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Tamoxifen promotes white matter recovery and cognitive functions in male mice after chronic hypoperfusion

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ABSTRACT

Cerebral white matter lesions (WMLs) induced by chronic cerebral hypoperfusion are one of the major components of stroke pathology and closely associated with cognitive impairment. However, the repair and related pathophysiology of white matter after brain injury remains relatively elusive and underexplored. Successful neuroregeneration is a method for the potential treatment of central nervous system (CNS) disorders. A non-steroidal estrogen receptor modulator, Tamoxifen, is an effective inhibitor of cell-swelling-activated anion channels and can mimic neuroprotective effects of estrogen in experimental ischemic stroke. However, its remains unclear whether Tamoxifen has beneficial effects in the pathological process after WMLs. In the present study, we investigated the efficacy of Tamoxifen on multiple elements of oligovascular niche of the male C57BL/6 mice brain after bilateral carotid artery stenosis (BCAS) - induced WMLs. Tamoxifen was injected intraperitoneally once daily from 1 day after BCAS until 1 day before sacrificed. Following chronic hypoperfusion, BCAS mice presented white matter demyelination, loss of axon-glia integrity, activated inflammatory response, and cognitive impairments. Tamoxifen treatment significantly facilitated functional restoration of working memory impairment in mice after white matter injury, thus indicating a translational potential for this estrogen receptor modulator given its clinical safety and applicability for WMLs, which lack of currently available treatments. Furthermore, Tamoxifen treatment reduced microglia activation and inflammatory response, favored microglial polarization toward to the M2 phenotype, enhanced oligodendrocyte precursor cells proliferation and differentiation, and promoted remyelination after chronic hypoperfusion. Together, our data indicate that Tamoxifen could alleviate white matter injury and play multiple targets protective effects following chronic hypoperfusion, which is a promising candidate for the therapeutic target for ischemic WMLs and other demyelination diseases associated cognitive impairment.

1. Introduction

The term vascular cognitive impairment (VCI) includes the spectrum of all cognitive disorders from mild cognitive impairment to dementia, and ischemic white matter lesions (WMLs) are one of the major causes (Garde et al., 2000). WMLs are a devastating and complex clinical condition of human ischemic stroke (Ercan et al., 2016; Miyamoto et al., 2015). A cascade of molecular and cellular changes, such as disruption of the blood-brain barrier (BBB), vascular dysfunction, metabolic failure, ionic dysfunction, inflammation, oxidative stress, demyelination, reactive gliosis and apoptosis caused by ischemic WMLs may generate a non-permissive environment for cell survival and axonal regeneration. Therefore, the complex set of events activated after WMLs require therapeutic approaches that target multiple elements of the damage cascade and produce a permissive environment for cellular survival and white matter regeneration.

The use of estrogen as a neuroprotective hormone is well known in the treatment of experimental neurological deficits, whereas the clinical

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use of exogenous estrogen is limited because of its peripheral feminizing and potentially dangerous proliferative effects (Guptarak et al., 2014). Tamoxifen is a selective non-steroidal estrogen receptor modulator and lack estrogen's systemic adverse effects, which has been found to display neuroprotective properties in the treatment of ischemic stroke, spinal cord injury and retinal photoreceptor degeneration (Wakade et al., 2008; Zhang et al., 2007; Zhang et al., 2005; Kimelberg et al., 2000; Kimelberg et al., 2003; Colon and Miranda, 2016; Tian et al., 2009). Tamoxifen exhibits multi-potential neuroprotective effects, including inhibiting excitatory amino acid release (Rutledge et al., 1998), decreasing formation of peroxynitrated proteins and nitric oxide synthase (NOS) (Osuka et al., 2001), circumventing inner membrane leakiness (Pavon et al., 2017), scavenging reactive oxygen species (ROS) (Wiseman et al., 1993), and stimulating an anti-inflammatory response (Suuronen et al., 2005; Tapia-Gonzalez et al., 2008). Meanwhile, Tamoxifen could regulate proliferation and maturation of oligodendrocyte progenitor cells (OPCs) and prevent delayed apoptotic death of oligodendrocytes (OLs) (Marin-Husstege et al., 2004). In addition, Tamoxifen has an ability to cross the BBB and accumulate in the brain, which could further facilitate its prominent neuroprotective effects after injury (Biegon et al., 1996; Osuka et al., 2001; Feustel et al., 2004). However, the therapeutic effects of Tamoxifen on white matter (WM) ischemic damage have not been studied to date.

The goal of the present study was to address the potential role of Tamoxifen in the pathogenesis of chronic cerebral hypoperfusion induced WM injuries. The results showed that Tamoxifen could modulate anti-inflammatory response and promote microglial polarization. We further demonstrated that Tamoxifen could promote oligodendrogenesis and rescue WM deficits. The data provide evidence that Tamoxifen may be a viable target for attenuating the demyelination and cognitive decline associated with chronic cerebral hypoperfusion, which is a promising candidate for the therapeutic treatment for ischemic WM injuries and other demyelination diseases.

2. Materials and methods

2.1. Ethics and animal preparation

All animal experimental procedures were performed according to the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and approved by the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology. Adult male C57BL/6 mice (10–12 weeks old) weighing between 20 and 28 g were randomly assigned into sham-operated (n = 20), vehicle treatment (n = 24), and Tamoxifen treatment (n = 34) groups. All efforts were made to minimize the number of animals used and their suffering. Randomization was performed by a person not associated with the study. One experimenter performed the operation and another one who was blind to the treatment groups applied the drug, conducted behavioral tests and tissue preparations. Observers were blinded to the grouping and experimental design during sample collection and analysis. All mice were given access to a 12-h light-dark cycle in a temperature-controlled facility, with free access to food and water.

All mice underwent the same surgical procedures. BCAS mice were under anesthesia with isoflurane (1%–2% by face mask) and then applied wire microcoils (Sawane Spring Co, Shizuoka, Japan) with an internal diameter of 0.18 mm around both common carotid arteries of mice, as previously described (Shibata et al., 2004). The fatality of this study was 2%. More than 95% of mice could pass the perioperative period safely. Mice with any neurological deficiency, such as visual field defect and limb hemiplegia would be excluded. Sham-operated mice only received bilateral exposure of the common carotid arteries. All mice were returned to the original cages after recovery.

Immediately after BCAS operation, all mice were randomly classified into either vehicle or Tamoxifen treatment groups. Tamoxifen (Sigma, Chemical Co., St. Louis, MO, USA) was dissolved by sonication in 10% EtOH/90% corn oil to reach the final concentrations. The same dosage of 10% EtOH/90% corn oil was injected into adult mice in vehicle group. All animals were randomly intraperitoneally (i.p) with different dosage of Tamoxifen (2.5, 5, or 10 mg/kg) and 10% EtOH/90% corn oil starting at 1 day after BCAS and daily until 1 day before sacrificed.

2.2. Bromodeoxyuridine labelling

All mice received daily i.p injection with the thymidine analog 5′-bromo-2′-deoxy-uridine (BrdU, 50 mg/body weight in sterile saline, Sigma Chemical Co., St. Louis, MO, USA) for 2 weeks, and sacrificed at 4 weeks after BCAS in order to evaluate the proliferation of oligodendrocytes precursor cells. Sham-operated mice received the same BrdU pulse and euthanized at the same time points. The number and phenotype of BrdU incorporated cells were determined by immunohistochemistry.

2.3. Immunohistochemical staining

Mice were sacrificed under deep anesthesia at determined time points (3 days, 2 weeks, 4 weeks and 12 weeks after BCAS injury). Mice were transcardially perfused with 20 ml ice-cold normal saline, followed by 20 ml 4% paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline (PBS). After perfusion, brain tissues were removed immediately and post-fixed in 4% PFA overnight and then cryoprotected in gradient sucrose for another 2 days at 4 °C until they sank. Twelve-micrometer-thick coronal sections (bregma +0.26 mm to +0.94 mm) were serially cut by cryostat and collected on poly-L-lysine-coated slides.

