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Wnt-3a alleviates neuroinflammation after ischemic stroke by modulating the responses of microglia/macrophages and astrocytes

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ABSTRACT

Neuroinflammation crucially influences functional recovery after ischemic stroke. Wnt-3a, a novel Wnt protein that specifically promotes Wnt/β-catenin signaling pathway, has been shown to regulate apoptosis and cell proliferation, but how it affects ischemic stroke-induced toxic brain inflammation remains unknown. Using a transient middle cerebral artery occlusion (tMCAO) mouse model in this study, we found that intranasal Wnt-3a-treated tMCAO mice had apparently reduced infarct volume and decreased brain water content after being allowed to recover for 72 h, as well as better neurologic outcomes on days 3, 7, and 14. Mice received Wnt-3a had significantly fewer tMCAO-induced peri-infarct TUNEL-positive cells compared with those received vehicle. Further, Wnt-3a-delivered tMCAO mice had notably fewer peri-infarct CD68-positive cells and lower ionized calcium-binding adapter molecule (Iba)-1 protein level. Wnt-3a significantly downregulated the expression of inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF)-α, and upregulated the expression of arginase 1 (Arg1) and CD206. Finally, Wnt-3a obviously decreased the number of tMCAO-induced peri-infarct glial fibrillary acidic protein (GFAP)/C3-positive cells, increased the number of GFAP/S100A10-positive cells, attenuated the protein levels of GFAP and interleukin 15 (IL15), and elevated IL33 protein level. Our findings suggest that intranasal Wnt-3a could ameliorate toxic responses of microglia/macrophages and astrocytes in ischemic brain injury, supporting that Wnt-3a might be potentially appropriate for ischemic stroke treatment functioning as an immunomodulatory agent.

1. Introduction

Ischemic stroke is a leading cause of disability and mortality globally. Acute ischemic injury induces rapid activation of microglia/macrophages and astrocytes [1,2]. It's well defined that reactive microglia/macrophages polarize into pro-inflammatory M1 phenotype and anti-inflammatory M2 phenotype [3]. In analogy to the M1/M2 microglia/macrophage nomenclature, recent studies identified two different phenotypes of reactive astrocytes, which were termed A1 (toxic) and A2 (protective) [4–7]. Although the dynamic of stroke-induced reactive astrocyte polarization has not been investigated, novel and clinically translatable strategy which could modulate activation and polarization of microglia/macrophages and astrocytes may be a promising candidate for ischemic stroke treatment.

Glycogen synthase kinase-3 beta (GSK-3β) inhibition has been revealed to protect against ischemic stroke through several mechanisms, such as attenuating apoptosis, oxidative stress and rtPA-induced hemorrhagic transformation, and stimulating neurogenesis [8–12]. Recently, studies showed that GSK-3β inhibition ameliorated the activation of microglia/macrophages and astrocytes in models of neonatal hypoxic-ischemic brain injury and taupathy [13,14]. Since GSK-3β inhibition leads to the stabilization of β-catenin, which is the key downstream molecule of the Wnt signaling [15], Wnt/GSK-3β/β-catenin pathway may participate in regulating stroke-induced activation and polarization of microglia/macrophages and astrocytes.

Of the identified nineteen Wnt proteins, the novel glycolipoprotein

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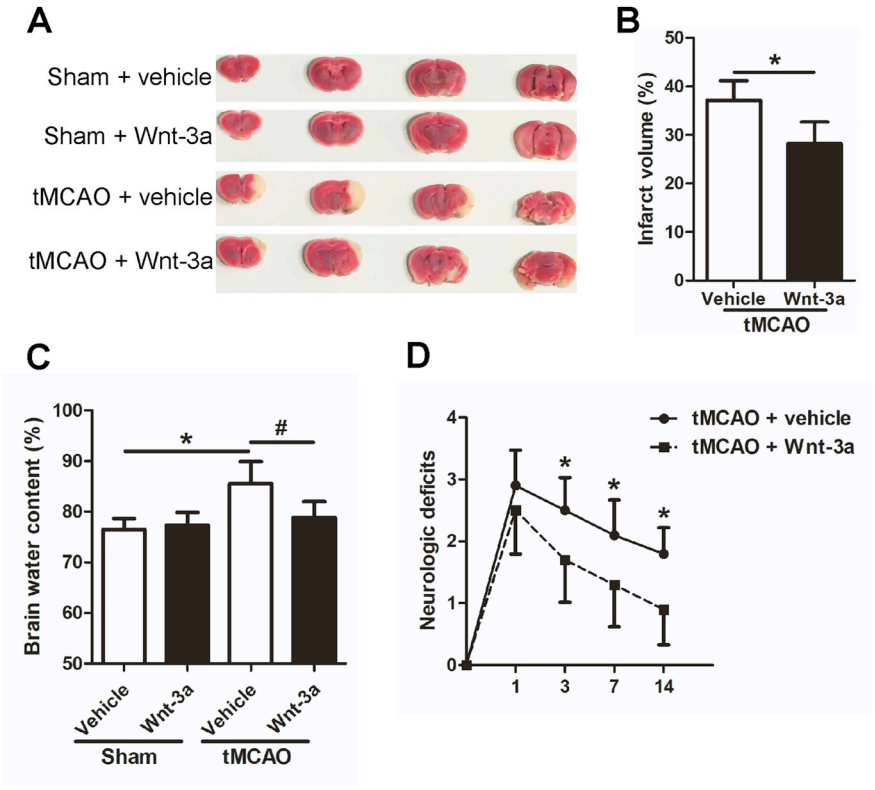

