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TNF- α /PAD4 axis drives hyperhomocysteine-induced white matter injury after acute ischemic stroke

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Running headline: HHcy impairs WM repair after stroke

Abbreviation: CitH3, citrullinated histone 3; CTX, cortex; EC, external capsule; HHcy, Hyperhomocysteine; I/R, ischemia/reperfusion; PAD4, protein arginine deiminase 4; PDGFR α , platelet-derived growth factor- α ; MCAO, middle cerebral artery occlusion; MBP, myelin basic protein; MyRF, Myelin regulator; OGD/R, oxygen-glucose deprivation/reoxygenation; OLs, oligodendrocytes; OPCs, oligodendrocyte precursor cells; STR, striatum; TNF- α , tumor necrosis factor- α .

Abstract

Neural functional impairment following stroke is strongly linked to the loss of white matter (WM) integrity, a process critically dependent on the successful differentiation of oligodendrocyte precursor cells (OPCs). Given that hyperhomocysteine (HHcy) aggravates stroke prognosis, we hypothesized that it impairs OPC differentiation during the recovery period. Using *in vivo* (MCAO) and *in vitro* (OGD/R) models, we showed that HHcy hinders functional recovery, impairs OPC differentiation, and compromises WM integrity. Mechanistically, HHcy acts by upregulating tumor necrosis factor- α (TNF- α), which subsequently promotes the nuclear translocation of protein arginine deiminase 4 (PAD4). This leads to the upregulation of nucleosomal citrullinated histone 3 (CitH3) and the subsequent downregulation of *myelin regulatory factor* (*MyRF*), resulting in the observed inhibition of OPCs differentiation. Crucially, pharmacological inhibition of PAD4 using the pharmacological inhibitor YW-356 effectively promoted OPC differentiation and enhanced WM repair after ischemic stroke. Therefore, our findings identify the TNF- α /PAD4 pathway as a novel therapeutic target for reversing HHcy-induced white matter impairment and improving neurological outcomes after stroke.

Introduction

Hyperhomocysteinemia (HHcy) is a significant and common risk factor for ischemic stroke^[1] and is also associated with poor prognosis and increased mortality following the event^[2, 3]. Neural functional recovery after stroke relies heavily on the reconstruction of white matter integrity. Ischemia and hypoxic injury in acute stroke affect not only the gray matter but also the brain white matter, which is notably more sensitive to such insult, often accounting for about half of the infarct volume. This damage forms a crucial pathological basis for long-term neurological impairment ^[4]. In addition, clinical evidence suggests a particularly close association between HHcy and the disruption of white matter integrity in ischemic stroke patients^[5, 6]. For instance, HHcy predicts the severity of white matter injury in older patients with lacunar cerebral infarction^[6]. This raises the hypothesis that HHcy may affect the prognosis of stroke patients by interfering with the repair of white matter integrity. However, the specific role and underlying mechanism of HHcy in white matter integrity repair during stroke recovery remain unexplored.

OLs are highly vulnerable to ischemic injury, and their dysfunction or death leads to myelin loss and subsequent chronic neurological deficits^[7]. The remodeling of white matter injury is fundamentally dependent on the regeneration of myelin, which requires the proliferation, migration, and differentiation of oligodendrocyte precursor cells (OPCs) into mature oligodendrocytes (OLs)^[8, 9]. The differentiation capacity of OPCs in the adult brain is often insufficient to fully support white matter repair after ischemia ^[10]. The differentiation of OPCs and central nervous system myelination are critically dependent on the myelin regulatory transcription factor, Myelin Regulatory Factor (MyRF)^[11]. MyRF is a membrane-bound protein whose expression is essential for the transcription of genes encoding myelin structural proteins, such as myelin basic protein (MBP), during OPC differentiation^[12]. Inhibition of MyRF expression can directly block the formation of mature oligodendrocytes. The initial transcription levels of myelin-related genes are often mediated by changes in chromatin structure during the early stages of OPC differentiation.

Epigenetic remodeling, particularly histone modification, plays a crucial role in regulating

OPC differentiation^[13]. Citrullination of histone H3 (CitH3), a specific type of modification, can influence transcription and regulate cell function by altering chromatin structure^[14]. The enzyme responsible for this modification is protein arginine deiminase 4 (PAD4), which converts arginine residues into citrulline residues on the polypeptide chain^[15]. PAD4 regulates gene expression via histone citrullination and is involved in cell differentiation and apoptosis^[15].

Following acute cerebral ischemia, PAD4 is significantly upregulated in the peri-infarct region and has been implicated in impairing vascular remodeling in stroke recovery^[16]. Furthermore, in demyelinating conditions such as multiple sclerosis, the nuclear translocation of PAD4 leads to nucleosome histone hypercitrullination of nucleosomal histones (*i.e.*, elevated CitH3 levels), a process linked to blocked OLs maturation and potential OLs apoptosis^[17]. Elevated CitH3 levels are also strongly associated with widespread white matter hyperintensity in ischemic stroke patients ^[18]. Nevertheless, the specific function of PAD4-mediated histone citrullination in the differentiation of OPCs and the subsequent repair of white matter integrity following stroke recovery is unknown.

In this study, we examined the effects of HHcy on OPCs differentiation and white matter repair and investigated the underlying mechanism using *in vitro* and *in vivo* models. We also assessed the impact of PAD4 inhibitor YW-356 on OPCs differentiation and white matter repair after ischemic stroke.

Materials and Methods

Animals

All animal experiments conducted in this research were approved by the Institutional Animal Care and Use Committee of Capital Medical University and were strictly performed in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Male wild-type C57BL/6J mice (6–8 weeks old) were procured from Beijing Vital River Laboratory Animal Technology. The animals were maintained under standardized housing conditions with a 12-hour light/12-hour dark cycle at a constant ambient temperature of $23 \pm 1^{\circ}\text{C}$ and were provided with ad libitum access to food and water. Throughout the experimental process, stringent measures were implemented to uphold ethical principles of animal welfare, including optimization of experimental design to reduce the number of animals used and application of procedures to alleviate potential suffering. All mice were sequentially numbered and randomly assigned to different groups using a randomization tool. The surgeon performing the procedures remained blinded to the group allocations. Behavioral assessments and histopathological analyses were conducted by researchers who were unaware of the experimental group assignments.

Surgery

A middle cerebral artery ischemia/reperfusion (I/R) model was performed as previously described^[19]. Mice were anesthetized with isoflurane (4% for induction, 1.5–2% for maintenance) and maintained at 37°C throughout the procedure. The common carotid artery (CCA) was carefully isolated, followed by further separation of its branches, the external carotid artery (ECA) and internal carotid artery (ICA). After temporarily clamping the CCA and ICA, a small oblique cut was made in the ECA, and a pre-marked suture with a silicon-coated tip was inserted. The suture was advanced through the CCA bifurcation into the ICA. After 60 minutes of ischemia, the incision was reopened, and the suture was gently removed, thereby completing the I/R model.

Drug treatment

L-Methionine (M9625, Sigma, USA) was diluted with dd H₂O (2% (w/v)). To induce hyperhomocysteine (HHcy), 6-week-old male C57BL/6J mice were administered 2% (w/v) L-methionine in their drinking water for two weeks.

Synthesis of YW3-56

The PAD4 inhibitor YW3-56 used in this study, whose structure is shown in Figure SM1, was synthesized following previously reported procedures^[20]. The chemical structure of the compound was confirmed by ¹H nuclear magnetic resonance (¹H NMR) and electrospray ionization mass spectrometry (ESI-MS) (see Supporting Information, Figures SM2 and SM3). YW3-56 was provided as a powder, dissolved in DMSO to prepare a stock solution for use, aliquoted, and stored protected from light at -20°C.

YW3-56 was diluted with 0.5% DMSO saline (0.5 mg/ml). Administration of YW3-56 (5 mg/kg) was initiated via tail vein injection immediately upon reperfusion, followed by subsequent injections every day for a total duration of 14 days.

Rotarod test

Mice were first acclimated to the behavioral testing room for 1 hour. The rotarod test was then conducted using a rotarod apparatus (ZS-RDM-XS, Beijing zhongshidichuang, China) with protocol set to accelerate uniformly from 4 rpm to 40 rpm over a period of 300 seconds. During the training phase, mice were placed on the rod facing opposite to the direction of rotation. The acceleration program (4 to 40 rpm in 300 seconds) was then initiated for a maximum duration of 5 minutes. The latency to fall was automatically recorded by the apparatus. If a mouse remained on the rod for the entire 300-second period, it was removed and assigned a maximum latency score of 300 seconds. Each mouse underwent three trials per day with inter-trial intervals of 15–30 minutes to prevent fatigue. Training continued for 3 days until the fall latency of the control group reached a stable plateau, indicating successful task acquisition. During the testing phase, the identical acceleration protocol was repeated, and the average latency to fall from multiple trials was calculated for each mouse as the final performance metric to enhance data stability and reliability^[21].

Corner Test

Mice were gently placed at the apex of a 30° corner (constructed from two boards with a 3-mm gap). Each mouse performed 10 trials (30-second interval), and turns (left/right) were recorded. The apparatus was cleaned with ethanol between mice. The total number of right turns for each mouse was quantified, and group comparisons were performed for statistical analysis.