To investigate the histological damage of white matters, a Luxol Fast Blue (LFB) staining was employed as we described previously (Chen et al., 2017). White matter injuries were evaluated in the middle region of corpus callosum in each mouse. The grading system is as follows: grade 0 (normal), grade 1 (derangement of nerve fibers), grade 2 (formation of marked vacuoles) and the grade 3 (disappearance of myelinated fibers).

For immunofluorescences staining, sections were washed three times with PBS, permeabilized in 0.5% Triton X-100 in PBS, blocked in 10% bovine serum albumin to bind nonspecific antigen for 1 h at room temperature, then incubated with primary antibodies for 1–2 days at 4 °C. Then sections were rinsed in PBS, treated with appropriately corresponding secondary antibodies (all 1:500, Jackson ImmunoResearch Laboratories, USA) for 1 h. Cell nuclei were labeled with DAPI (1 μg/ml, Sigma, USA) for 15 min at room temperature. For BrdU staining, sections were incubated in primary antibody followed by 30 min 2N HCl incubation at 37 °C. Then sections were blocked again and incubated in anti-BrdU (1:100; Santa Cruz, USA) primary antibody followed by secondary antibody incubation. Primary antibodies used in the test included: rat anti-MBP (1:100, Millipore, USA), mouse anti-MAG (1:200, Millipore, USA), rabbit anti-SMI32 (1:200; Abcam, USA), rabbit anti-Iba1 (1:200; Wako, Japan), rabbit anti-olig2 (1:200; Abcam, USA), rabbit anti-NG2 (1:200, Millipore, USA), rat anti-CD16/32 (1:100; BD bioscience, USA), mouse anti-CD206 (1:100; Merck Millipore, USA), rabbit anti-NG2 (1:200, Millipore, USA), rabbit anti-GST-π (1:200, Medical and Biological Laboratories, Japan).

Images were observed blindly under an Olympus BX51 fluorescent microscope (Olympus, Japan) or laser scanning confocal microscope (Olympus, FV500, Japan). Immunopositive cell counts present as the mean number of cells per square millimeter were quantified by a blinded investigator using Image J software (National Institute of Health, Bethesda, MD, USA).

2.4. Electron microscopy

Mice were perfused by perfusion with ice-cold normal saline followed by 4% PFA/2.5% glutaraldehyde buffered with 0.1 mol/L phosphate buffer. Corpus callosum were sectioned into 1 mm thick tissues and then post-fixed in 2.5% glutaraldehyde for 2 h. Subsequently, samples were fixed in 1% OsO4 and block-stained in 1% uranyl acetate for 1 h. Following dehydrations, samples were embedded in epoxy resin and cut with an EM UC7 ultramicrotome (Leica Microsystems (UK) Ltd, Milton Keynes) into 60 nm ultrathin transverse sections, stained with uranyl acetate and lead citrate, and viewed using a Tecnai G2 20 TWIN transmission electron microscope (FEI UK Ltd., Cambridge, England). G-ratios were calculated by dividing the diameter of the axon by the diameter of the entire myelinated fiber, which were key measures of white matter health (Furusho et al., 2012). Approximately 1000 axons were measured for each group by Image J.

2.5. Behavioral test

We evaluated neurological functional deficits and recovery using an eight-arm radial maze test at 4 weeks after BCAS (Kim et al., 2010). The test was conducted consecutively for over 7 training days and then 9 test days. Before the initial pre-training, mice were deprived of food until the body weight was reduced to 80–85% of the initial level. Mice were first given 3 daily sessions of pre-training to facilitate habituation to the apparatus. Each mouse was placed in the central starting platform and allowed to explore and consume food pellets. In spatial working memory task of the 8-arm radial maze, all 8 arms were baited with food pellets. In reference memory task, only 4 arms were baited with food pellets and a trial was terminated immediately after one pellet was consumed. The arms with pellets were called working arms and the others were called reference arms. Mice who re-entered into a previously visited arm would be recorded for the Working memory errors (WME). Reference memory errors (RME), which occurred when the mice entered the reference arm for the first time, were recorded. The test was completed when the mouse found food in all eight arms or 15 min had elapsed.

2.6. Western blot

Mice were sacrificed and perfused quickly with ice-cold heparinized saline at 4 weeks post operation. Corpus callosumbrain tissues were freshly excised for western blots. Samples containing 30 μg proteins were electrophoresed on 10% SDS-PAGE, separated and transferred to nitrocellulose membrane (0.45 μm, Millipore, USA). The non-specific bindings were blocked by 5% fat-free dry milk in Tris-buffered saline (TBS) containing 0.25% Tween 20 (Amresco, Solon, OH, USA) for 1 h at room temperature, and incubated at 4 °C overnight with the following primary antibodies: rabbit anti-β- actin (1:1000; Santa Cruz, USA), rat anti- MBP (1:500, Millipore, USA), mouse anti- MAG (1:500, Millipore, USA), mouse anti-SMI32 (1:1000, Abcam, USA), rabbit anti-IL-1β (1:500, Santa Cruz, USA), mouse anti-TNF-α (1:500, Santa Cruz, USA), rabbit anti-NOX2 (1:1000, Abcam, USA), mouse anti-Hif-1α (1:500, Abcam, USA), and mouse anti-β-actin (1:5000, Abcam, USA). Thereafter, the membranes were washed and incubated with the appropriate horseradish peroxidase (HRP) -conjugated secondary antibodies for 1 h at room temperature. Finally, all blots were detected with enhanced chemiluminescence (ECL) kits (Thermo Fisher Scientific, Pierce, CAN) and the resulting digital images were analyzed by Image J. Protein was normalized relative to β-actin as expressed as a ratio compared to sham mice.

2.7. Statistical analysis

All measurements were performed by experimenters blinded to each experimental group and condition. Data are expressed as the mean ± SEM and were analyzed using GraphPad Prism 6.0 (GraphPad Software, Inc, La Jolla, CA). Significance of difference between animal groups was determined by variance (ANOVA) with repeated analysis, Mann–Whitney *U* test, two-tailed, or two-way ANOVA followed by Dunnett's post-hoc test. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Evaluation the effects of different doses of Tamoxifen on mice after BCAS injury

We firstly investigated the effects of Tamoxifen with various concentrations (2.5, 5, or 10 mg/kg) based on previous reports (Zhang et al., 2007; Tian et al., 2009), confirming its ability to protect against ischemic WM injury in mice after BCAS. At 4 weeks after operation and daily injection of different concentrations of Tamoxifen, we performed immunofluorescence staining of different types of glia (microglia and oligodendrocytes) in the corpus callosum (CC) and evaluated the cognitive functions using 8 arm test to find the optimal concentration. The results obtained from immunofluorescence showed that Tamoxifen (2.5 and 5 mg/kg) attenuated BCAS-induced microglial activation and number of Iba1 positive cells in a dose-dependent manner compared with that in vehicle group, confirming its ability to reduce inflammation (Fig. 1A, C, $P < 0.05$). However, Tamoxifen (10 mg/kg, i.p) gave no effect on the microglia activation (Fig. 1A, C). Moreover, the numbers of olig2 positive OLs decreased after BCAS which were alleviated after low doses of Tamoxifen (2.5 and 5 mg/kg) treatment (Fig. 1B, D; $P < 0.05$). Next, we observed whether Tamoxifen could attenuate neurological functions by an eight-arm maze test. BCAS mice showed significant increased revisiting errors in the behavioral test. Tamoxifen (5 mg/kg) did the most significantly improvement in working memory, as demonstrated by a decreased number of revisiting errors and an increased number of different arm choices in the first eight entries compared with the vehicle-treated group (Fig. 1E1, E2; $P < 0.05$). However, no significant differences were found in the spatial reference memory task among all groups (Fig. 1E3; $P > 0.05$). The results demonstrated that Tamoxifen with doses tested could improve the cognitive impairment at different level. Taken the protective effects as a whole, we chose 5 mg/kg Tamoxifen concentration in the following study.

