2-AG signaling exactly at its synthesis site. This targeted approach has the potential to circumvent the broader systemic effects associated with MAGL inhibitors while providing neuroprotection and facilitating functional behavioral recovery. Furthermore, the integration of ABHD6-mediated lipid metabolism with the Ddit3/Bnip3 autophagic cascade represents a novel finding. Previous literature has predominantly framed endocannabinoid hydrolyzing enzymes within the context of receptor-mediated lipid signaling. By elucidating the ABHD6-Ddit3/Bnip3 axis, this study conceptually bridges postsynaptic lipidomics with the transcriptional regulation of autophagic pathways, offering new insights into the multifaceted pathophysiology of IS.

Abbreviations

ABHD6 Alpha/beta hydrolase domain 6

CBRs Cannabinoid receptors

CCK-8 Cell Counting Kit-8

CNS Central nervous system

ECS Endocannabinoid system

ELISA Enzyme-Linked Immunosorbent Assay

EPM Elevated plus maze

4-MUH 4-methylumbelliferyl-heptanoate

FUA Forelimb use asymmetry

GSIS Glucose-stimulated insulin secretion

IS Ischemic stroke

MAGL Monoacylglycerol lipase

MCAO/R Middle cerebral artery occlusion/reperfusion

mNSS Modified neurological severity score

OGD/R Oxygen-glucose deprivation/reoxygenation

2-AG 2-arachidonoylglycerol

Declarations

Supplementary Information Supporting Information is available online or from the author.

Availability of data and materials The sequencing data have been deposited in the Genome Sequence Archive of the National Genomics Data Center, China National Center for Bioinformation (<https://ngdc.cncb.ac.cn/omix/>), under accession number PRJCA070873. Other data will be made available on reasonable request.

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Author contributions D.L., Z.C., L.Z. have contributed equally to this work. D.L., Z.C., L.Z. analyzed all the experimental results and wrote the original draft. K.Y. and Z.T. conducted cell experiments and prepared mechanism diagrams. K.Q., Z.Z. and Y.W. established animal models. H.X. conducted RNA-seq analysis. X.L., X.G. and P.L. designed the overall study and revised the initial draft. All authors reviewed and approved the final manuscript.

Consent to publish statement/form All the authors of this article have consented to publication.

Informed consent from participants All patients involved in the article were informed and signed informed consent.

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Competing interests The authors declare no competing interests.

Ethical approval All animal studies were conducted following the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of the Fourth Military Medical University (No. 20250071).

References

1. Martin SS, Aday AW, Almarzooq ZI et al (2024) 2024 heart disease and stroke statistics: A report of us and global data from the american heart association[J]. *Circulation* 149(8):e347–e913
2. Sharma R, Lee K (2025) Advances in treatments for acute ischemic stroke[J]. *BMJ*, 389:e076161
3. Joy MT, Carmichael ST (2021) Encouraging an excitable brain state: Mechanisms of brain repair in stroke[J]. *Nat Rev Neurosci* 22(1):38–53
4. Martinez Ramirez CE, Ruiz-Pérez G, Stollenwerk TM et al (2023) Endocannabinoid signaling in the central nervous system[J]. *Glia* 71(1):5–35
5. Mechoulam R, Parker LA (2013) The endocannabinoid system and the brain[J]. *Annu Rev Psychol* 64:21–47
6. Wang Q, Peng Y, Chen S et al (2009) Pretreatment with electroacupuncture induces rapid tolerance to focal cerebral ischemia through regulation of endocannabinoid system[J]. *Stroke* 40(6):2157–2164
7. Wang Q, Li X, Chen Y et al (2011) Activation of epsilon protein kinase c-mediated anti-apoptosis is involved in rapid tolerance induced by electroacupuncture pretreatment through cannabinoid receptor type 1[J]. *Stroke* 42(2):389–396
8. Su B, Dong H, Ma R et al (2009) Cannabinoid 1 receptor mediation of spinal cord ischemic tolerance induced by limb remote ischemia preconditioning in rats[J]. *J Thorac Cardiovasc Surg* 138(6):1409–1416
9. Baggelaar MP, Maccarrone M, van der Stelt M (2018) 2-arachidonoylglycerol: A signaling lipid with manifold actions in the brain[J]. *Prog Lipid Res* 71:1–17
10. Chen C (2023) Inhibiting degradation of 2-arachidonoylglycerol as a therapeutic strategy for neurodegenerative diseases[J]. *Pharmacol Ther*, 244:108394
11. Zhou H, Zhang Z, Wei H et al (2013) Activation of stat3 is involved in neuroprotection by electroacupuncture pretreatment via cannabinoid cb1 receptors in rats[J]. *Brain Res*, 1529(154 – 64.
12. England TJ, Hind WH, Rasid NA et al (2015) Cannabinoids in experimental stroke: A systematic review and meta-analysis[J]. *J Cereb Blood Flow Metab* 35(3):348–358
13. Savinainen JR, Saario SM, Laitinen JT (2012) The serine hydrolases magl, abhd6 and abhd12 as guardians of 2-arachidonoylglycerol signalling through cannabinoid receptors[J]. *Acta Physiol (Oxf)* 204(2):267–276
14. Schlosburg JE, Blankman JL, Long JZ et al (2010) Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system[J]. *Nat Neurosci* 13(9):1113–1119