Foot fault test

Mice were tested for foot failure after 3, 7, 14, and 28 days of reperfusion. Mice were placed on a 106 cm × 110 cm mesh frame (containing a 9 cm² grid) with a height of 50 cm, and placed on a suspended grid without external interference. After 1 minute of acclimatization, the number of errors in one hundred strides of the left forelimb was recorded.

Neurological scores

The neurological function of mice after right middle cerebral artery occlusion was assessed using an 8-point scoring scale: 0, no deficit; 1, failure to fully extend the left forepaw; 2, weakened grip of the left forelimb when the tail is pulled; 3, spontaneous movement in all directions but circling to the left when the tail is pulled; 4, spontaneous circling or walking to the left; 5, walking only when stimulated; 6, loss of consciousness with no response to stimulation; and 7, death. This scale systematically quantifies neurological deficits ranging from mild left forelimb motor impairment to complete loss of consciousness and death.

Magnetic resonance imaging (MRI)

Brain structural assessment in mice was performed using small-animal magnetic resonance imaging (PharmaScan 70/16 US, BR, Germany). Before scanning, mice were anesthetized with isoflurane and securely positioned within a dedicated radiofrequency coil, with body temperature maintained using a controlled warming system. MRI was conducted on a 7.0T high-field scanner. The core imaging protocol included T2-weighted imaging to visualize cerebral infarction (hyperintense regions) and assess chronic-phase brain atrophy. Following image acquisition, infarct volume or volumetric changes in specific brain regions were quantified using professional analysis software via manual or semi-automated segmentation.

Diffusion tensor imaging (DTI)

Utilizing the Bruker PharmaScan 70/16 US small-animal magnetic resonance imaging system

for mouse brain diffusion tensor imaging, the procedure can be summarized as follows: Mice are anesthetized with isoflurane and securely positioned to ensure head stability at the isocenter of the scanning coil, while respiratory rate and body temperature are maintained throughout the scan. Subsequently, a multi-directional diffusion gradient encoding sequence (typically with a b-value ranging from 800 to 1500 s/mm² and at least 30 directions) is configured in Paravision software to acquire whole-brain diffusion-weighted images. Finally, the data are processed and subjected to tensor calculation using DSI Studio software (<http://dsi-studio.labsolver.org/>) to generate the fractional anisotropy map—a core parameter map reflecting white matter fiber integrity—which is then employed for quantitative analysis of microstructural changes in specific brain white matter regions.

Cell culture

OLN93, rat oligodendrocytes (Fig. S1a), were grown in high glucose DMEM (C11995500BT, Gibco, USA) with 10% FBS (FSP500, ExCell, UY) in a humidified incubator at 37°C with 5% CO₂. OLN93 were treated with L-homocysteine (H9633, Sigma-Aldrich) at a final concentration of 500 μM in DMEM for the indicated durations^[22, 23].

Oxygen-Glucose Deprivation/Reoxygenation (OGD/R) Model

When cells reached 70–80% confluency, the culture medium was removed, and the cells were gently washed twice with PBS. Cells were then incubated in glucose-free DMEM medium (L160KJ, BasalMedia, China) and placed in a hypoxia incubator chamber (MIC-101, Billups-Rothensberg, USA), which was flushed with 95% N₂ + 5% CO₂ at a flow rate of 5–10 L/min for 15 min before sealing. The sealed chamber was transferred to a 37 °C incubator for a 6-hour OGD treatment. After OGD, the cells were promptly removed from the hypoxic environment, the glucose-free medium was aspirated, and the cells were washed once with PBS, followed by the addition of normal complete culture medium for reoxygenation^[24].

Transfection of PAD4 siRNA

Transfection of PAD4 siRNA (sense: GGACCCAAGACCUCAUCAATT; antisense: UUGAUGAGGUCUUGGGUCCTT) was performed using Lipofectamine 2000 (11668019,

Thermo Fisher, USA). Cells were seeded into 6-well plates one day before transfection, reaching 50–70 % confluency at the time of transfection. For each well, 100 pmol of siRNA (final concentration 100 nM) was diluted in 125 μ L of Opti-MEM, while 5 μ L of Lipofectamine 2000 was diluted separately in 125 μ L of Opti-MEM. After incubating at room temperature for 5 minutes, the two solutions were combined and allowed to stand for an additional 20 minutes to form complexes. The complexes were then added to wells containing 1.8 mL of antibiotic-free medium, gently mixed, and incubated at 37°C for 6 hours before being replaced with complete medium containing 10% FBS. The knockdown efficiency of PAD4 was validated by western blot (Fig. S1b, c).

TNF- α neutralization in vitro

Cells were seeded at an appropriate density in multi-well plates and cultured in complete medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂. After overnight incubation to allow attachment, the medium was replaced, and cells were subjected to the following experimental conditions for 72 h: Normoxia, OGD/R, OGD/R + TNF- α neutralizing antibody, OGD/R + L-homocysteine, OGD/R + L-homocysteine + TNF- α neutralizing antibody, L-homocysteine. TNF- α neutralizing antibody (14-7423-81, Invitrogen, USA) was added at a final concentration of 10 ng/mL where indicated [25]. OGD/R was performed as previously described. The medium containing the antibody was refreshed every 24 h to maintain activity.

Immunofluorescence Staining

Following rehydration and PBS washing, tissue sections were permeabilized with 0.3% Triton X-100 in PBS for 30 min, blocked with 5% BSA (A8020, Solarbio, China) in permeabilization buffer for 2 h, and then incubated with primary antibody at 4 °C overnight. After thorough PBS washes, sections were incubated with fluorophore-conjugated secondary antibody for 1 h in the dark, washed again, and counterstained with DAPI. Finally, sections were mounted with antifade medium and imaged under a fluorescence or confocal microscope. Primary antibodies used in the study: mouse anti-CC1 (ab16794, Abcam, UK), rabbit anti-PDGFR α (3174S, CST, USA), rabbit anti-MBP (78896S, CST, USA), rabbit anti-PAD4 (ab96758, Abcam, UK), rabbit

anti-CitH3 (ab5103, Abcam, UK), rabbit anti-MyRF (OB-PRB007, Oasis biofarm, China), rabbit anti-H3 (17168-1-AP, Proteintech, China), mouse anti-GADPH (BM3876, Boster, China)^[26]. The antibodies used are listed in Table 1.

Western Blot

Protein samples were lysed on ice for 30 min using RIPA buffer supplemented with protease and phosphatase inhibitors, followed by centrifugation to collect the supernatant^[27]. Protein concentration was determined by the BCA assay, and equal amounts of protein (20–50 µg) were denatured in loading buffer at 95 °C for 10 min. Proteins were separated by SDS-PAGE using appropriate gel concentrations (e.g., 10% or 12%) under constant voltage (80 V in stacking gel, 120 V in separating gel) and transferred onto PVDF membranes at 200 mA for 90 min in an ice bath. After transfer, membranes were blocked with 5% non-fat milk in TBST for 2 h at room temperature, then incubated overnight at 4 °C with primary antibodies diluted in blocking buffer. Following three washes with TBST, membranes were probed with HRP-conjugated secondary antibodies for 1 h at room temperature. After extensive washing, protein bands were visualized using an ECL substrate and imaged with a chemiluminescence detection system. Band intensities were quantified using ImageJ software, and target protein expression levels were normalized to the corresponding loading control.

RNA isolation and quantitative real-time PCR (qRT-PCR)

The samples were lysed and homogenized with TRIzol (15596018CN, Invitrogen, USA), followed by the addition of chloroform for phase separation through shaking. After centrifugation, the upper aqueous phase was collected, and RNA was precipitated with isopropanol. The RNA pellet was then washed with ethanol, air-dried at room temperature, and finally dissolved in RNase-free water for long-term storage at -80°C. Subsequently, 1 µg of total RNA was used with ReverAid First Strand cDNA Synthesis Kit (K1622, Thermo Scientific, USA) for reverse transcription (42°C for 60 minutes, 70°C for 5 minutes) to obtain cDNA. Quantitative real-time PCR (qPCR) was performed according to the kit manufacturer's instructions (Q341-02, Vazyme, China) under the following cycling conditions: initial

denaturation at 95°C for 30 seconds, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. GAPDH was used as the internal reference gene, and the relative expression of target genes was calculated using the $2^{-\Delta\Delta C_t}$ method^[28]. The primers used are listed in Table 2.

Chromatin Immunoprecipitation (ChIP)-qPCR

Chromatin immunoprecipitation (ChIP) was performed using the ChIP assay kit (P2078, Beyotime, China) according to the manufacturer's instructions. Cells at 80–90 % confluence were cross-linked with 1% formaldehyde for 10 min at room temperature, quenched with 125 mM glycine for 5 min, washed with ice-cold PBS, and collected. Pellets were lysed, and chromatin was sheared by sonication to 200–700 bp fragments. Sheared chromatin (25–50 µg DNA) was precleared with protein G magnetic beads, then incubated overnight at 4°C with specific antibody or IgG control. Immune complexes were captured with protein G beads, washed sequentially (low-salt, high-salt, LiCl, TE buffers), eluted, reverse cross-linked overnight at 65°C with proteinase K, and DNA purified using columns. qPCR was performed in triplicate with SYBR Green on a QuantStudio system; enrichment was calculated using the $2^{-\Delta\Delta C_t}$ method. Data represent mean ± SEM. The primers used are listed in Table 2.