3.2. Tamoxifen attenuates the degradation of white matter integrity induced by BCAS

The white matter integrity measured by the immunofluorescence staining and western blot in the CC revealed that chronic cerebral hypoperfusion gave rise to significant white matter degradation. The expression of myelin associated glycoprotein (MAG), a marker for myelin-axon integrity, as well as the expression of myelin basic protein (MBP), a marker for myelin and axon integrity, respectively, were quantified at 4 weeks and 12 weeks post-injury in the BCAS group using immunochemistry. MAG and MBP showed a significant decline in the BCAS group at 4 weeks after injury. However, the relative expression of MAG and MBP elevated at week 12. The reduction in MAG expression in BCAS mice at 4 weeks and 12 weeks post-injury was partly reversed by treatment with Tamoxifen, which indicates reversal of the disruption to myelin-axon integrity (Fig. 2 A, B, C2-, D2-). However, administration of Tamoxifen induced no detectable changes of MBP expression (Fig. 2 A, B, C1-, D1-). Concurrently, the expression of SMI32, a marker for the injury of axonal, exhibited an increase after BCAS, which was ameliorated after Tamoxifen treatment at week 4 and week 12 after injury (Fig. 2 A, B, C3, D3).

Additionally, WM disruption was rescued by these treatments, as assessed by LFB staining. LFB staining is a more sensitive marker of myelin loss. The sham group exhibited labeling intensity and

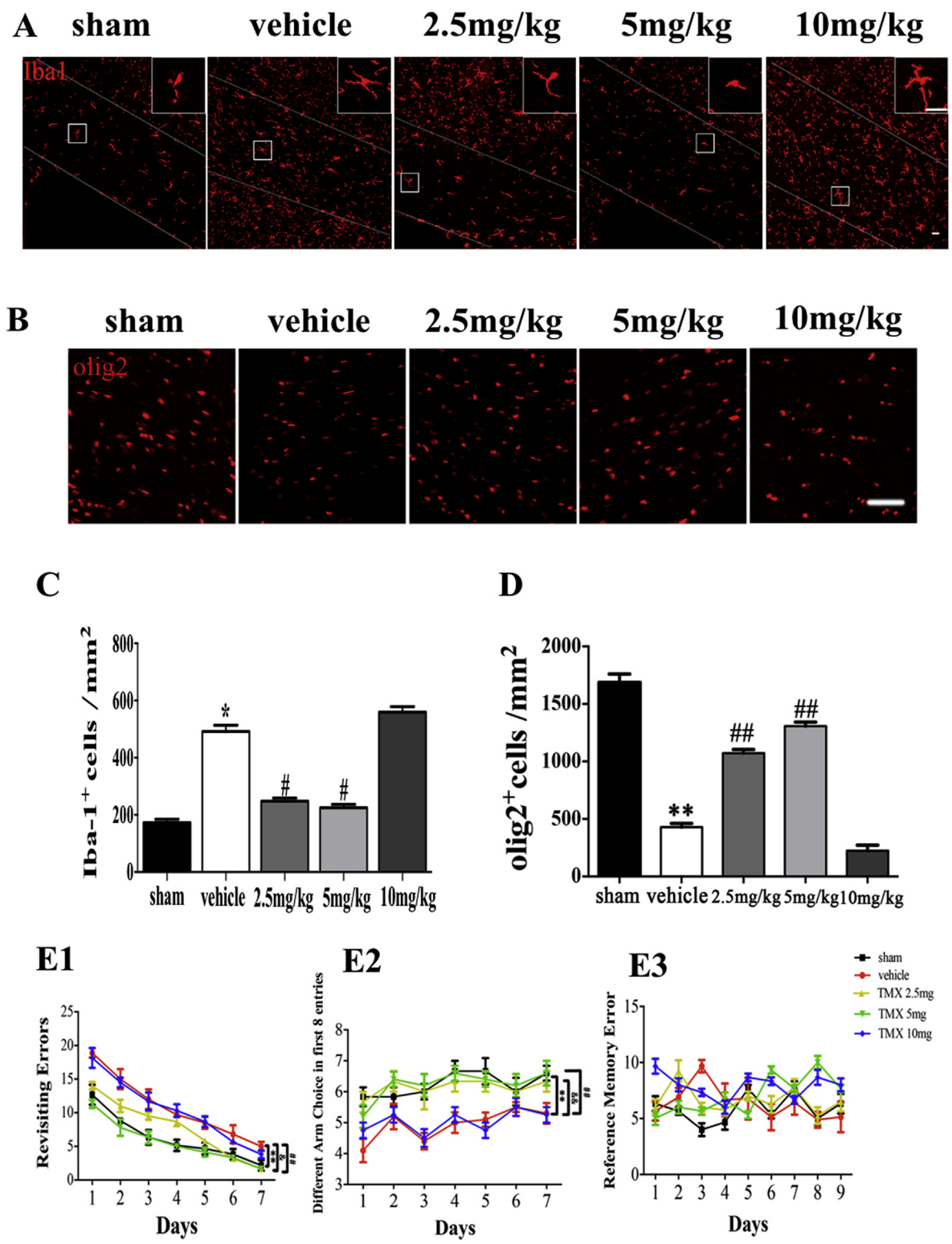

Fig. 1. Evaluation the effects of different doses of Tamoxifen on mice after chronic hypoperfusion. **(A)** Representative images depicting immunofluorescent labeling of microglia (Iba1, red) in the corpus callosum (CC) of sham-operated mice and mice in different doses of Tamoxifen (2.5 mg/kg, 5 mg/kg and 10 mg/kg) groups post BCAS. Scale bar represents 50 μm. Right upper white boxes are high-power magnified images of the dashed line boxes. Scale bar represents 20 μm. **(B)** Representative images depicting immunofluorescent labeling of oligodendrocytes (olig2, red) in the corpus callosum (CC) of sham-operated mice and mice in different doses of Tamoxifen (2.5 mg/kg, 5 mg/kg and 10 mg/kg) groups post BCAS. Scale bar represents 50 μm. **(C–D)** Quantitative analysis of results in (A–B) was performed as cells/mm^2. Two-way ANOVA with Dunnett's post-hoc test. Values are expressed as mean ± SEM (n = 4/group). $^{*}P < 0.05$, $^{**}P < 0.01$, vs sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs vehicle group. **(E1-E3)** The cognitive decline was reversed in mice treated with Tamoxifen (2.5 mg/kg and 5 mg/kg) at 4 weeks post BCAS. The revisiting errors in Tamoxifen (2.5 mg/kg and 5 mg/kg) treated group were much less **(E1)** and much more different arm choices was made in the first 8 entries **(E2)** compared to vehicle-treated mice ($P < 0.05$), while no obvious difference of reference memory errors was observed **(E3)** ($P > 0.05$). Values are expressed as mean ± SEM, Two-way analysis of variance (ANOVA) with repeated analysis, n = 10/group. $^{*}P < 0.05$, $^{**}P < 0.01$, vs sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs vehicle group. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, vs vehicle group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