15. Marrs WR, Blankman JL, Horne EA et al (2010) The serine hydrolase abhd6 controls the accumulation and efficacy of 2-ag at cannabinoid receptors[J]. *Nat Neurosci* 13(8):951–957
16. Straiker A, Hu SS, Long JZ et al (2009) Monoacylglycerol lipase limits the duration of endocannabinoid-mediated depolarization-induced suppression of excitation in autaptic hippocampal neurons[J]. *Mol Pharmacol* 76(6):1220–1227
17. Farah SI, Hilston S, Tran N et al (2022) 1-, 2- and 3-ag as substrates of the endocannabinoid enzymes and endogenous ligands of the cannabinoid receptor 1[J]. *Biochem Biophys Res Commun*, 591(31 – 6.
18. Cao JK, Kaplan J, Stella N (2019) Abhd6: Its place in endocannabinoid signaling and beyond[J]. *Trends Pharmacol Sci* 40(4):267–277
19. Zhao S, Mugabo Y, Iglesias J et al (2014) A/ β -hydrolase domain-6-accessible monoacylglycerol controls glucose-stimulated insulin secretion[J]. *Cell Metab* 19(6):993–1007
20. Tanaka M, Moran S, Wen J et al (2017) Wwl70 attenuates pge(2) production derived from 2-arachidonoylglycerol in microglia by abhd6-independent mechanism[J]. *J Neuroinflammation* 14(1):7
21. Zhang Z, Gao X, Tian Z et al (2024) Preso enhances mglur1-mediated excitotoxicity by modulating the phosphorylation of mglur1-homer1 complex and facilitating an er stress after traumatic brain injury[J]. *Cell Death Discov* 10(1):153
22. Chiang T, Messing RO, Chou W-H (2011) Mouse model of middle cerebral artery occlusion [M]. 1940-087X
23. Miralpeix C, Reguera AC, Fosch A et al (2021) Carnitine palmitoyltransferase 1c negatively regulates the endocannabinoid hydrolase abhd6 in mice, depending on nutritional status[J]. *Br J Pharmacol* 178(7):1507–1523
24. Zou P, Li T, Cao Z et al (2025) High-altitude hypoxia aggravated neurological deficits in mice induced by traumatic brain injury via bach1 mediating astrocytic ferroptosis[J]. *Cell Death Discov* 11(1):46
25. Naydenov AV, Horne EA, Cheah CS et al (2014) Abhd6 blockade exerts antiepileptic activity in ptz-induced seizures and in spontaneous seizures in r6/2 mice[J]. *Neuron* 83(2):361–371
26. Xue Z, Ye L, Ge J et al (2023) Wwl70-induced abhd6 inhibition attenuates memory deficits and pathological phenotypes in appswe/ps1de9 mice[J]. *Pharmacol Res*, 194(106864
27. Wen J, Jones M, Tanaka M et al (2018) Wwl70 protects against chronic constriction injury-induced neuropathic pain in mice by cannabinoid receptor-independent mechanisms[J]. *J Neuroinflammation* 15(1):9
28. Zhao J, Brault JJ, Schild A et al (2008) Coordinate activation of autophagy and the proteasome pathway by foxo transcription factor[J]. *Autophagy* 4(3):378–380
29. Lin Q, Li S, Jiang N et al (2021) Inhibiting nlrp3 inflammasome attenuates apoptosis in contrast-induced acute kidney injury through the upregulation of hif1a and bnip3-mediated mitophagy[J]. *Autophagy* 17(10):2975–2990

30. Zecchini S, Giovarelli M, Perrotta C et al (2019) Autophagy controls neonatal myogenesis by regulating the gh-igf1 system through a nfe2l2- and ddit3-mediated mechanism[J]. *Autophagy* 15(1):58–77

31. Xu W, Wang Q, Li M et al (2025) Screening and validation of autophagy-related mrnas and lncrnas in bone nonunion based on transcriptome sequencing analysis[J]. *Hum Cell* 39(1):2

32. Wei M, Yang L, Su F et al (2024) Abhd6 drives endocytosis of ampa receptors to regulate synaptic plasticity and learning flexibility[J]. *Prog Neurobiol*, 233(102559

33. Kim TW, Cheon C, Ko SG (2020) Sh003 activates autophagic cell death by activating atf4 and inhibiting g9a under hypoxia in gastric cancer cells[J]. *Cell Death Dis* 11(8):717