Statistical analysis

Experimental data are expressed as the mean±standard error of the mean (SEM). Two-way ANOVA with repeated measures was used for behavior results including neurological scores, Rotarod tests and the foot-fault-placing assessment. The factors were the treatment administered and testing time (days). One-way ANOVA was used to compare the difference between two groups on the same day. One-way or two-way analysis of variance (ANOVA) was used to assess the quantification of PCR, Western blot and immunofluorescence results. The threshold for statistical significance was set at $P<0.05$. GraphPad Prism 10.6.0 (GraphPad Software, USA) served as the primary tool for statistical computation and figure preparation, complemented by IBM SPSS Statistics 25 for analytical verification.

Results

HHcy hinders the recovery of neurological function after ischemic cerebral ischemia

HHcy is clinically associated with poor prognosis after ischemic stroke^[2,3], although supporting evidence from animal models has been lacking. To address this, C57BL/6J mice (6 weeks old) were pre-treated for two weeks with either normal drinking water (Vehicle group) or methionine-enriched water (HHcy group) to induce elevated plasma homocysteine (Hcy) levels. Following the pre-treatment, mice were subjected to the middle cerebral artery occlusion (MCAO) model to induce ischemic stroke. Neurological scores, behavioral tests, and cerebral infarction and atrophy were assessed at different stages of reperfusion. Subsequent results showed that the HHcy group, which confirmed a significant increase in plasma homocysteine (Hcy) levels in 8-week-old mice compared to the vehicle group, exhibited worse outcomes (Fig. 1a-e). Specifically, T2-weighted MRI revealed that HHcy mice had an increased cerebral infarction area on day 2 (Fig. 1a, c) and aggravated brain atrophy on day 28 post-MCAO (Fig. 1b, d). Supplementation with 2% methionine in drinking water resulted in significant elevations in plasma Hcy ($30.6360 \pm 2.6307 \mu\text{M}$ in methionine-treated group versus $9.10508 \pm 1.5114 \mu\text{M}$ in control group) (Fig. 1e). Furthermore, the HHcy group displayed significantly deteriorated sensorimotor impairments (Fig. 1f-h) and diminished memory and learning abilities (Fig. S2a-d) across different stages of reperfusion. These findings suggest that HHcy hinders the recovery of neural function following ischemic cerebral ischemia and support the clinical observation of an aggravated poor prognosis.

HHcy impedes the repair of white matter integrity and the differentiation of OPCs after cerebral ischemia

Cerebral ischemia frequently leads to significant white matter damage, which can account for up to half of the total infarct volume. In the present study, diffusion tensor imaging (DTI) was used to detect white matter injury and repair after cerebral ischemia, with fractional anisotropy (FA) revealing significant white matter damage in the external capsule (EC) and striatum (STR) on the ischemic(I) hemisphere compared to the non-ischemic hemisphere (NI), an effect further

exacerbated by elevated HHcy (Fig. 2a, b). Myelin basic protein (MBP) was assessed in three specific white matter brain regions: the cortex (CTX), EC, and STR on day 14 after MCAO. The MBP intensity and expression levels indicated that HHcy aggravated myelin sheath injury compared with the vehicle group in the I hemisphere after ischemia (Fig. 2c, d, g). CC1 (a marker of OLs) staining revealed that HHcy exhibited a more severe reduction in the number of CC1-positive cells 14 days after ischemia (Fig. 2e, f). Additionally, HHcy increased platelet-derived growth factor- α (PDGFR α , a marker of OPCs) expression compared with vehicle mice (Fig. 2g), suggesting that HHcy hindered OPCs differentiation. *In vitro*, OLN93 cells were treated with homocysteine followed by OGD/R. The results for PDGFR α and MBP revealed that HHcy significantly reduced MBP and PDGFR α levels under OGD/R, suggesting that HHcy impedes OLs formation (Fig. 2h). Taken together, these results indicate that HHcy compromises the repair of post-ischemic white matter integrity by hindering the differentiation of OPCs into OLs.

HHcy up-regulates the expression of PAD4 during convalescence from ischemic stroke

HHcy is known to upregulate the expression of protein arginine deiminase 4 (PAD4) in neutrophils [29]. In the current study, the expression of related differential genes was evaluated by comparing the RNA-seq expression profiles of brain tissues in the convalescent brain tissues of mice treated with HHcy, and the molecules associated with the proliferation and differentiation of OPCs (Olig2, PDGFR α , MBP, MyRF, ID2) were analyzed, and the results showed that HHcy treatment promoted the increase of PAD4 compared with the vehicle group (Fig. 3a). Immunofluorescence assessment of PAD4 levels in the CTX, EC, and STR brain regions on day 14 post-MCAO revealed that, compared to the vehicle group, HHcy promoted PAD4 expression in the OPCs in the ischemic hemisphere following ischemia (Fig. 3b, c). Western blots results confirmed that HHcy increased PAD4 expression in the ischemic hemisphere (Fig. 3d). Since PAD4 catalyzes the post-translational conversion of arginine residues to citrulline, we also examined the levels of CitH3, and our results showed that HHcy promoted increased expression of CitH3 in MCAO and OGD/R models (Fig. 3e, h). In addition, nuclear-cytoplasmic segregation and fluorescence staining of PAD4 indicated that HHcy

exacerbates the increase in PAD4 nuclear expression levels under the OGD/R model (Fig. 3f, g). *In vitro*, PAD4 knockdown effectively decreased HHcy-induced impairment of OPCs differentiation under OGD/R (Fig. 3i). Taken together, these results suggest that HHcy promotes PAD4 expression and its mediated histone citrullination, as well as PAD4 nuclear translocation. Importantly, PAD4 knockdown decreased the HHcy-induced impairment of OPCs differentiation.

PAD4 inhibitor YW3-56 improves neurological recovery, white matter integrity repair, and OPCs differentiation after acute cerebral ischemia

In the current study, the PAD4 inhibitor YW3-56 was used to pharmacologically inhibit PAD4 as previously described^[30]. Compared with the vehicle group, the YW3-56 group had a significant decrease in neurological scores (Fig. 4a). The results of the foot fault test and rotarod test showed that the motor function of mice in the YW3-56 group was significantly improved compared with the vehicle group (Fig. 4b-c). Water maze results revealed that YW3-56 improved learning and memory dysfunction after cerebral ischemia (Fig. S3a-d). Collectively, these results suggest that the PAD4 inhibitor YW3-56 can improve neurological recovery after cerebral ischemia.

Due to the close relationship between learning and memory ability and white matter integrity, in the current study, the effect of YW3-56 treatment on MBP expression in the CTX, EC, and STR brain regions was evaluated following 14 days of ischemia. Results showed that YW3-56 promoted increased MBP expression in the ischemic hemisphere compared with the vehicle group (Fig. 4d, f). CC1 immunofluorescence and PDGFR α expression results indicated that inhibition of PAD4 increased the number of CC1-positive cells (Fig. 4e) and reduced PDGFR α expression after cerebral ischemia (Fig. 4g), suggesting that inhibition of PAD4 can promote the OPCs differentiation. Additionally, the expression of citrullinated histones showed that inhibition of PAD4 reduced histone citrullination (Fig. 4h), indicating that inhibition of PAD4 and histone citrullination can promote white matter integrity and OPCs differentiation and suggesting that the PAD4/histone citrullination pathway negatively regulates white matter integrity and OPC differentiation following cerebral ischemia.

HHcy promotes PAD4 nucleation and decreases MyRF expression by up-regulating TNF- α during the recovery period of cerebral ischemia

In the current study, we investigated the mechanism by which HHcy may impair white matter repair following cerebral ischemia, focusing on the role of TNF- α and PAD4. Previous research indicated that TNF- α treatment causes PAD4 to move from the cytoplasm to the nucleus in OPCs^[17] and that HHcy increases TNF- α levels in the mouse aorta^[31]. We hypothesized that HHcy promotes PAD4 nuclear expression and translocation via TNF- α during the cerebral ischemia recovery phase. Consistent with this, in the current study, both *in vitro* and *in vivo* experiments confirmed an increase in TNF- α expression following HHcy treatment (Fig. 5a-b). Blockade of TNF- α with a specific neutralizing antibody prevents HHcy-induced PAD4 nuclear translocation, confirming that TNF- α is required for HHcy-driven PAD4 activation (Fig. 5c-e). In addition, TNF- α neutralizing antibody treatment also increased OPCs differentiation under HHcy (Fig. 5f-h). Given that nuclear PAD4 can elevate citH3, which alters nuclear transcription, we examined the expression of genes related to OPC differentiation. ChIP-qPCR assays using an anti-CitH3 antibody demonstrate that HHcy increased CitH3 enrichment at the *MyRF* enhancer under OGD/R (Fig. 6a, b). In addition, our results demonstrated a significant decrease in the expression of the myelin regulator *MyRF* in HHcy-treated cells and ischemic mouse brains (Fig. 6c-h). Furthermore, inhibiting PAD4 rescued *MyRF* expression after cerebral ischemia (Fig. 6i-k). Those suggesting that HHcy impairs OPCs differentiation and subsequent white matter repair by hindering *MyRF* transcription through PAD4-mediated citH3 elevation.