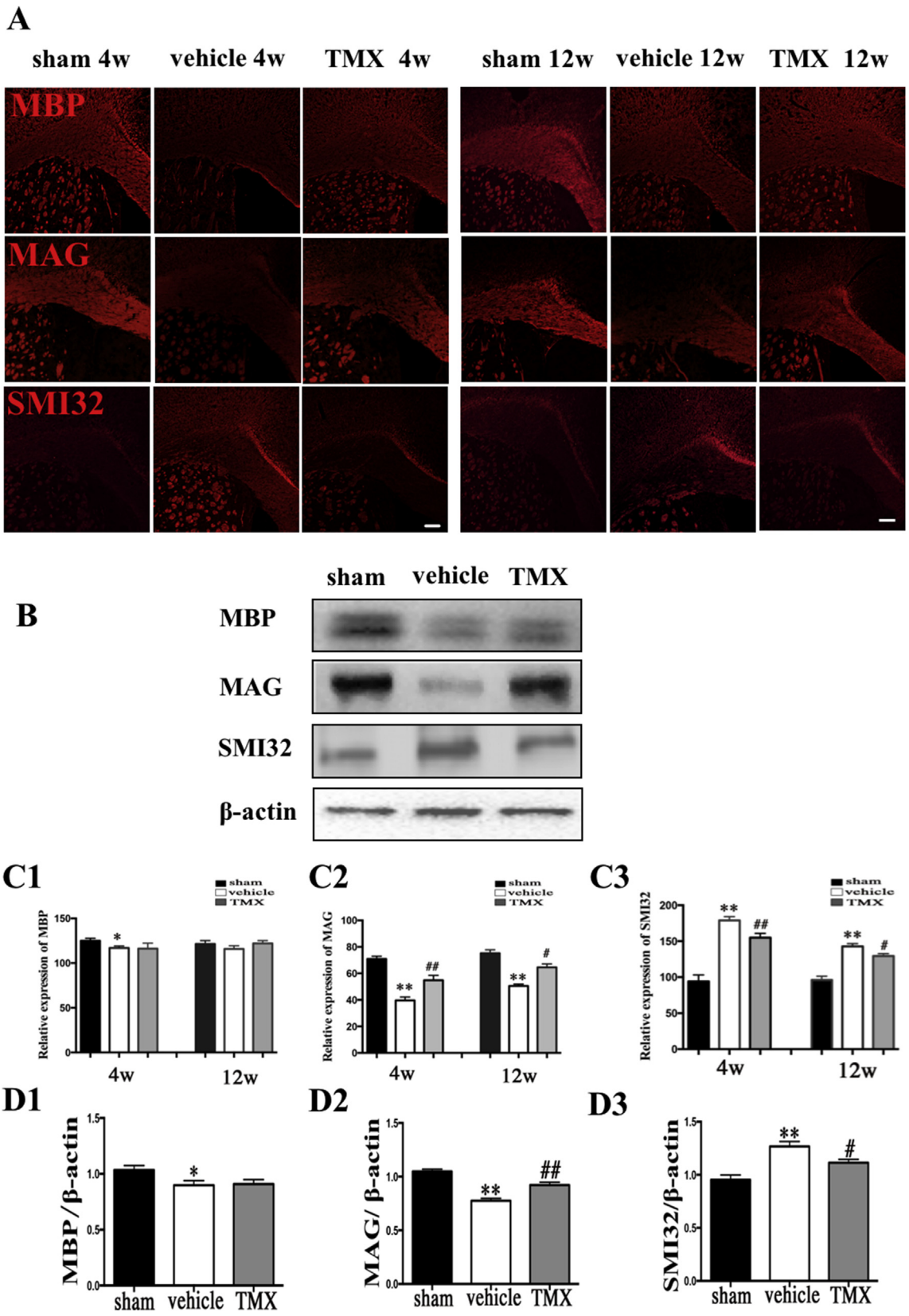

Fig. 2. Tamoxifen rescues white matter disruption in mice after chronic hypoperfusion. **(A)** Representative confocal images of coronal sections labeled with MBP, MAG and SMI32 from sham, vehicle and Tamoxifen treatment groups at 4 weeks and 12 weeks post BCAS, n = 4/group. Scale bar represents 100 μm. **(B)** The expression of MBP, MAG and SMI32 was determined by western-blots in mice from sham-operated, vehicle treated, and Tamoxifen treated group at 4 weeks post BCAS, n = 5/group. **(C1–C3)** Quantitative analysis of **(A)** was performed. Two-way ANOVA with Dunnett's post-hoc test. Values are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, vs sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs vehicle group. **(D1-D3)** Quantitative analysis of **(B)** was performed. Two-way ANOVA with Dunnett's post-hoc test. Values are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, vs sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs vehicle group.

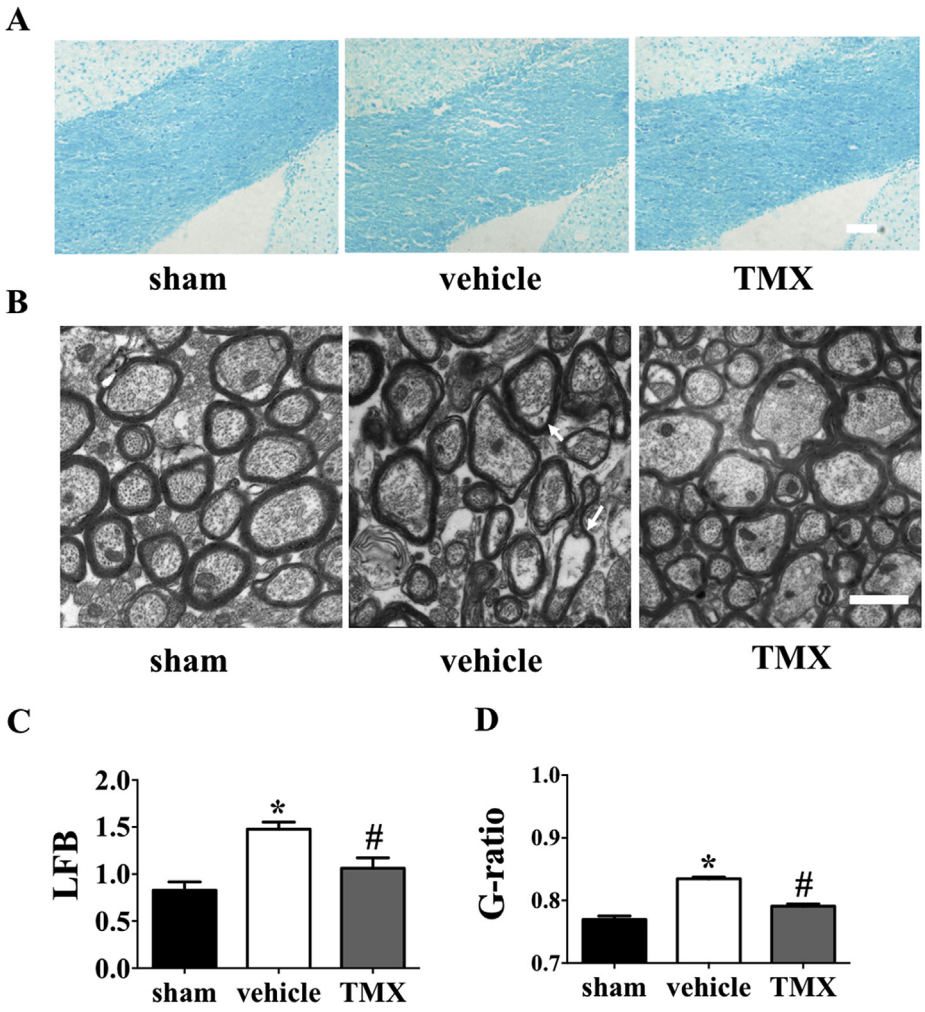

Fig. 3. Tamoxifen attenuates the degradation of myelin integrity induced by chronic hypoperfusion. (A) Representative LFB staining in the corpus callosum (CC) of sham, vehicle and Tamoxifen treatment groups at 4 weeks post BCAS, n = 5/group. Scale bar represents 50 μm. **(B)** Representative electron microscopy images revealed that axons in the corpus callosum of sham-operated mice were wrapped with well-defined lamellar myelin sheath. However, with the process of hypoperfusion, there was an increasingly disruption of myelinated structure, as vacuole or grid like changes. n = 4/group. Scale bar represents 1 μm. **(C)** Quantitative analysis of LFB staining was performed. Mann–Whitney *U* test, two-tailed. Values are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, vs sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs vehicle group. **(D)** Quantitative analysis of G-ratios which indicated that the inner axonal diameter to the total outer diameter confirmed that 4 weeks of hypoperfusion significantly increased this ratio, indicating a reduction of myelin sheath. Two-way ANOVA with Dunnett's post-hoc test. Values are expressed as mean ± SEM $^{*}P < 0.05$, $^{**}P < 0.01$, vs sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs vehicle group.

characteristic of normal myelination in middle of corpus callosum (CCm), whereas it was lighter and there were many vacuoles in the BCAS group at 4 weeks after operation (Fig. 3A). However, the BCAS-induced white matter demyelination was partially attenuated after Tamoxifen treatment (Fig. 3A, C, $P < 0.05$ vs. vehicle group). Furthermore, we used electron microscopy to determine whether Tamoxifen rescued ultrastructure abnormalities of myelination. Disruption of myelinated structures, manifested as vacuoles and grid-like changes, were detected in the CC area at 4 weeks after BCAS (Fig. 3B), while the axons remained intact. Consistently, the increase of G-ratio indicated evident reduction of myelin thickness without any axons changes in BCAS mice, and these changes were significantly attenuated by Tamoxifen treatment. (Fig. 3B, D; $P < 0.05$ vs. vehicle group).