Figures

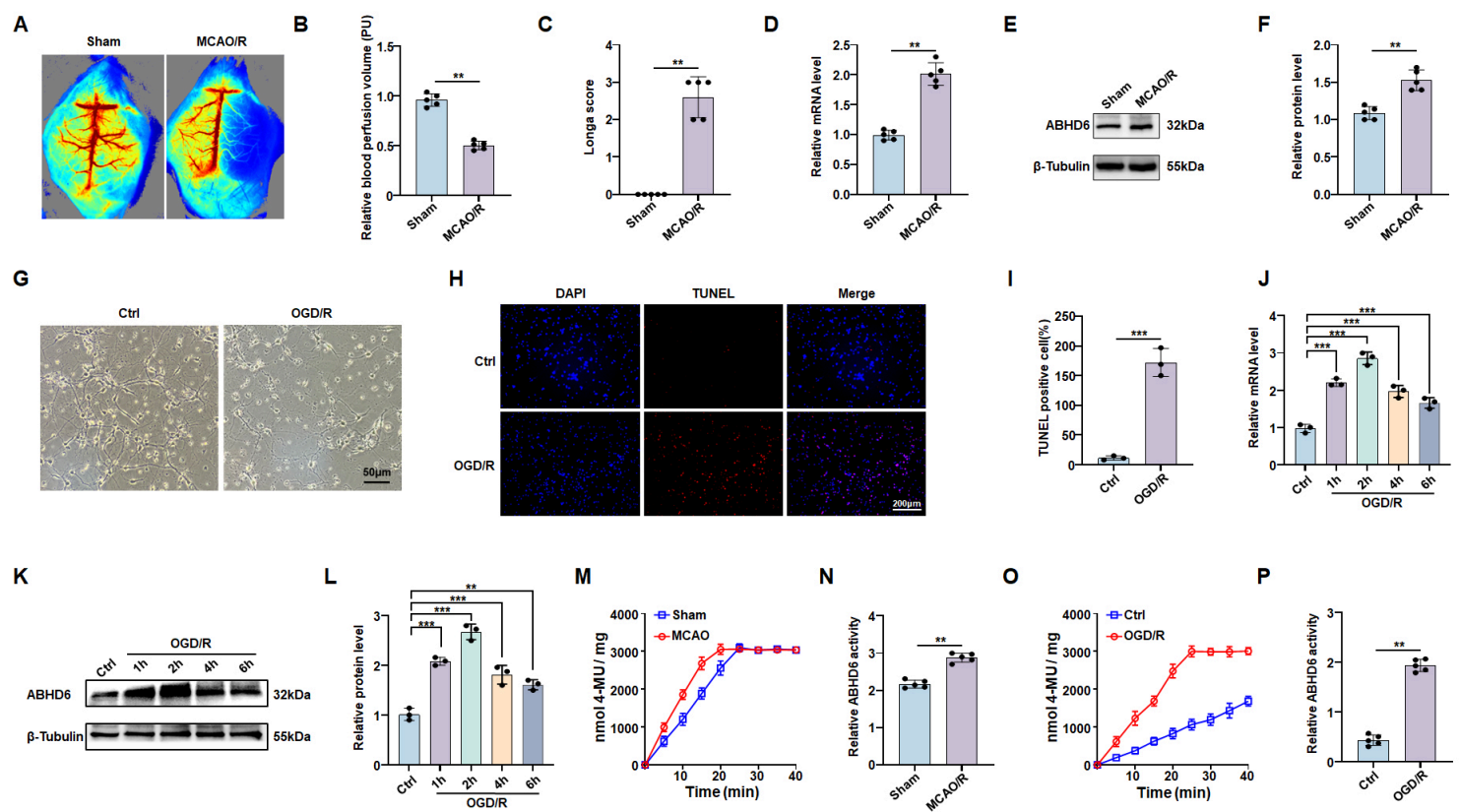


Figure 1

The expression level and enzymatic activity of ABHD6 increased after IS. A) Representative laser speckle contrast imaging of brain in healthy and MCAO/R mice. B) The volume of cerebral blood perfusion was quantified (n=5). C) The longa score of healthy and MCAO/R mice (n=5). D) qRT-PCR analyses of the levels of ABHD6 in mice brain tissue (n=5). E) The expression of ABHD6 in mice brain tissue was determined by western blotting. F) The expression of ABHD6 protein in mice brain tissue was quantified (n=5). G) Representative imaging of primary neurons deprived of oxygen glucose for 1 h and then restored to oxygen glucose for 24 h. Scale bar: 50 μ m. H) TUNEL staining of primary neurons

deprived of oxygen glucose for 1 h and then restored to oxygen glucose for 24 h were performed. Red: TUNEL. Blue: DAPI. Scale bar: 200 μ m. I) The TUNEL positive cells were quantified (n=3). J) qRT-PCR analyses of the levels of ABHD6 in primary neurons deprived of oxygen glucose for different hours and then restored to oxygen glucose for 24 h were performed (n=3). K) The expression of ABHD6 in primary neurons deprived of oxygen glucose for different hours and then restored to oxygen glucose for 24 h was determined by Western blotting. L) The expression of ABHD6 in primary neurons treated with OGD/R was quantified (n=3). M) ABHD6 hydrolyses 4-MUH producing 4-MU, a highly fluorescent compound (excitation 355 nm; emission 460 nm). Time course of 4-MUH hydrolysis by cells from healthy and MCAO/R mice brain (n=5). N) Relative activity of ABHD6 in cells from healthy and MCAO/R mice brain was quantified (n=5). O) Time course of 4-MUH hydrolysis by primary neurons treated with OGD/R (n=5). P) The activity of ABHD6 in primary neurons treated with OGD/R was quantified (n=5). The data were presented as the mean $\pm$ SEM. **, $P < 0.01$; ***, $P < 0.001$.