Discussion

HHcy predicts a poor prognosis after ischemic stroke [2, 3], and white matter repair is important for neural functional recovery[7]. HHcy may impair the functional recovery through disrupting the white matter repair. Therefore, understanding the mechanism underlying HHcy disrupting white matter repair and developing strategies to promote OPCs differentiation after ischemic stroke are crucial. Using a mouse MCAO and a cellular OGD model, we investigated the impact and mechanism of HHcy on OPCs differentiation and white matter repair, as well as the effect of PAD4 inhibitor YW-356. Our key findings are: 1) HHcy impairs OPCs differentiation, white matter integrity, and hinders functional recovery. 2) HHcy promotes PAD4 nuclear translocation and nucleosome histone hypercitrullination by upregulating TNF- α , subsequently downregulating MyRF and thus inhibiting OPCs differentiation. 3) Inhibition of PAD4 with YW-356 promotes OPCs differentiation and white matter repair after ischemic stroke. In conclusion, our results demonstrate that HHcy induced impairment of white matter repair by PAD4-mediated CitH3 signal pathway after ischemic stroke (Fig. 7).

HHcy is a significant and common risk factor for ischemic stroke^[1], and clinical studies consistently link it to poor prognosis and increased mortality following the event^[2, 3]. For instance, patients with HHcy have been shown to face a 3.6-fold increased risk of death during hospitalization and a 1.58-fold increased risk of disability and death at discharge^[32]. While clinical evidence strongly associates HHcy with disrupted white matter integrity^[5, 6], the specific mechanisms by which it hinders neural recovery have remained elusive. Since the differentiation of OPCs into OLs is the cornerstone of white matter repair^{[7] [33]}, identifying the pathways that hinder this process is clinically paramount. In the current study, we provide the first experimental evidence that HHcy directly impairs neural functional recovery by disrupting the TNF- α /PAD4/CitH3 signaling axis, which subsequently suppresses the essential transcription factor MyRF and halts OPC differentiation.

The transition of OPCs to mature OLs is tightly governed by transcriptional regulation, with MyRF serving as a master regulator of myelin structural protein expression^[11, 12]. Our RNA

sequencing analysis revealed that HHcy significantly downregulates genes associated with this maturation process, specifically targeting *MyRF*. To identify the upstream drivers of this suppression, we investigated chromatin modifications, which dictate gene accessibility during early differentiation^[13]. We focused on PAD4-mediated CitH3, a modification known to alter chromatin structure^[14]. While HHcy was previously known to upregulate PAD4 in neutrophils^[29], our *in vivo* and *in vitro* data confirm that HHcy similarly induces PAD4 expression and nuclear translocation within OPCs during the stroke recovery period. This elevation in CitH3, already linked to white matter hyperintensities in clinical cohorts^[18], represents a pathological shift in the epigenetic landscape that actively suppresses the myelination program.

The mechanism driving this nuclear shift appears to be mediated by TNF- α . Aligning with prior research showing that HHcy elevates inflammatory cytokines^[31], our data suggests that HHcy-induced TNF- α promotes the translocation of PAD4 from the cytoplasm to the nucleus. This finding is critical because it highlights a functional divergence between PAD isoforms: while cytosolic PAD2 supports physiological myelination during development ^[34], the nuclear translocation of PAD4 under inflammatory stress leads to hypercitrullination at the enhancers of key genes such as *MyRF*, effectively "locking" the OPCs in an immature state^[17].

We then tested the therapeutic potential of targeting this pathway using the pharmacological inhibitor YW-356. While PAD4 inhibition has previously been studied in spinal cord injury ^[35], its role in post-stroke OPC differentiation was unexplored. Our findings demonstrate that YW-356 treatment successfully rescues *MyRF* expression, promotes OPC differentiation, enhances white matter repair, and improves functional recovery. While YW3-56 is a potent PAD4 inhibitor, it also exhibits activity against PAD2 ^[20]. In the central nervous system, PAD2-mediated citrullination is essential for normal oligodendrocyte differentiation and myelination during development ^[34]. However, in the context of multiple sclerosis, both PAD2 and PAD4 are pathologically overexpressed, leading to an aberrant increase in citrullinated proteins^[36]. Because both isoforms contribute to the disease phenotype, any off-

target inhibition of PAD2 by YW3-56 would likely complement the inhibition of PAD4, further reducing the levels of citrullinated substrates such as CitH3. Therefore, the potential dual-inhibitory nature of YW3-56 does not compromise our data interpretation; rather, it reinforces the conclusion that reducing total PAD activity is beneficial in this model.

To understand the upstream mechanism of *MyRF* downregulation, we investigated chromatin structure modification, specifically histone citrullination, which plays a pivotal role in regulating transcription during early OPC differentiation [13, 14]. Our *in vivo* and *in vitro* experiments confirmed that HHcy upregulates both PAD4 expression and CitH3 levels in OPCs during stroke recovery. Critically, PADs exhibit isoform-specific roles in this process. While previous studies have shown that physiological PAD2 activity, predominantly cytosolic, promotes oligodendrocyte differentiation and myelination during development through MBP citrullination [34], our study reveals an inhibitory role for PAD4 under pathological conditions. Unlike PAD2, PAD4 uniquely undergoes nuclear translocation, allowing it to drive excessive CitH3 at the enhancers of key myelination genes such as *MyRF*, thereby suppressing the transition from OPCs to mature OLs.

The current study bridges a significant gap by elucidating the trigger for this pathological shift: our data, consistent with previous reports [17][31], suggests that HHcy-induced TNF- α serves as the primary trigger for PAD4 nuclear translocation. We confirmed that HHcy treatment increases TNF- α expression in the brain during stroke recovery, likely by increasing oxidative stress and ROS levels [37], NF- κ B [38], or hydrogen sulfide [23] via activating resident microglia and astrocytes [39] or infiltrating macrophages [23, 37] and monocytes [40]. This elevation in TNF- α promotes the movement of PAD4 from the cytoplasm to the nucleus, leading to increased nucleosomal CitH3 and the subsequent blockade of OL maturation. This mechanism aligns with findings in other demyelinating conditions where TNF- α -induced translocation leads to impaired remyelination [17].

The regional differences in HHcy-exacerbated white matter injury, most prominent in the EC, STR, and CTX, likely reflect a combination of intrinsic cellular and vascular factors [41].

The EC and STR are particularly vulnerable due to their dense population of myelinated fibers, high metabolic demands, and relatively poor collateral circulation^[42]. This makes them more susceptible to the synergistic detrimental effects of ischemia and HHcy, leading to pronounced reductions in fractional anisotropy. In contrast, the CTX may benefit from better vascular perfusion, which partially mitigates the severity of white matter disruption. Ultimately, regional heterogeneity in pathological manifestations is driven by local variations in OPC density, collateral flow, and inflammatory microenvironments.

While this study elucidates how HHcy exacerbates white matter injury, several limitations regarding clinical translation remain. Unlike the uniform HHcy levels in our animal model, clinical patients display significant inter-individual heterogeneity and often present with confounding comorbidities—such as hypertension and diabetes—that may independently modulate pathology ^[43] ^[44]. Additionally, as we exclusively used young male mice, future studies must consider how sex and age influence injury severity and therapeutic efficacy^[45]. Finally, while the OLN-93 cell line provides a stable and reproducible model, as an immortalized line it may not fully recapitulate the maturation potential or signaling pathways of primary OPCs ^[46]. Because these *in vitro* results reflect a simplified system, caution is necessary when extrapolating them to *in vivo* differentiation; future research should incorporate primary OPC cultures to further validate these findings.

In summary, we demonstrate that HHcy impairs white matter repair by activating a novel TNF- α /PAD4-mediated CthH3 signaling pathway after ischemic stroke. We provide a mechanistic link between metabolic dysregulation and impaired endogenous brain repair. Furthermore, pharmacological inhibition of PAD4 can promote OPC maturation and improve functional outcomes suggest that PAD4 represents a high-value therapeutic target. Targeting this pathway offers a promising strategy to promote white matter remodeling and enhance the prognosis for stroke patients with comorbid hyperhomocysteine.

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Author contributions: X.J. conceived the concept, designed the research, interpreted data, and wrote the manuscript. C.G. wrote the manuscript. Y.W. designed the YW3-56 research and revised the manuscript, W.D., H G, Y. W., X.H., J.Z., P.G., conducted the research and/or analyzed data.

Declaration of Interest

X.J., P.G. W.D., Y.W. and are inventors on a patent application related to this work filed to the National Intellectual Property Administration, the People's Republic of China (no. 202610013444.1; filed 7 Jan 2026). The other authors declare that they have no competing interests.