Taken together, using several different approaches, we found that disruption of WM integrity following BCAS was reversed by the administration of Tamoxifen. Overall, these results point to Tamoxifen as a contributor to rescue myelin loss after cerebral hypoperfusion.

3.3. Tamoxifen inhibits microglia activation and inflammation reaction after BCAS

Microglia activation-mediated oxidative stress and inflammatory responses may exacerbate ischemic white matter injury, including increase the susceptibility of OLs to excitotoxicity (Chen et al., 2014; Orellana et al., 2011). In this study, we used immunofluorescence staining of microglial marker Iba1 to observe microglia activation in the corpus callosum. As shown in Fig. 4A, Iba-1-positive microglia were barely observed in the CC area of sham mice. While, strongly Iba-1 positive microglia, displaying the characteristic ramified appearance of the activated state with hypertrophy of cell body as well as thick branches (Fig. 1A), were increased in the subcortical white matter already at 3 days, 2 weeks and 4 weeks after BCAS. Administration with Tamoxifen remarkably decreased the number of Iba1-positive microglia compared with that in the vehicle group (Fig. 4A and B; $P < 0.05$).

As a marker for hypoxic conditions, hypoxia-inducible factor 1-alpha (HIF-1α) was increased after BCAS and attenuated by Tamoxifen (Fig. 4C, E; $P < 0.05$ vs. vehicle group). NOX is a common mediator of microglia–mediated white matter damage (Choi et al., 2015). Western blots analysis showed a significantly increase of NOX2 expression in the corpus callosum at week 4 after BCAS, while they were significantly reversed after Tamoxifen treatment (Fig. 4C, E; $P < 0.05$ vs. vehicle group). Meanwhile, proinflammatory cytokines like TNF-α and IL-1β contribute to impaired remyelination in models of demyelination (Arnett et al., 2001; Mason et al., 2001). As shown in Fig. 4D and F, the contents of TNF-α as well as IL-1β proteins in the CC of mice after BCAS were significantly higher than those in the sham-operated group. Tamoxifen significantly attenuated TNF-α and IL-1β expression compared with the vehicle group ($P < 0.05$).

3.4. Tamoxifen promotes a switch from M1-to M2-dominant response occurred in microglia

Microglia with different phenotypes have distinct impacts on the survival and differentiation of oligodendrocyte lineage cells (Miron et al., 2013). M1 microglia are characterized by pro-inflammatory effects and lead to demyelination, whereas M2 microglia are anti-

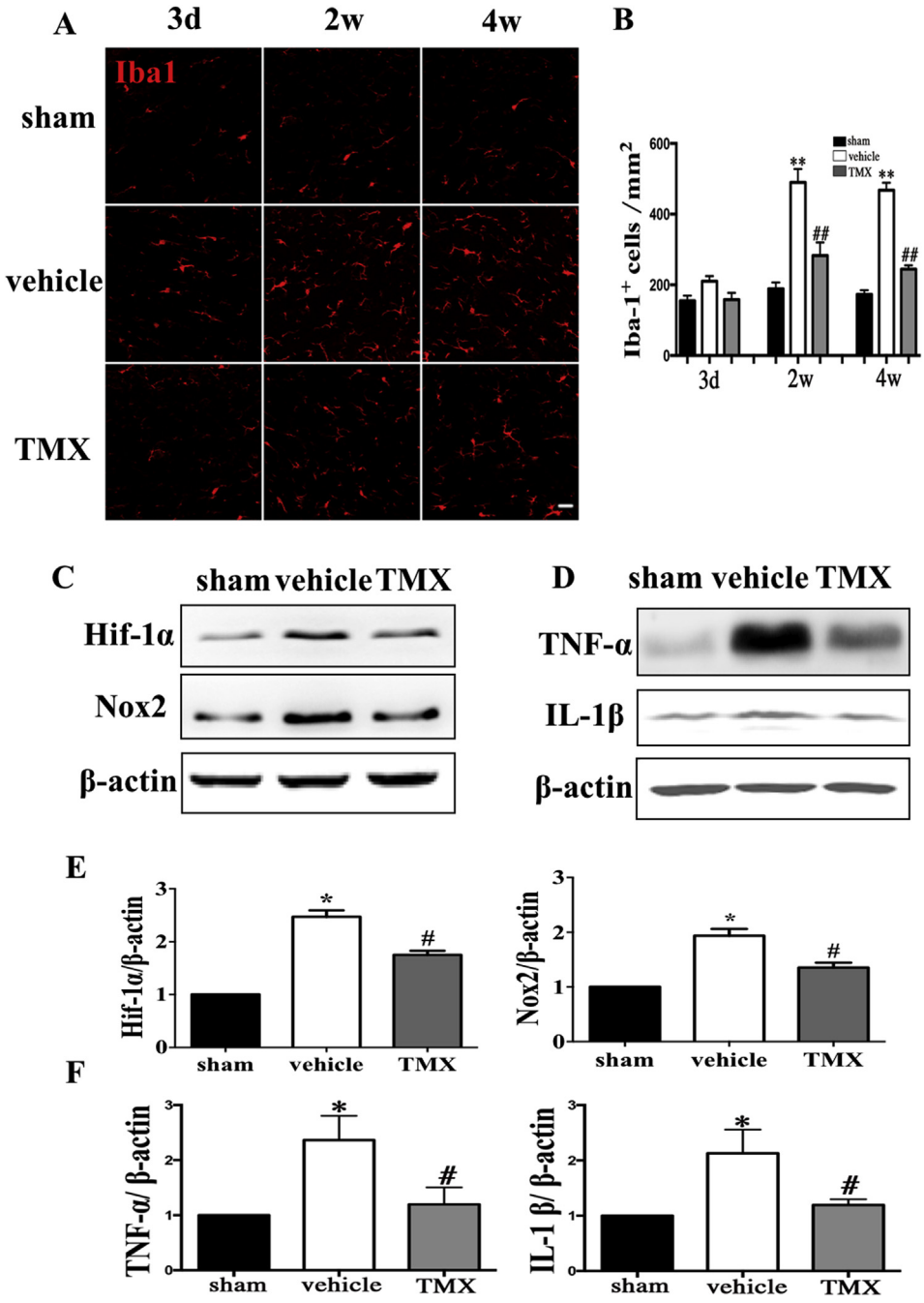

Fig. 4. Tamoxifen inhibits the microglia activation and inflammation reaction after BCAS. **(A)** Representative images depicting immunofluorescent labeling of microglia (Iba1, red) in the corpus callosum (CC) in sham, vehicle and Tamoxifen treatment groups at different time points (3 days, 2 weeks, and 4 weeks) post BCAS, n = 5/group. Scale bar represents 50 μm. **(B)** Quantitative analysis of Iba1 in sham, vehicle and Tamoxifen treatment groups at different time points (3 days, 2 weeks, and 4 weeks) post BCAS, expressed as cells/mm^2. Two-way ANOVA with Dunnett's post-hoc test. Values are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, vs sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs vehicle group. **(C-D)** Representative western blots of NOX2, Hif-1α, TNF-α, IL-1β, and β-actin expression in the CC area of sham, vehicle and Tamoxifen treatment groups at 4 weeks post BCAS, n = 5/group. **(E-F)** Quantitative analysis of **(C–D)** were performed. Two-way ANOVA with Dunnett's post-hoc test. Values are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, vs sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs vehicle group. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

inflammatory and facilitate tissue repair (Hu et al., 2015). We further tested whether Tamoxifen exerts its anti-inflammatory effects by affecting microglia phenotype. Double staining for the microglia/macrophage marker Iba1 and M1-associated marker CD16/32 or M2-associated marker CD206 in the CC were performed after BCAS (Fig. 5A and B). M1 microglia were increased after BCAS but significantly inhibited by Tamoxifen (Fig. 5A, C), whereas the M2 microglia was further enhanced by Tamoxifen (Fig. 5B, D). Collectively, these findings suggest that Tamoxifen contributes to microglia polarization toward beneficial M2 phenotype.