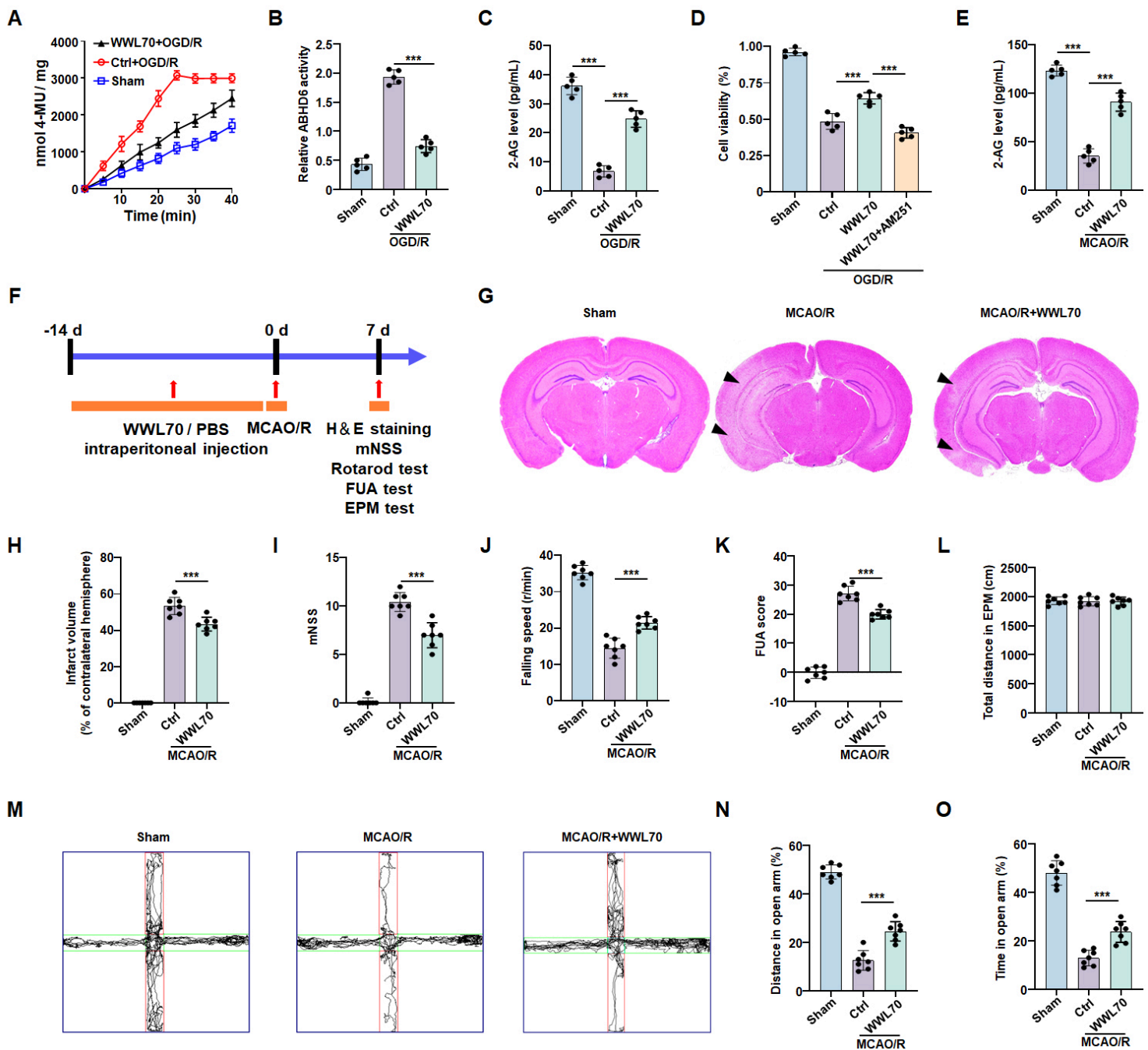


Figure 2

ABHD6 exacerbated brain injury following IS by hydrolyzing 2-AG. A) Time course of 4-MUH hydrolysis by primary neurons treated with OGD/R and WWL70 (n=5). B) Relative activity of ABHD6 in primary neurons treated with OGD/R and WWL70 was quantified (n=5). C) The 2-AG level in primary neurons treated with OGD/R and WWL70 was determined (n=5). D) The cell viability of primary neurons was quantified (n=5). E) The 2-AG level in brain tissue of mice was determined. F) Diagram showing the experimental design. G) Brain tissue was H&E-stained 7 days after MCAO/R treatment. H) The brain's infarct size was calculated (n=7). I) At day 7 post-MCAO/R, mNSS in mice receiving different treatments was quantified (n=7). J) Mice were tested 7 days after MCAO/R treatment for the speed at which they fell off the rotarod (n=7). K) Mice were tested 7 days after MCAO/R treatment by FUA test (n=7). L-O) At

day 7 post-MCAO/R, mice receiving different treatments were tested using EPM tests. L) The total distance of mouse activity in EPM test (n=7). M) Typical trajectory plots of EPM tests. Red: open arm. N) Percentage of the distance in the open arm to the total distance (n=7). O) Percentage of the time in the open arm to the total time (n=7). The data are presented as the mean $\pm$ SEM. ***, $P < 0.001$.