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Figure Legends

Figure 1. Hyperhomocysteine (HHcy) exacerbates brain injury and sensorimotor dysfunction after middle cerebral artery occlusion (MCAO). **a** Representative T2-weighted imaging-magnetic resonance imaging (MRI) scan (the infarcted area is outlined in red) on day 2 after MCAO. X-axis numbers 1-5 correspond to five representative images from rostral to caudal. **b** Representative T2-weighted imaging-MRI scans on day 28 after MCAO. X-axis numbers 1-5 correspond to five representative images from rostral to caudal. **c** Quantitative analysis of cerebral infarction indicated that HHcy aggravated the infarct area on day 2 after MCAO (** $P < 0.01$ vs. Vehicle group, $n = 4$). **d** Quantitative analysis of cerebral atrophy indicated that HHcy aggravated the atrophy area on day 28 after MCAO (* $P < 0.05$, ** $P < 0.01$ vs. Vehicle group, $n = 4$). **e** Measurement of plasma homocysteine (Hcy) levels in 8-week-old mice revealed a significant increase in the HHcy group compared to the vehicle group (** $P < 0.01$ vs. Vehicle group, $n = 6$). **f-h** Neurological scores (**f**), corner turn test (**g**), and foot fault test (**h**) demonstrated that the HHcy group exhibited worse sensorimotor dysfunction at different time points after ischemia and reperfusion (* $P < 0.05$, ** $P < 0.01$ vs. Vehicle group, $n = 5 \sim 6$). Data are expressed as mean $\pm$ SEM.

Figure 2. HHcy impedes oligodendrocyte precursor cells (OPCs) differentiation and white matter integrity repair after cerebral ischemia. **a-b.** Representative images of diffusion tensor imaging (DTI)-magnetic resonance imaging (MRI) scans (**a**) and fractional anisotropy (FA) measurements in the external capsule (EC) and striatum (STR) on day 7 after MCAO (**b**) indicated that HHcy exhibited significantly reduced FA values in several scanning planes compared with vehicle group (* $P < 0.05$ vs. Vehicle group, $n = 4 \sim 5$). **c-d** Representative images (**c**) and quantitative analysis (**d**) showed that HHcy significantly decreases myelin basic protein (MBP) expression in the ischemic hemisphere (I) on day 14 after ischemia (* $P < 0.05$ vs. NI of the Vehicle group, # $P < 0.05$ vs. I of the Vehicle group, $n = 3$). **e-f** Representative immunofluorescence images (**e**) and quantitative analysis (**f**) of the oligodendrocytes (OLs) marker CC1 demonstrated that HHcy impede the differentiation of OPCs into OLs on day 14

after MCAO (* P <0.05 vs. NI of the Vehicle group, # P <0.05 vs. I of the Vehicle group, n =3).

g Representative western blot images and quantitative analysis demonstrated that HHcy significantly decreases MBP and increases platelet-derived growth factor- α (PDGFR α) expression in the I hemisphere compared with the vehicle group on day 14 after MCAO (* P <0.05 vs. NI of the Vehicle group, # P <0.05 vs. I of the Vehicle group, n =5~6. NI, non-ischemic hemisphere; I, ischemic hemisphere. **h** Representative western blot images and quantitative analysis indicated that HHcy exacerbates the decline of MBP and PDGFR α under oxygen-glucose deprivation/reoxygenation (OGD/R) (* P <0.05, ** P <0.01 vs. Normoxia, n =3~4). Data are expressed as mean $\pm$ SEM.

Figure 3. HHcy orchestrates the dysregulation of the protein arginine deiminase 4 (PAD4)/citrullinated histone 3 (CitH3) axis after cerebral ischemia. **a** Heatmap of RNA array profiles showing differentially expressed RNAs (>1.4-fold change, FDR<0.15) in the ischemic brain of HHcy compared with the Vehicle group at 7 days post-ischemia (Red and green represent significant upregulation and downregulation, n =3/group). **b-c** Representative immunofluorescence images (b) and quantitative analysis (c) demonstrated that PAD4 in the OPCs in the ischemic hemisphere (I) cortex (CTX), external capsule (EC), and striatum (STR) was significantly increased in the HHcy group compared to the vehicle (* P <0.05 vs. NI of the Vehicle group, # P <0.05 vs. I of the Vehicle group, n =3). **d** Representative western blot and quantitative analysis demonstrated that HHcy significantly increases PAD4 expression in the I hemisphere compared with the vehicle on day 14 after MCAO (* P <0.05 vs. NI of the Vehicle group, # P <0.05 vs. I of the Vehicle group, n =5). **e** Representative western blot and quantitative analysis demonstrated that HHcy significantly increases CitH3 expression in the I hemisphere compared with the vehicle on day 14 after MCAO (* P <0.05 vs. NI of the Vehicle group, # P <0.05 vs. I of the Vehicle group, n =5). **f**. Representative western blot indicated that HHcy increases the nuclear expression of PAD4 in oligodendrocyte lineage under OGD/R. **g** Representative immunofluorescence images indicated that HHcy increases the nuclear expression of PAD4 under OGD/R. **h** Representative western blot images indicated that HHcy increases CitH3 expression under OGD/R (* P <0.05 vs. normoxia, # P <0.05 vs. OGD/R, n =3).

i Representative western blot images and quantitative analysis indicated that PAD4 knockdown effectively rescued HHcy-induced decline of MBP and PDGFR α under OGD/R ($*P<0.05$ vs. Normoxia, $\#P<0.05$ vs. OGD/R+HHcy, $n=3$). Data are expressed as mean $\pm$ SEM.

Figure 4. Protein arginine deiminase 4 (PAD4) inhibitor YW3-56 enhances sensorimotor and cognitive recovery, white matter integrity repair, and OPCs differentiation after cerebral ischemia. **a-c** Neurological scores (**a**), foot fault (**b**), and rotarod test (**c**) demonstrated that YW3-56 treatment significantly promotes the recovery of sensorimotor function on days 3, 7, and 14 post-ischemia ($*P<0.05$, $**P<0.01$ vs. Vehicle group, $n=8\sim10$). **d** Representative images and quantitative analysis of MBP intensity showed that YW3-56 significantly increases MBP expression in the ischemic hemisphere (I) on day 14 after ischemia ($*P<0.05$ vs. NI of the Vehicle group, $\#P<0.05$ vs. I of the Vehicle group, $n=3$). **e** Representative immunofluorescence images of the oligodendrocytes (OLs) marker CC1 demonstrated that YW3-56 promotes OPCs differentiation on day 14 after MCAO. **f-h** Representative Western blot images and quantitative analysis of MBP, PDGFR α , and CitH3 demonstrated that YW3-56 significantly increases MBP, decreases PDGFR α and CitH3 expression in the I hemisphere compared with the vehicle group on day 14 after ischemia ($*P<0.05$ vs. NI of the Vehicle group, $\#P<0.05$ vs. I of the Vehicle group, $n=4\sim5$). Data are expressed as mean $\pm$ SEM. NI, non-ischemic hemisphere; I, ischemic hemisphere; cortex, CTX; external capsule, EC; striatum, STR).

Figure 5. HHcy inhibits OPCs differentiation via tumor necrosis factor- α (TNF- α), driving PAD4 nuclear translocation. **a-b** HHcy significantly increased TNF- α mRNA levels in the ischemic hemisphere compared to the vehicle after cerebral ischemia, and HHcy significantly increased TNF- α expression under OGD/R ($*P<0.05$ vs. NI of the Vehicle group, $\#\#P<0.01$ vs. I of the Vehicle group, $n=3$). **c** Schematic illustration of the cell treatment involving TNF- α neutralizing antibody. **d-e** Representative immunofluorescence images and western blot images indicated that TNF- α neutralizing antibody attenuated the HHcy-induced nuclear expression of PAD4 under OGD/R. **f-h** Representative western blot images and quantitative analysis indicated that TNF- α neutralizing antibody effectively rescued HHcy-

induced the decline of MBP and PDGFR α under OGD/R (* P < 0.05, * P < 0.01 vs. Normoxia, n =3). Data are expressed as mean $\pm$ SEM.

Figure 6. HHcy inhibits *myelin regulator* (*MyRF*) via citrullinated histone 3 (CitH3) to impede post-ischemic repair. **a** Schematic illustration of CitH3 recruitment to the enhancer region of *MyRF*. **b** ChIP-qPCR analysis demonstrated that HHcy enhanced the recruitment of CitH3 to the enhancer region of *MyRF*, inhibiting *MyRF* transcription under OGD/R (** P < 0.01 vs. Normoxia, ## P < 0.01 vs. OGD/R, n =3). **c** HHcy significantly decreased *MYRF* mRNA levels under OGD/R (** P < 0.01 vs. normoxia, # P < 0.05 vs. OGD/R, n =3). **d-e** Representative images and quantitative analysis of *MyRF* intensity (**d**) and *MyRF* expression (**e**) showed that HHcy significantly decreases *MyRF* level under OGD/R (** P < 0.01 vs. normoxia, # P < 0.05 vs. OGD/R, n =3). **f** HHcy significantly decreased *MYRF* mRNA in the ischemic hemisphere compared to the vehicle group after cerebral ischemia (** P < 0.01 vs. NI of the Vehicle group, n =3). Representative images and quantitative analysis of *MyRF* intensity (**g**) and *MyRF* expression (**h**) showed that HHcy significantly decreases *MyRF* expression in the ischemic hemisphere on day 14 after ischemia (* P < 0.05 vs. NI of the Vehicle group, n =3). **i** YW3-56 significantly increased *MYRF* mRNA levels in the ischemic hemisphere compared to the vehicle group after cerebral ischemia. **j-k** Representative images and quantitative analysis of *MyRF* intensity (**j**) and *MyRF* expression (**k**) showed that YW3-56 significantly increases *MyRF* expression in the I hemisphere compared with the vehicle group on day 14 after MCAO (* P < 0.05 vs NI of the Vehicle, # P < 0.05 vs. I of the Vehicle, n =3~5, NI, non-ischemic hemisphere; I, ischemic hemisphere). Data are expressed as mean $\pm$ SEM.