3.5. Tamoxifen promotes OPCs proliferation and differentiation of OLs following chronic cerebral hypoperfusion

The loss of myelin in the central nervous system, produced by OLs, leads to cognitive deficits (Lopez Juarez et al., 2016). Regeneration of mature oligodendrocytes is essential for remyelination and functional recovery (Zhang et al., 2013). Thus, we sought to evaluate whether Tamoxifen treatment acted on oligodendrocyte lineage development after BCAS, thereby facilitating white matter restoration. Staining of the OPC marker NG2 indicates a progressively larger and more intensely stained cell population in chronic cerebral hypoperfusion, and this endogenous response was more drastic after Tamoxifen treatment (Fig. 6 A, B). Coincidence with that increase in NG2-positive cells, the number of GST-π-positive mature oligodendrocytes and olig2-positive total oligodendrocyte lineage cells was dramatically reduced in the CC areas after BCAS, while administration of Tamoxifen attenuated the reduction (Fig. 6 A, C, D; $P < 0.05$).

BrdU incorporation assay demonstrated that ischemia stimulated oligodendrogenesis proliferation in the CC areas, as reflected by increased numbers of BrdU/NG2, BrdU/olig2 and BrdU/GST-π cells in vehicle-treated groups compared with the sham control group,

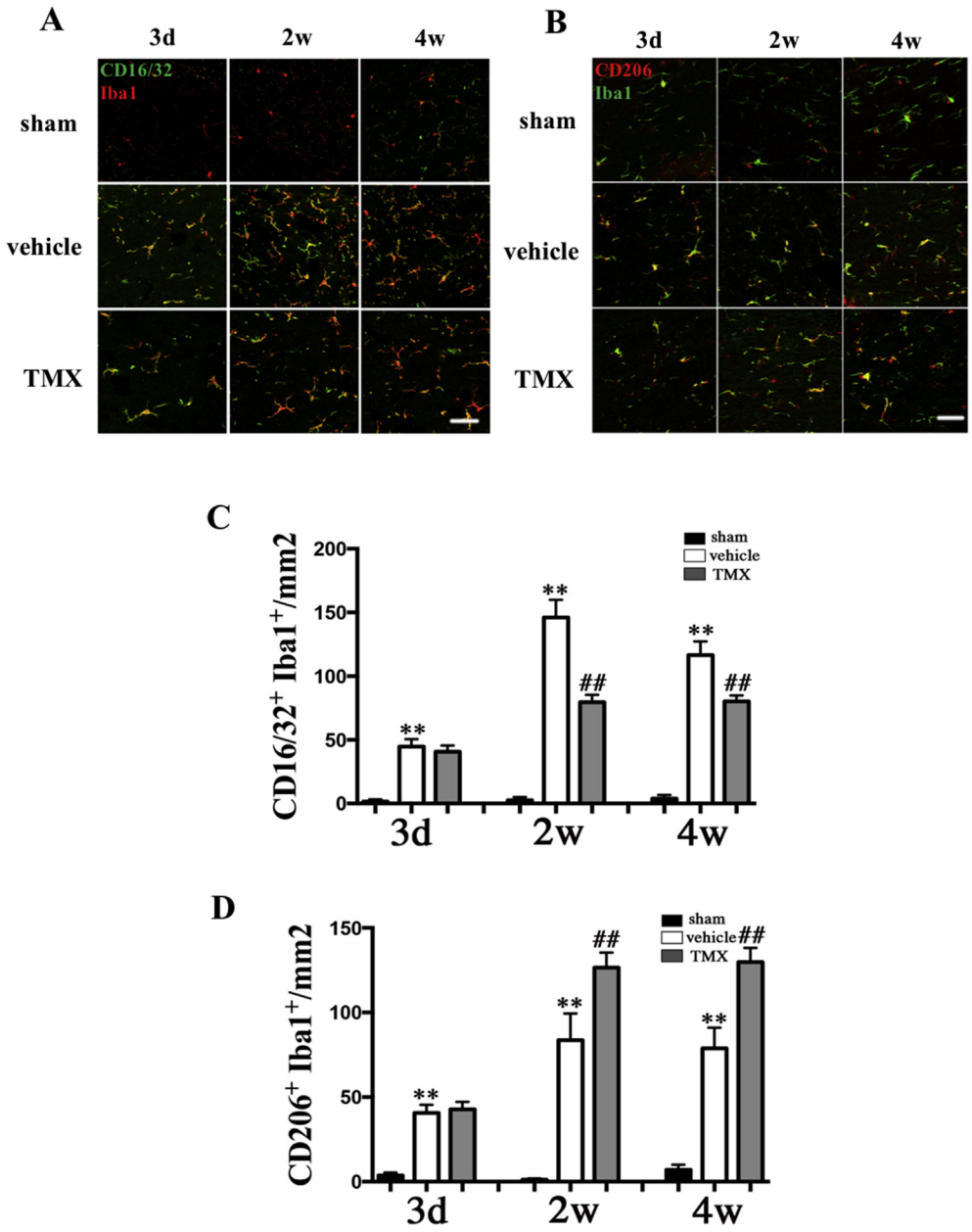

Fig. 5. Tamoxifen promotes a switch from an M1-to an M2-dominant response occurred in microglia. **(A)** M1 type microglia in the corpus callosum (CC) at 4 weeks was detected by double staining of CD16/32 (green) and Iba1 (red) in sham, vehicle and Tamoxifen treatment groups at different time points (3 days, 2 weeks, and 4 weeks) post BCAS. M2 phenotype was detected by double staining of CD206 (red) and Iba1 (green) in sham, vehicle and Tamoxifen treatment groups at different time points (3 days, 2 weeks, and 4 weeks) post BCAS. n = 5/group. Scale bar represents 50 μm. **(B–C)** Quantitative analysis of immunofluorescent staining of CD16/32/Iba1 and CD206/Iba1 in the CC of sham, vehicle and Tamoxifen treatment groups at different time points (3 days, 2 weeks, and 4 weeks) post BCAS, expressed as cells/mm^2. n = 5/group. Two-way ANOVA with Dunnett's post-hoc test. Values are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, vs sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs vehicle group. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

suggesting that spontaneous generation of new oligodendrocytes occurs at 4 weeks after BCAS (Fig. 6). Tamoxifen treatment facilitated the increase of OPC proliferation, as revealed by increased numbers of NG2-positive OPCs with BrdU incorporation in CC areas (Fig. 6 A, B). Moreover, Tamoxifen treatment further augmented oligodendrocyte replacement, as evidenced by greater colocalization of BrdU/GST-π new mature OLs and BrdU/olig2 new oligodendrogenesis were detected (Fig. 6 A, C, D). These data indicate that Tamoxifen treatment promoted the proliferation and differentiation of OPCs into mature OLs after chronic cerebral hypoperfusion.