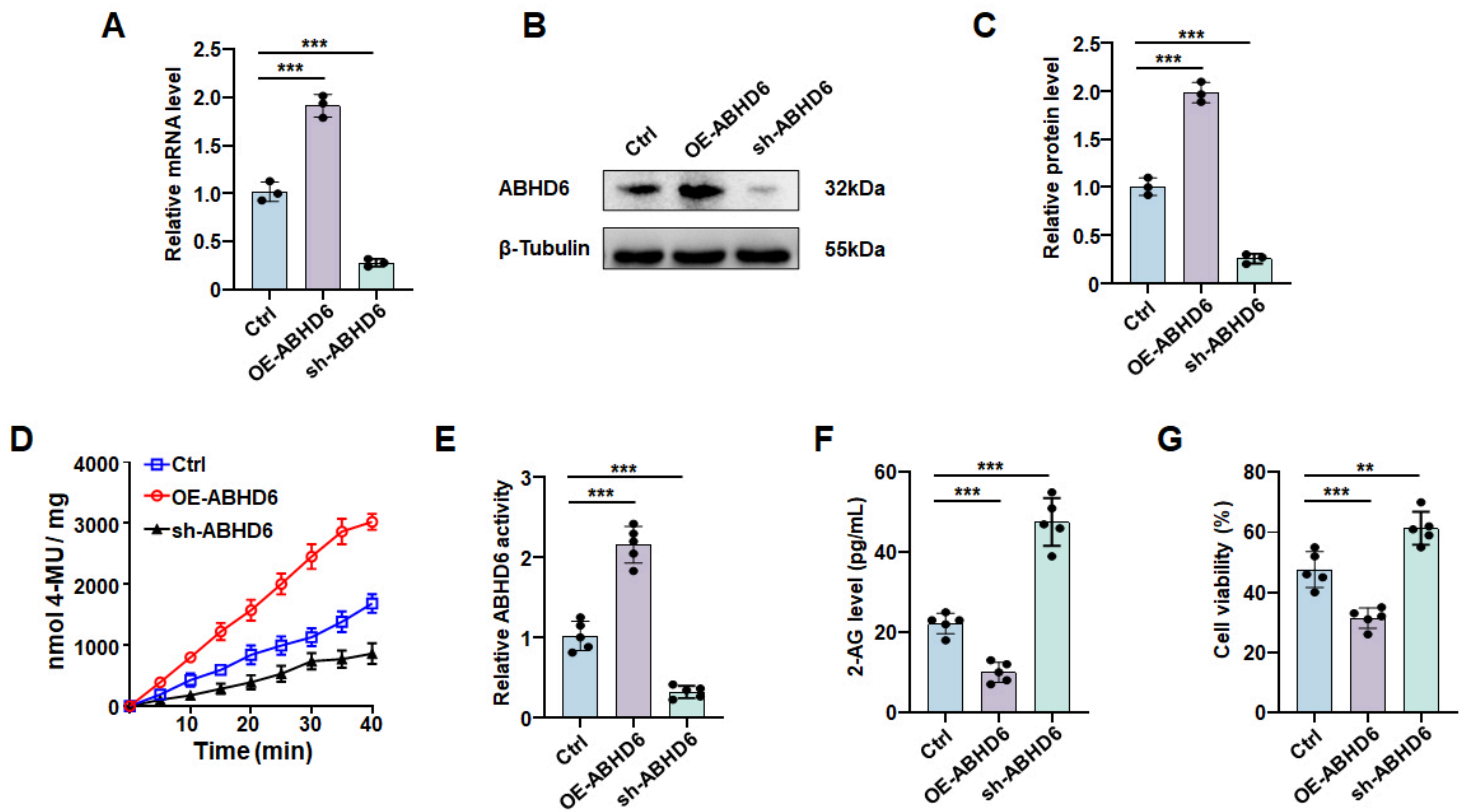


Figure 3

Knockdown of ABHD6 improved the cell viability of HT22 cells after OGD/R. A) The mRNA level of ABHD6 in HT22 cells was quantified (n=3). B) The protein level of ABHD6 was determined by western blot. C) The protein level of ABHD6 was quantified (n=3). D) Time course of 4-MUH hydrolysis by HT22 cells treated with OGD/R (n=5). E) The activity of ABHD6 in HT22 cells treated with OGD/R was quantified (n=5). F) The level of 2-AG in HT22 cells treated with OGD/R was measured (n=5). G) The cell viability of HT22 cells treated with OGD/R was quantified (n=5). The data were presented as the mean $\pm$ SEM. **, $P < 0.01$; ***, $P < 0.001$.