Figure 7. Summary

Hyperhomocysteine (HHcy) increases tumor necrosis factor- α (TNF- α), which promotes the nuclear translocation of protein arginine deiminase 4 (PAD4) in oligodendrocyte precursor cells (OPCs), resulting in increases of citrullinated histone 3 (CitH3), which further decreases the transcription of the transcription factor *myelin regulator* (*MyRF*), thereby inhibiting the differentiation of OPCs into oligodendrocytes (OLs), hindering the repair of white matter integrity and neural functional recovery.

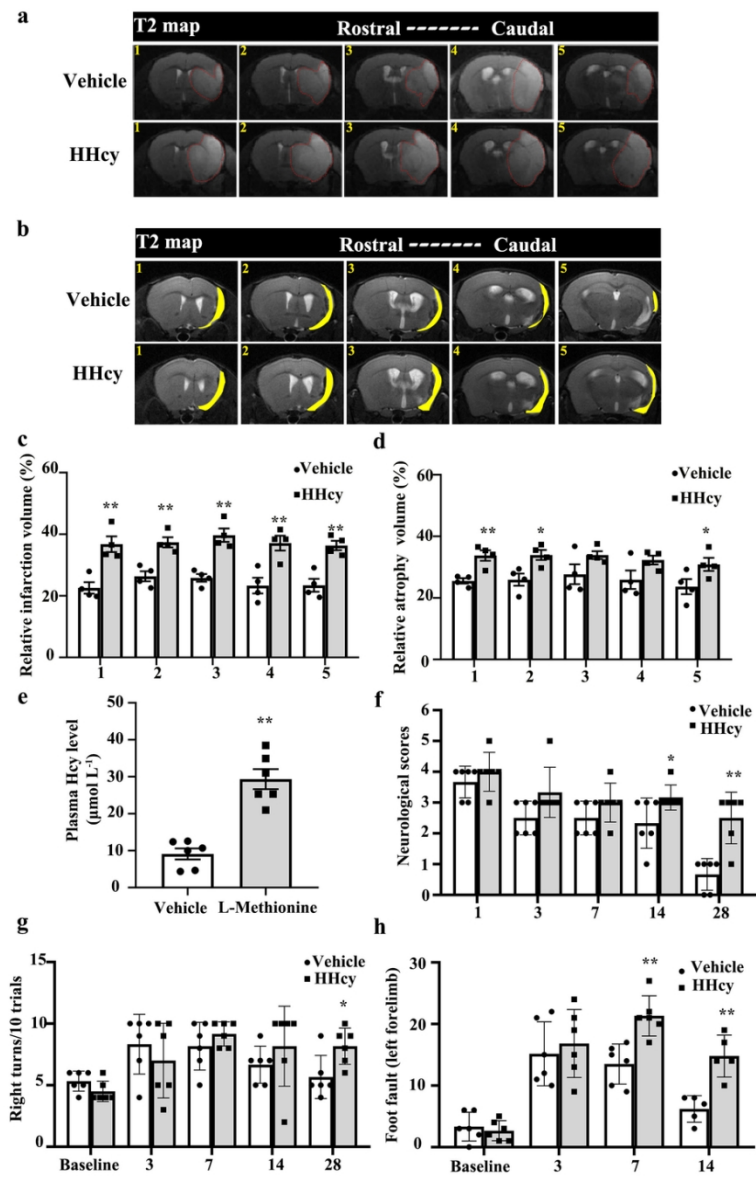


Figure 1

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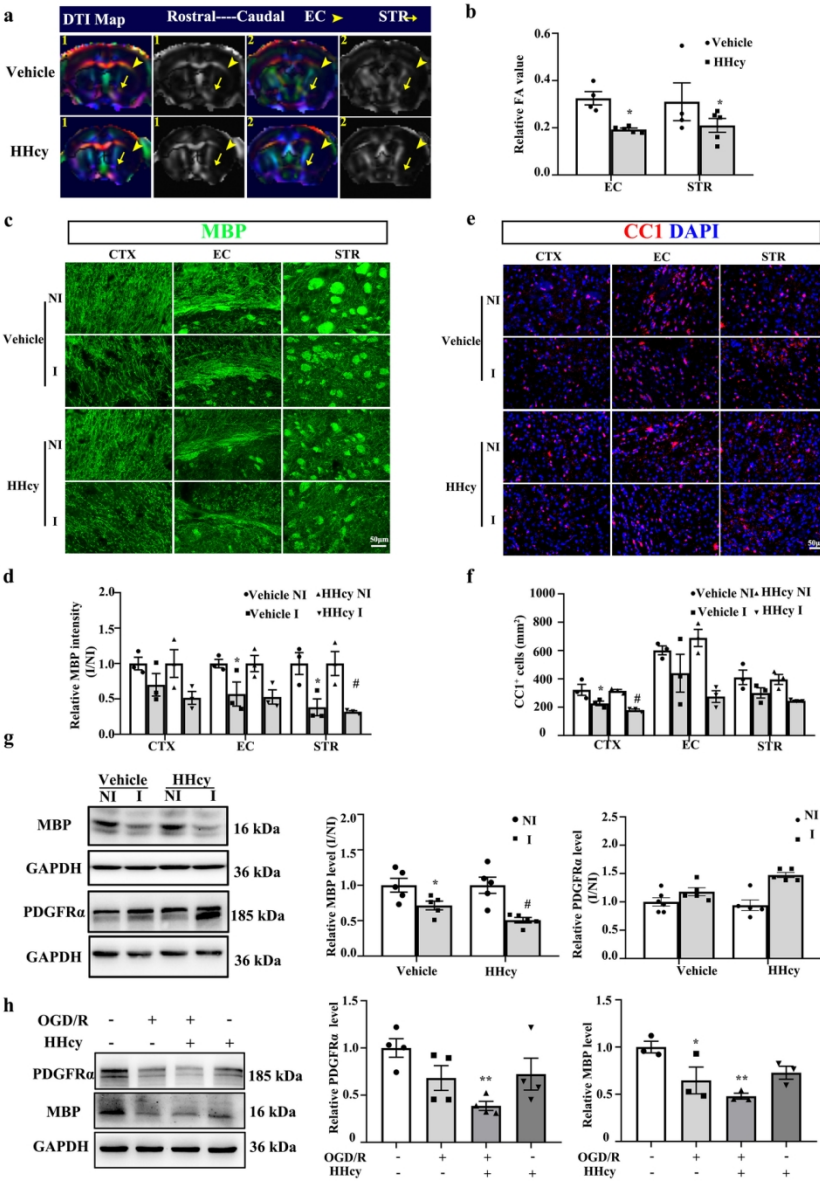


Figure 2

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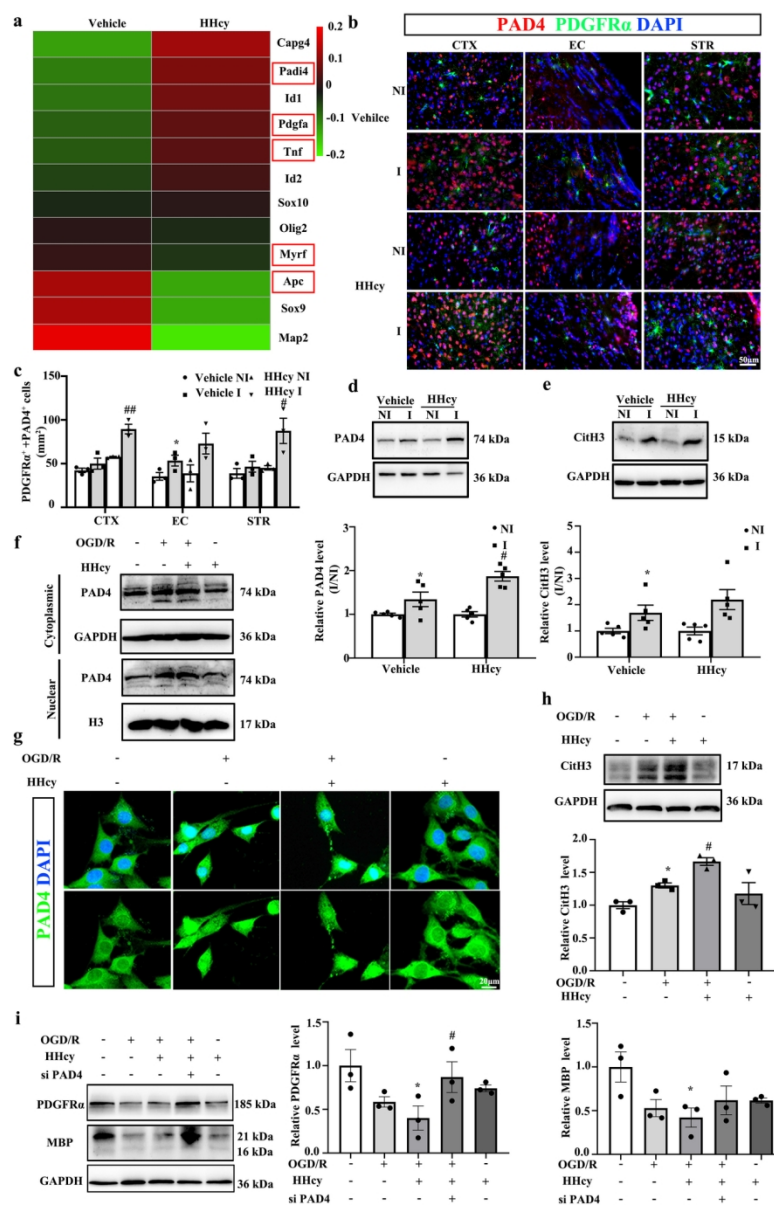