4. Discussion

Anatomically distinct strokes confined entirely to white matter constitute 25% of all strokes (Matute et al., 2013), and both gray and white matter are involved in the majority of ischemic strokes. Furthermore, subcortical white matter degeneration changes due to slowly developing vascular compromise, which is very common in elderly populations and associated with vascular cognitive impairment (VCI) (Shibata et al., 2004). It is well established that the pathophysiology of ischemic injury is different in white and gray matter (Zhang and Sejnowski, 2000). However, no clear therapeutic strategies for improving clinical outcome after acute or chronic white matter injury have emerged. Ischemic white matter injuries induced by chronic cerebral hypoperfusion are a complex process involving multiple cellular mechanisms and are associated with cognitive decline and other neurological deficits (Iadecola, 2013; Mathiesen et al., 2004; Farkas et al., 2007). Traditional studies of white matter injuries have been focused on the oligodendrocytic death and axonal damage. However, multiple cell types and intercellular signaling cascades contribute to the maintenance of white matter integrity and connectivity (Hayakawa and Lo, 2016). Thus, a “cocktail” protective strategies that targets multiple cellular elements of the brain may yield more opportunities for stroke treatment in the future (Wang et al., 2016). The present study examined the effect of Tamoxifen against white matter injuries under chronic hypoperfusion. Our results showed that Tamoxifen treatment reduced microglia activation and inflammatory response, enhanced OPCs proliferation and differentiation, and promoted remyelination after chronic

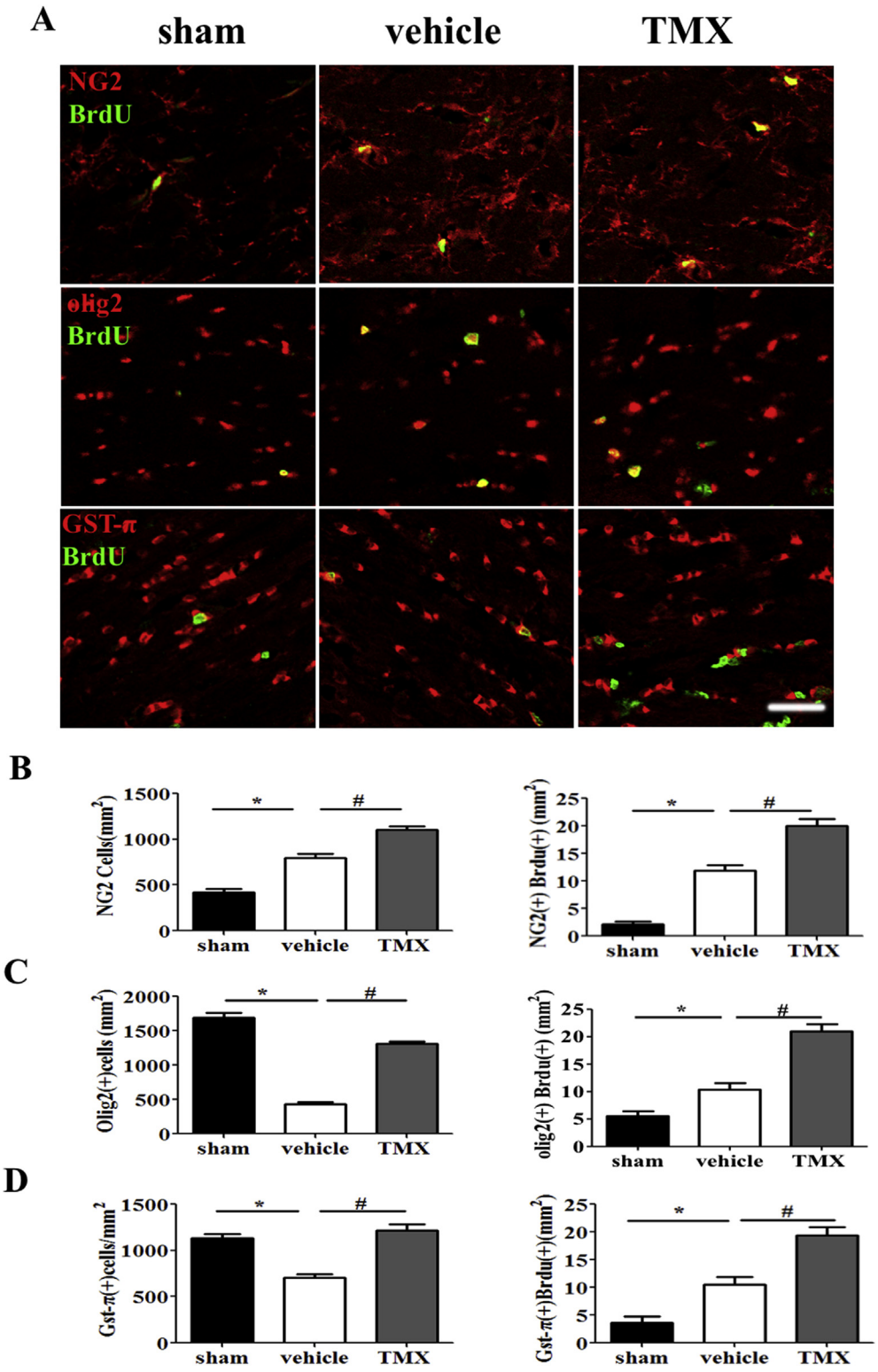

Fig. 6. Tamoxifen promotes OPCs proliferation and differentiation of OLs following chronic cerebral hypoperfusion. (**A**) Representative image showing the co-localization of bromodeoxyuridine (BrdU; green) and NG2/olig2/GST-π (red) staining in the corpus callosum (CC) of mice in sham, vehicle and Tamoxifen treatment groups at 4 weeks after operation. Scale bar represents 50 μm. **(B)** Numbers of NG2 labeled oligodendrocyte progenitor cells (OPCs), olig2 labeled the oligodendrocyte (OL) lineage cells, GST-π labeled mature OLs, BrdU/NG2-dual labeled OPCs, BrdU/olig2-dual labeled the OL lineage cells, and BrdU/GST-π labeled mature OLs in the CC were expressed as cells/mm^2, n = 5/group. Two-way ANOVA with Dunnett's post-hoc test. Values are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, vs sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs vehicle group. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

hypoperfusion. Furthermore, Tamoxifen treatment significantly facilitated functional restoration of working memory impairment after white matter injury. Together, our observations suggest that Tamoxifen exert a key role in maintaining the health of cerebral white matter integrity in the face of chronic cerebral hypoperfusion.

As a selective estrogen receptor modulator, Tamoxifen is a well-established and FDA approved drug widely used for the treatment of estrogen receptor-positive metastatic breast cancer. Tamoxifen has the ability to across BBB (Biegon et al., 1996) and short-term use of Tamoxifen in the clinical neurological disease may be considerably safe (Mosquera et al., 2014). Tamoxifen has been shown to play diverse neuroprotective effects through several cellular mechanisms including inhibition of neuronal nitric oxide synthase, reactive oxygen species, inflammatory reaction, and swelling-induced release of excitatory amino acids (Feustel et al., 2004; Osuka et al., 2001; Phillis et al., 1998; Feng et al., 2004; Ismailoglu et al., 2010; Wiseman et al., 1993; Obata and Kubota, 2001). In addition, Tamoxifen has potent anti-oxidant, anti-apoptotic, and anti-gliotic effects (Guptarak et al., 2014; Ismailoglu

et al., 2010; Zhang et al., 2007; Mosquera et al., 2014; Wei and Ma, 2014; De La Torre Valdovinos et al., 2016). It is reported that Tamoxifen treatment could reduce oligodendrocyte cell death, spared mature oligodendrocytes and increased myelin levels and remyelination (Cao et al., 2010; Li et al., 2007; Tian et al., 2009). Consistent with these protective effects, the key observation of the present study supported that administration of Tamoxifen could promote white matter remyelination and neurological regeneration after cerebral hypoperfusion induced white matter injury.