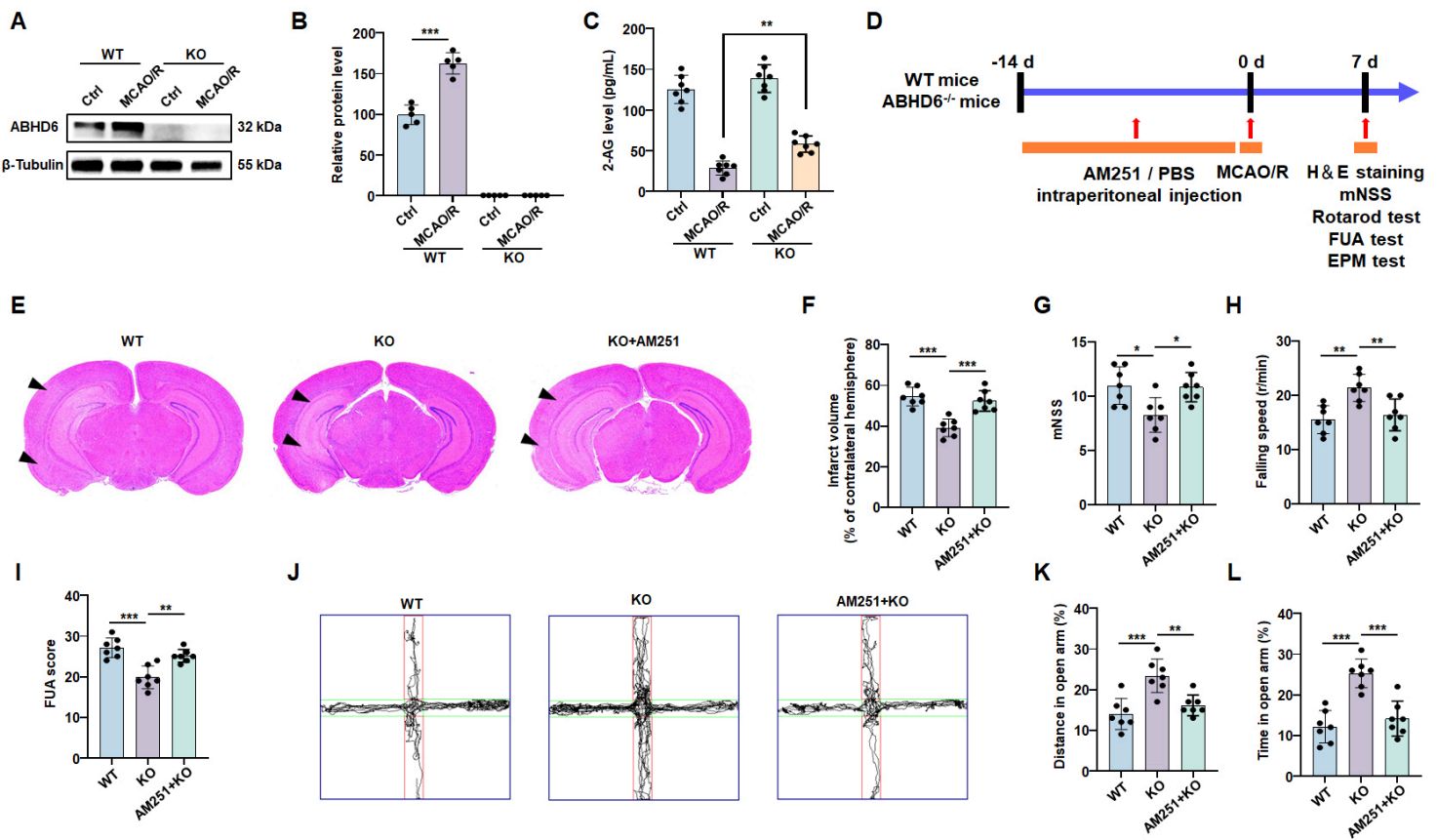


Figure 4

Knockdown of ABHD6 improved neurological function after MCAO/R in mice. A) The protein level of ABHD6 in the brain tissue of WT and ABHD6-KO mice treated with MCAO/R was determined by western blot. B) At day 7 post-MCAO/R, the protein level of ABHD6 in the brain tissue of WT and ABHD6-KO mice was quantified (n=5). C) At day 7 post-MCAO/R, the level of 2-AG in the brain tissue of WT and ABHD6-KO mice was quantified (n=7). D) Diagram showing the experimental design. E) Brain tissue was H&E-stained 7 days after MCAO/R treatment. F) The brain's infarct size was calculated (n=7). G) At day 7 post-MCAO/R, mNSS in WT and ABHD6-KO mice receiving different treatments was quantified (n=7). H) WT and ABHD6-KO mice were tested 7 days after MCAO/R treatment for the speed at which they fell off the rotarod (n=7). I) WT and ABHD6-KO mice were tested 7 days after MCAO/R treatment by FUA test (n=7). J-L) At day 7 post-MCAO/R, mice receiving different treatments were tested using EPM tests. J) Typical trajectory plots of EPM tests. Red: open arm. K) Percentage of the distance in the open arm to the total distance (n=7). L) Percentage of the time in the open arm to the total time (n=7). The data are presented as the mean $\pm$ SEM. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

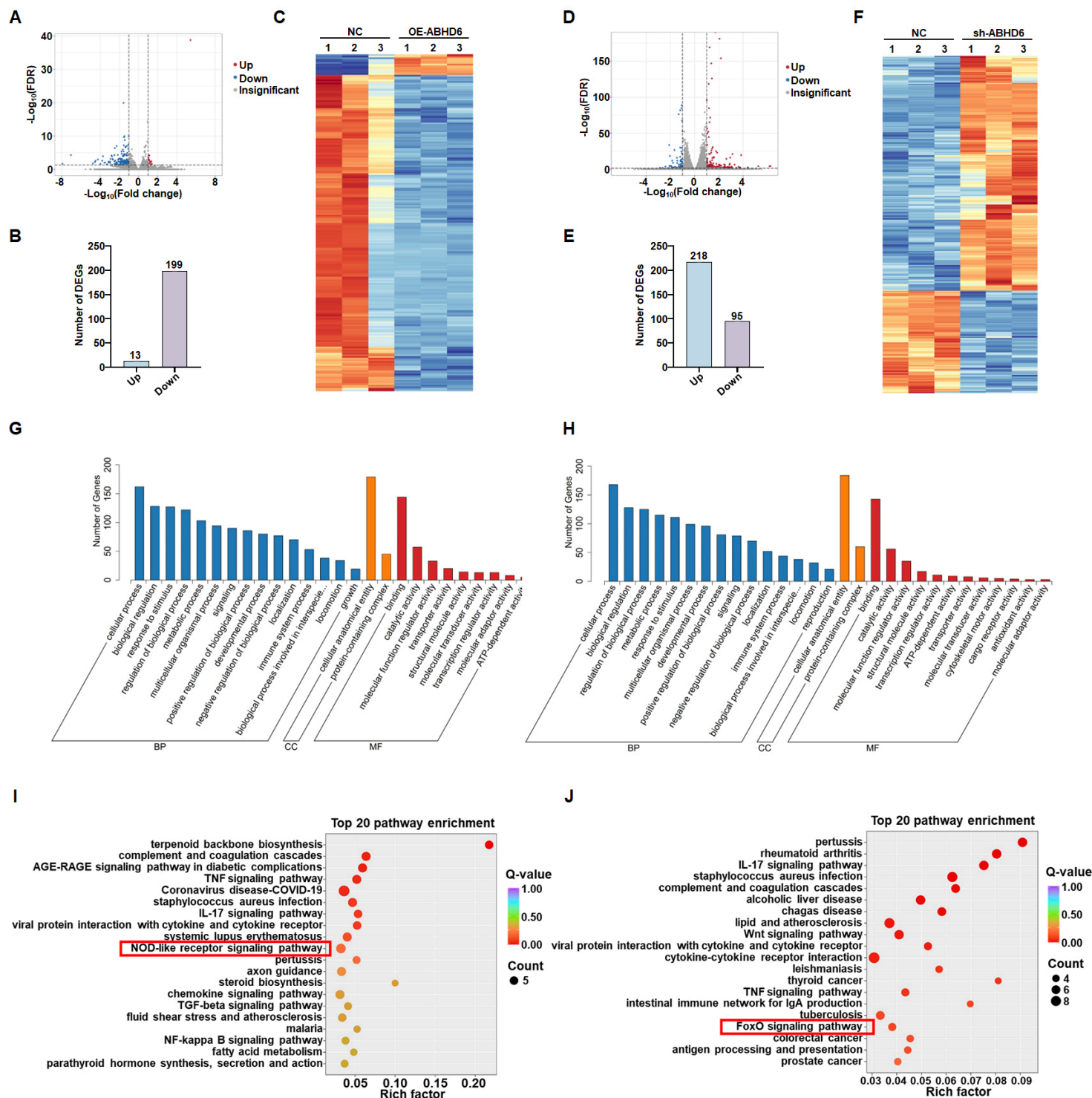


Figure 5

Mechanism of ABHD6 aggravating neuronal injury after IS. A) Primary neurons treated with OE-ABHD6 or NC were subjected to RNA-seq after OGD/R (n=3). The expression level of genes was shown by a volcano plot. 212 genes were identified with a cut-off of >1.0-fold for gene expression change and $P < 0.05$. B) 13 up-regulated genes and 199 down-regulated genes were identified. C) 212 differentially expressed genes were shown in a heatmap. D) Primary neurons treated with sh-ABHD6 or NC were subjected to RNA-seq after OGD/R (n=3). The expression level of genes was shown by a volcano plot.