Fig.3

114x176mm (300 x 300 DPI)

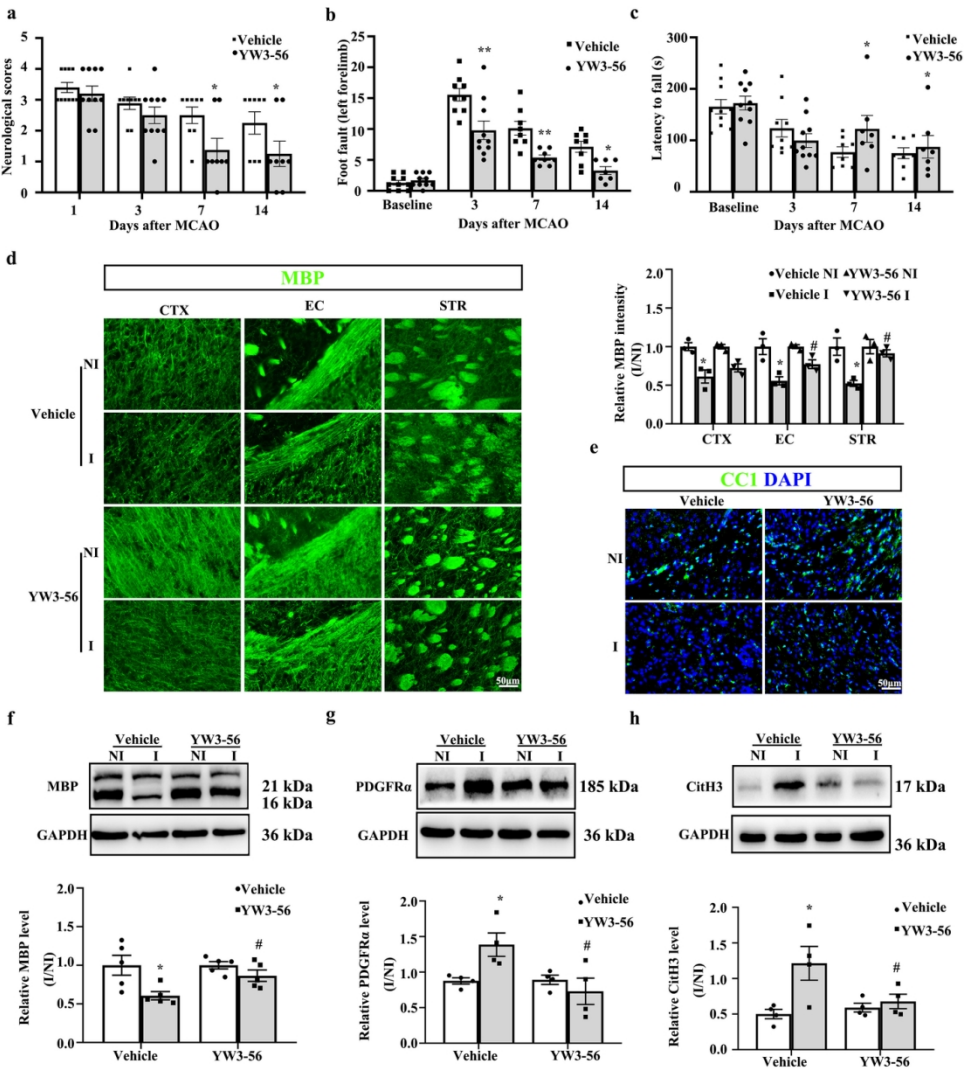


Figure 4

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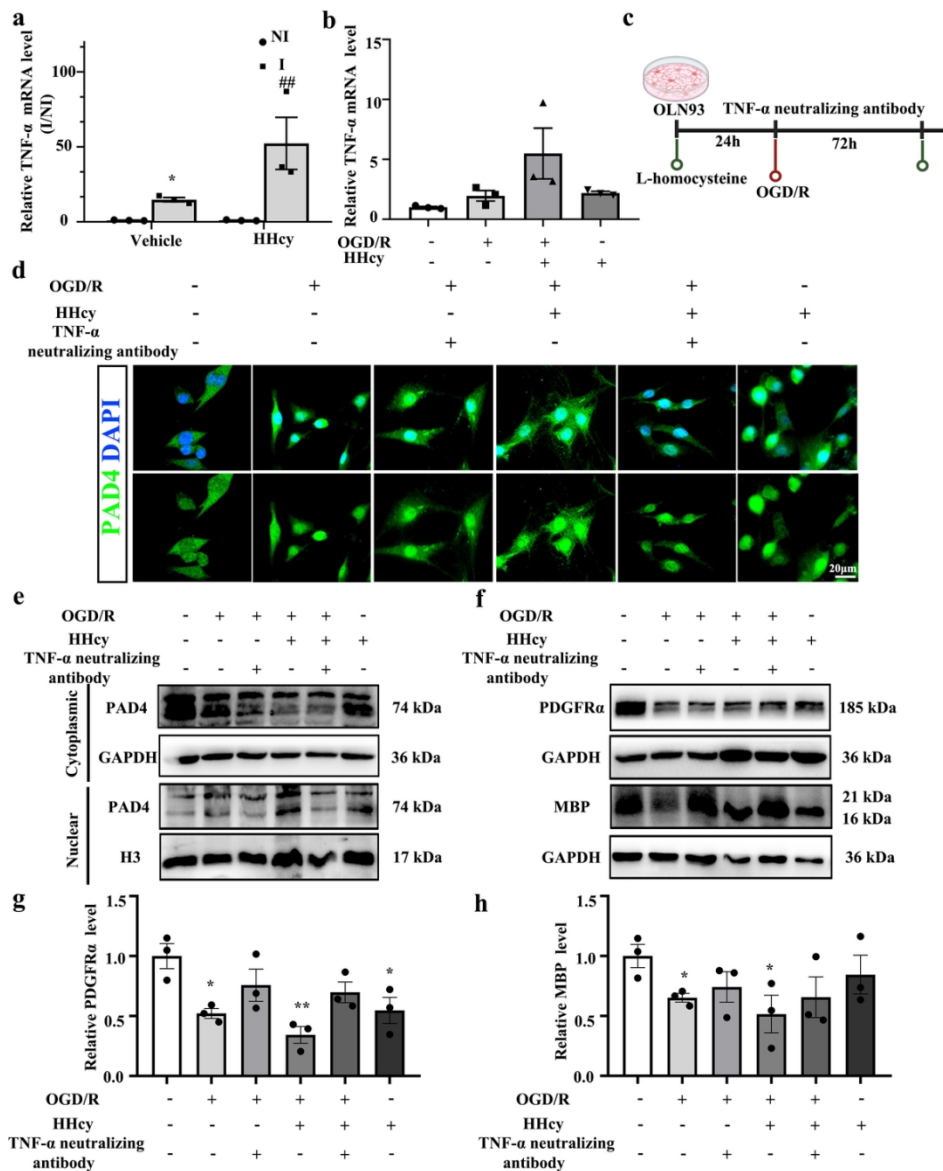
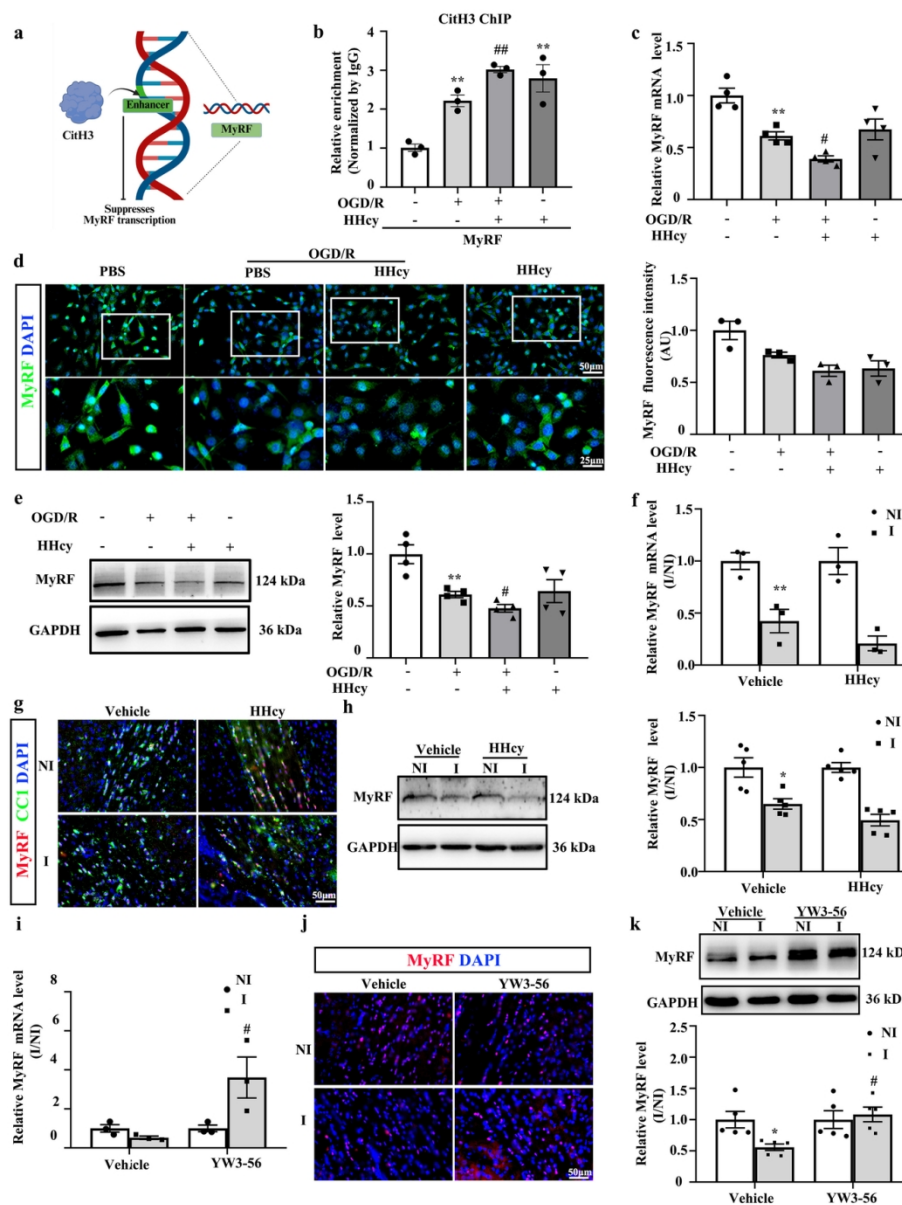