Microglial phagocytic activity is a prominent feature in the demyelination/remyelination during white matter injury (Moxon-Emre and Schlichter, 2010; Jalal et al., 2012; Chen et al., 2016). Stroke-induced cerebral inflammatory processes adversely impact white matter, including axon and myelin structural integrity, which is associated with long-term neurological deficits (Blasi et al., 2014; Scantlebury et al., 2011). Profound demyelination occurs precisely in the regions that display significant activation of microglia (Kipp et al., 2011). Inflammation-associated oxidative stress and nicotinamide adenine dinucleotide phosphate oxidase (NOX)-dependent reactive oxygen species (ROS) production in activated microglia is a major contributor to the pathogenesis of cerebral ischemia and neurological degenerative diseases (Milatovic et al., 2005; Kahles et al., 2010; Gilgun-Sherki et al., 2002), which trigger apoptotic pathways (Hur and Gray, 2011) and is responsible for the death of OPCs (Yu et al., 2017). Hypoxia-inducible factor 1-alpha (HIF-1α) has been reported to be an identified and sensitive marker for hypoxia-like metabolic tissue injury (Semenza, 2000), which is associated with myelin destruction and inflammatory demyelination (Le Moan et al., 2015). The expression of HIF-1α in the white matter lesions may reflect a hypoxia-like metabolic injury of the tissue as a common denominator of tissue injury. Tamoxifen is an effective antioxidant (Buelna-Chontal et al., 2016) and could impede the activation of the oxidative reaction as well as the oxidative-induced release of membrane-bound cytochrome (Buelna-Chontal et al., 2016; Pavon et al., 2017). Administration of Tamoxifen significantly suppressed microglia activation, possessed potent anti-inflammatory effects, reduced infarct size, and alleviated white matter injury after ischemic injury (Phillis et al., 1998; Kimelberg et al. 2000, 2003; Zhang et al., 2005; Tian et al., 2009; Ismailoglu et al., 2010). Wang et al. recently have shown that Tamoxifen could suppress retinal microglial activation and proinflammatory cytokine expression in models of photoreceptor degeneration (Wang et al., 2017). We previously have shown that administration of Tamoxifen significantly suppresses microglia activation mediated inflammatory response and myelin loss after spinal cord injury (Tian et al., 2009). Consistent with these studies, here we demonstrated that Tamoxifen significantly attenuated microglia activation and the expression of NOX, Hif-1α, TNF-α and IL-1β after chronic cerebral hypoperfusion induced white matter damage.

Microglia are highly plastic cells that can assume different phenotypes in response to microenvironmental signals and play a dual-faced role with detrimental (M1-like phenotype) or supportive (M2-like phenotype) functions in the progression of white matter injury after stroke (Suenaga et al., 2015; Han et al., 2015). Regenerative properties of macrophages following injury are associated with alternative or deactivation to M2-polarized phenotypes, which secrete anti-inflammatory cytokines and growth factors. In contrast, M1 'classically activated' phenotypes secret pro-inflammatory cytokines and reactive oxygen and nitrogen species (Edwards et al., 2006). M2 microglia/macrophages have been suggested to improve multiple brain repair responses such as neurogenesis, angiogenesis, and white matter repair by resolving inflammatory response and producing trophic factors (Hu et al., 2015). Miron has shown that M2 microglia and macrophages drive oligodendrocyte differentiation and promote white matter integrity during remyelination (Miron et al., 2013). By using double immunofluorescent staining of M1 marker CD16/32 or M2 marker CD206, administration of Tamoxifen dramatically decreased the number of M1 microglia and enhanced the expression of M2 microglia after chronic cerebral hypoperfusion. Consistent with these functional roles, M2 microglial/macrophage phenotype was correlated with myelin integrity in the present study. The results implicate that Tamoxifen is critical in decreasing NADPH oxidase-dependent generation of ROS as well as subsequent pro-inflammatory cytokines production in microglia by favoring microglial polarization toward the M2 phenotype, which could suppresseinflammatory reaction and promote tissue recovery by clearing up debris and secreting anti-inflammatory cytokines. However, microglia may exert their multiple roles in demyelination/remyelination rather than acting by solely M1/M2 phenotype transition. The present data suggest that Tamoxifen modulated microglial reactivity changes and made balance between beneficial and detrimental microglia which may facilitate a more permissive environment for remyelination after chronic cerebral hypoperfusion.

White matter damage defined as axonal degeneration and secondary demyelination induces disturbance of nerve impulse conduction and evokes serious neurological dysfunction (Mifsud et al., 2014). As the major component of white matter, OLs are exquisitely sensitive to ischemic injury (McIver et al., 2010). After brain injury, remyelination occurs to ensheathe demyelinated axons with new myelin and reduce functional deficits. Successful regeneration of OLs is essential for remyelination and axonal preservation after brain injuries. Although adult brain has limited capacity for full remyelination and white matter repair, the immature forms of OLs -oligodendrocyte precursor cells (OPCs) could proliferate and migrate towards the demyelinated axons and final differentiate into mature functional OLs (Miyamoto et al., 2015; Gregersen et al., 2001). In vitro study has shown that Tamoxifen could regulate OPCs proliferation and maturation and prevent delayed apoptotic death of OLs (Marin-Husstege et al., 2004). In the present study, NG2 positive OPCs respond to the cerebral hypoperfusion with proliferation while mature OLs were declined. Tamoxifen treatment improved the proliferation and differentiation of OPCs and OLs after cerebral hypoperfusion. Coincident with the decrease in NG2 cells, the number of mature myelinating OLs was increased, indicating a relatively reduced mature population. Meanwhile, Tamoxifen treatment further augmented oligodendrocyte replacement, as evidenced by that more colocation of new-differentiated mature OLs were detected. Altogether, Tamoxifen treatment enhances generation of new OLs from progenitor cells, which are critical for the control of the oligodendrogenesis and remyelination. These findings suggest a robust process of oligodendrocyte regeneration and a potential partial reparative response with Tamoxifen treatment following chronic cerebral hypoperfusion induced white matter injury.

In summary, our present data may provide new insights showing that Tamoxifen provides a novel multiple target–protective effects after chronic cerebral hypoperfusion-induced white matter lesions. The results demonstrated that Tamoxifen reduced the severity of myelin pathology and cognitive loss in a murine model of chronic brain–wide hypoperfusion. Although the underlying signaling mechanisms by which Tamoxifen exert their protective effects remain unknown, we therefore propose that Tamoxifen may comprise a candidate therapeutic agent for the treatment of demyelinating diseases.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Abbreviations

BCAS	bilateral carotid artery stenosis
VCI	vascular cognitive impairment
BBB	blood-brain barrier
NOS	nitric oxide synthase
ROS	reactive oxygen species
I.P.	intraperitoneally
BrdU	5′-bromo-2′-deoxy-uridine
PFA	paraformaldehyde
PBS	phosphate-buffered saline
LFB	Luxol Fast Blue
DAPI	4′,6-Diamidino-2-Phenylindole, Dihydrochloride
MBP	myelin basic protein
MAG	myelin associated glycoprotein
SMI32	non-phosphorylated neurofilaments
Olig2	oligodendrocyte transcription factor 2
NG2	neuron-glialantigen 2
WME	working memory errors
RME	reference memory errors
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	tris-buffered saline
NOX	nicotinamide adenine dinucleotide phosphate oxidase
IL-1β	interleukin-1β
TNF-α	tumor necrosis factor alpha
HIF	hypoxia-inducible factor
HRP	horseradish peroxidase
ECL	enhanced chemiluminescence
ANOVA	analysis of variance
OL	oligodendrocyte
CC	corpus callosum
OPC	oligodendrocyte precursor cells
WML	white matter lesions

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.104566.

Author contributions

MJX and DJP conceived the study and designed experiments. YXC and YYT, HT performed the experiments and wrote the manuscript. YYT, YW, WW and YKF analyzed the results. QBH contributed regents/materials/tools. All authors read and approved the final version of the manuscript.

Animal research

Animal protocols were approved by the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology.

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