313 genes were identified with a cut-off of >1.0-fold for gene expression change and $P < 0.05$. E) 218 up-regulated genes and 95 down-regulated genes were identified. F) 313 differentially expressed genes were shown in a heatmap. G) Differential gene GO annotation analysis strip histogram of biological process, cellular component, and molecular function (OE-ABHD6 vs. NC). H) Differential gene GO annotation analysis strip histogram of biological process, cellular component, and molecular function (sh-ABHD6 vs. NC). I) KEGG analysis was performed to identify differential pathway enrichment (OE-ABHD6 vs. NC). J) KEGG analysis was performed to identify differential pathway enrichment (sh-ABHD6 vs. NC). The data were presented as the mean $\pm$ SEM.

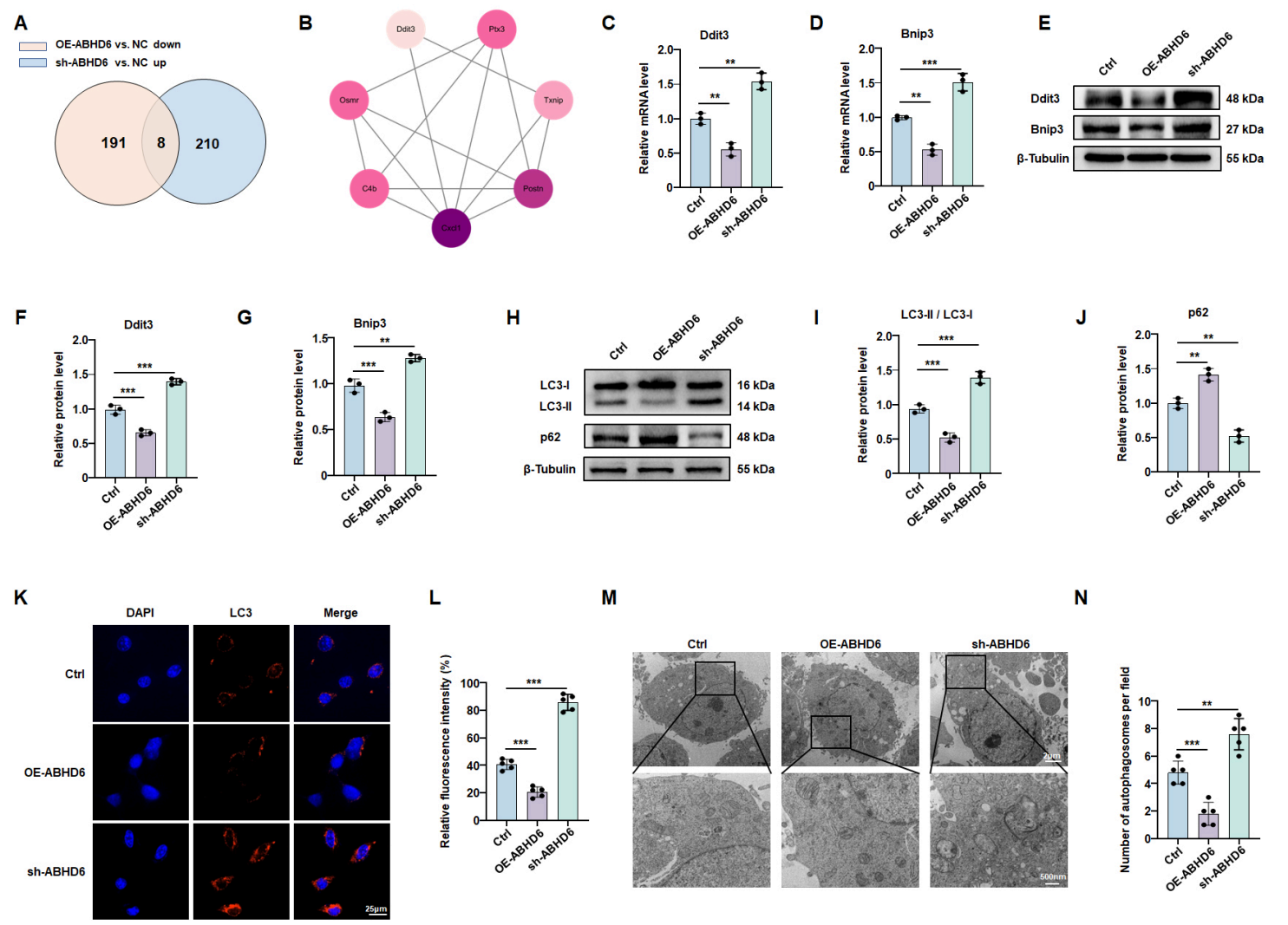


Figure 6

ABHD6 aggravate OGD/R injury by inhibiting autophagy through negatively regulating Ddit3/Bnip3 in HT22 cells. A) Venn diagram showing the intersection of downregulated DEGs in the OE-ABHD6 group and upregulated DEGs in the sh-ABHD6 group. Eight overlapping genes were identified. B) PPI network of the hub genes. C) RT-qPCR analysis of Ddit3 mRNA levels in HT22 cells transfected with OE-ABHD6 or sh-ABHD6 under OGD/R conditions (n=3). D) RT-qPCR analysis of Bnip3 mRNA levels in HT22 cells transfected with OE-ABHD6 or sh-ABHD6 under OGD/R conditions (n=3). E) Ddit3 and Bnip3 protein level

in HT22 cells transfected with OE-ABHD6 or sh-ABHD6 under OGD/R conditions was determined by Western Blot. F) Quantitative analysis of Ddit3 protein level (n=3). G) Quantitative analysis of Bnip3 protein levels (n=3). H) Western blot analysis of autophagy-related markers, including LC3-II/I ratio and p62 level in HT22 cells under OGD/R conditions. I) Quantification of LC3-II/I ratio in HT22 cells (n=3). J) Quantification of p62 level in HT22 cells (n=3). K) Representative immunofluorescence images of LC3 in HT22 cells under OGD/R conditions. Red: LC3. Blue: DAPI. Scale bar: 25µm. L) Fluorescence intensity of LC3 in HT22 cells under OGD/R conditions was quantified (n=5). M) Representative transmission electron microscopy images showing autophagic vacuoles in HT22 cells under OGD/R conditions. Scale bar: 2 µm and 500 nm. N) Quantification of the number of autophagosomes per field (n=5). The data were presented as the mean $\pm$ SEM. **, $P < 0.01$; ***, $P < 0.001$.