Figure 5

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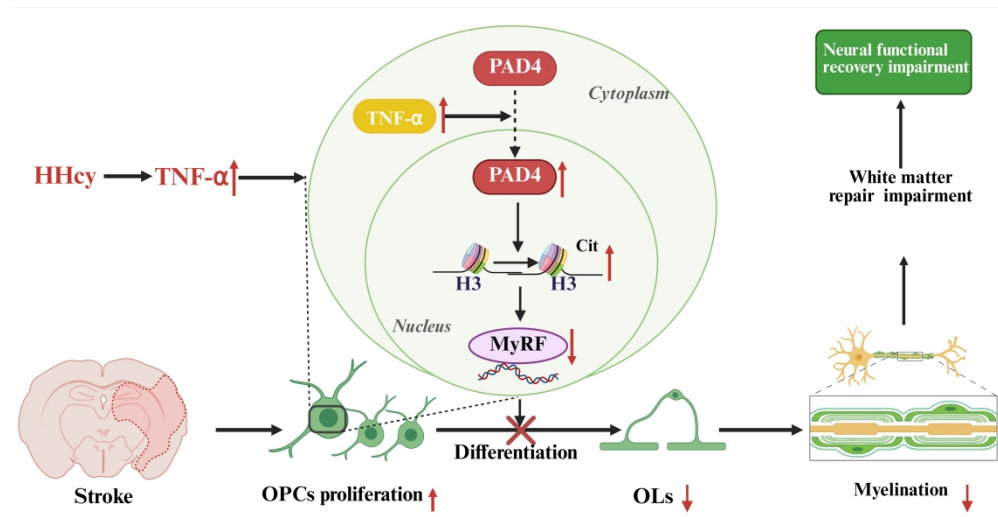


Figure 7

345x176mm (300 x 300 DPI)

Table 1. Antibodies for western blot , immunofluoresence and neutralization

Antibodies	Source	Identifier	Dilution
mouse anti-CC1	Abcam, Cambridge, UK	Cat#ab16794	1:100
rabbit anti-PDGFR α	CST, Danvers, USA	Cat#3174S	1:200
rabbit anti-MBP	CST, Danvers, USA	Cat#78896S	1:1000
rabbit anti-PAD4	Abcam, Cambridge, UK	Cat#ab96758	1:200
rabbit anti-CitH3	Abcam, Cambridge, UK	Cat#ab5103	1:500
rabbit anti-MyRF	Oasis biofarm, Hangzhou, China	Cat#OB-PRB007	1:1000
rabbit anti-H3	Proteintech, Wuhan, China	Cat#17168-1-AP	1:1000
mouse anti-GAPDH	Boster,Wuhan, China	Cat#BM3876	1:4000
TNF α Monoclonal Antibody (TN3-19.12)	Invitrogen, Carlsbad , USA	Cat#14-7423-81	
Alexa Fluor 488 goat anti-Mouse IgG	Invitrogen, Carlsbad , USA	Cat#A-11001	1:800
-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, C	Invitrogen, Carlsbad , USA	Cat#A-10521	1:800
Alexa Fluor 488 goat anti-Rabbit IgG	Invitrogen, Carlsbad , USA	Cat#A-11008	1:800
i-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, C	Invitrogen, Carlsbad , USA	Cat#A-10520	1:800
HRP-AffiniPure Goat Anti-Mouse IgG	Boster,Wuhan, China	Cat#BM3895	1:4000
HRP-AffiniPure Goat Anti-Rabbit IgG	Boster,Wuhan, China	Cat#BM3894	1:4000

Table 2. Primers for qRT-PCR.

Gene	Direction	Sequence (5'-3')
Mouse-TNF- α	Forward	CATCTTCTCAAAATTCGAGTGACAA
	Reverse	GGGTTGTACCTTGTCTACTCCCA
Mouse-MYRF	Forward	ATGGAGGTGGTGGACGAGA
	Reverse	GGGAAGCAGAGATCAGAGGC
Mouse-GAPDH	Forward	TTCACCACCATGGAGAAGGC
	Reverse	GGCATGGACTGTGGTCATGA
Rat-TNF- α	Forward	CACCATGAGCACGGAAAGCA
	Reverse	GCAATGACTCCAAAGTAGACC
Rat-MYRF	Forward	CTTCTTCGAAGGCCACGACA
	Reverse	GGTGCAGAGATCTCAGGGAAG
Rat-MYRF-enhancer	Forward	TCTCTGCTCTCGCCCACTTA
	Reverse	CAGGGCACAGGTCTTGTCAT
Rat-GAPDH	Forward	AAGCCCATCACCATCTTCCA
	Reverse	ATGGCATGGACTGTGGTCAT

Table 1. Antibodies for western blot , immunofluorescence

Antibodies	Source
mouse anti-CC1	Abcam, Cambridge, UK
rabbit anti-PDGFR α	CST, Danvers, USA
rabbit anti-MBP	CST, Danvers, USA
rabbit anti-PAD4	Abcam, Cambridge, UK
rabbit anti-CitH3	Abcam, Cambridge, UK
rabbit anti-MyRF	Oasis biofarm, Hangzhou, China
rabbit anti-H3	Proteintech, Wuhan, China
mouse anti-GAPDH	Boster, Wuhan, China
TNF α Monoclonal Antibody (TN3-19.12)	Invitrogen, Carlsbad , USA
Alexa Fluor 488 goat anti-Mouse IgG	Invitrogen, Carlsbad , USA
Anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, C	Invitrogen, Carlsbad , USA
Alexa Fluor 488 goat anti-Rabbit IgG	Invitrogen, Carlsbad , USA
Anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, C	Invitrogen, Carlsbad , USA
HRP-AffiniPure Goat Anti-Mouse IgG	Boster, Wuhan, China
HRP-AffiniPure Goat Anti-Rabbit IgG	Boster, Wuhan, China

e and neutralization

Identifier	Dilution
Cat#ab16794	1:100
Cat#3174S	1:200
Cat#78896S	1:1000
Cat#ab96758	1:200
Cat#ab5103	1:500
Cat#OB-PRB007	1:1000
Cat#17168-1-AP	1:1000
Cat#BM3876	1:4000
Cat#14-7423-81	
Cat#A-11001	1:800
Cat#A-10521	1:800
Cat#A-11008	1:800
Cat#A-10520	1:800
Cat#BM3895	1:4000
Cat#BM3894	1:4000

Table 2. Primers for qRT-PCR.

Gene	Direction	Sequence (5'-3')
Mouse-TNF- α	Forward	CATCTTCTCAAAATTCGAGTGACAA
	Reverse	GGGTTGTACCTTGTCTACTCCCA
Mouse-MYRF	Forward	ATGGAGGTGGTGGACGAGA
	Reverse	GGGAAGCAGAGATCAGAGGC
Mouse-GAPDH	Forward	TTCACCACCATGGAGAAGGC
	Reverse	GGCATGGACTGTGGTCATGA
Rat-TNF- α	Forward	CACCATGAGCACGGAAAGCA
	Reverse	GCAATGACTCCAAAGTAGACC
Rat-MYRF	Forward	CTTCTTCGAAGGCCACGACA
	Reverse	GGTGCAGAGATCTCAGGGAAG
Rat-MYRF-enhancer	Forward	TCTCTGCTCTCGCCCACTTA
	Reverse	CAGGGCACAGGTCTTGTCAT
Rat-GAPDH	Forward	AAGCCCATCACCATCTTCCA
	Reverse	ATGGCATGGACTGTGGTCAT