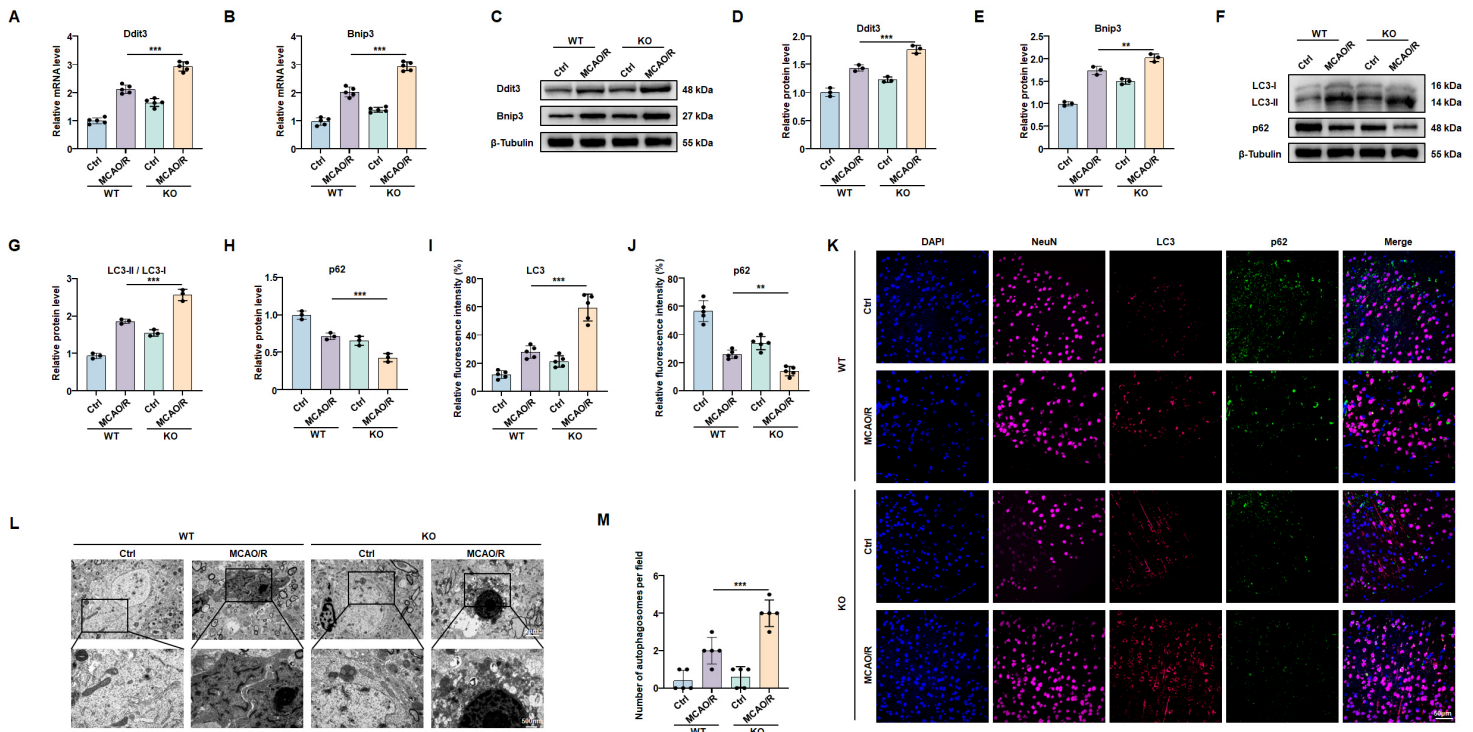


Figure 7

ABHD6 exacerbated MCAO/R injury by inhibiting neuronal autophagy through negatively regulating Ddit3/Bnip3. A) RT-qPCR analysis of Ddit3 mRNA levels in KO or WT mouse brains after MCAO/R (n=5). B) RT-qPCR analysis of Bnip3 mRNA levels in KO or WT mouse brains after MCAO/R (n=5). C) Ddit3 and Bnip3 protein level in KO or WT mouse brains after MCAO/R was determined by Western Blot. D) Quantitative analysis of Ddit3 protein level (n=5). E) Quantitative analysis of Bnip3 protein levels (n=5). F) Western blot analysis of autophagy-related markers, including LC3-II/I ratio and p62 level in KO or WT mouse brains after MCAO/R. G) Quantification of LC3-II/I ratio (n=3). H) Quantification of p62 level (n=3). I-K) The level of LC3 and p62 in neurons of KO or WT mouse brains after MCAO/R was determined by immunostaining. I) Fluorescence intensity of LC3 was quantified (n=5). J) Fluorescence intensity of p62 was quantified (n=5). K) Representative immunofluorescence images of LC3 and p62. Red: LC3. Green: p62. Pink: NeuN. Blue: DAPI. Scale bar: 50µm. L) Representative TEM images showing autophagic

vacuoles in KO or WT mouse brains after MCAO/R. Scale bar: 2 μ m and 500 nm. M) Quantification of the number of autophagosomes per field (n=5). The data were presented as the mean $\pm$ SEM. **, $P < 0.01$; ***, $P < 0.001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [ABHD6SupportingInformation20260909.docx](#)
- [WBrawdata.docx](#)
- [Onlinefloatimage1.png](#)