Fig. 1. Intranasal Wnt-3a influences infarct volume, brain water content and neurologic deficits after stroke. (A) Representative TTC-stained brain sections at 72 h post-surgery. Infarct area remains white. (B) Quantification of the corrected infarct volume showing as a percentage obtained from the described formula ($^{*}p < 0.05$, $n = 6$/group). (C) Quantification of the brain water content at 72 h post-surgery ($^{*}p < 0.05$ vs. Sham + vehicle group, $^{\#}p < 0.05$ vs. tMCAO + Wnt-3a group, $n = 6$/group). (D) Representative that intranasal Wnt-3a significantly improved neurologic outcomes on days 3, 7, 14 after tMCAO ($^{*}p < 0.05$, $n = 10$/group). Data are shown as mean ± SD.

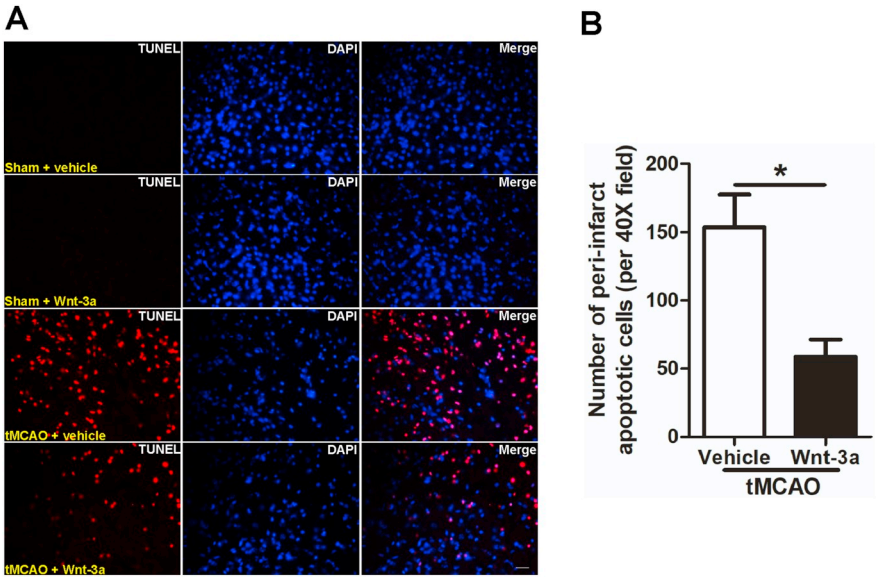

Fig. 2. Effect of intranasal Wnt-3a on peri-infarct apoptosis at 72 h after tMCAO. (A) Representative TUNEL-stained apoptotic cells in each group. Scale bar = 25 μm. (B) Quantification showed that intranasal Wnt-3a significantly decreased the number of stroke-induced peri-infarct apoptotic cells ($^{*}p < 0.05$, $n = 8$/group). Data are shown as mean ± SD.

Wnt-3a inhibits GSK-3β and specifically activates Wnt/GSK-3β/β-catenin pathway [16]. Wnt-3a could be naturally secreted by cells like mesenchymal stem cell in the body, and cross the blood-brain barrier (BBB) [17]. In the middle cerebral artery occlusion (MCAO) rat model, intranasal administration of exogenous recombinant Wnt-3a could protect neuron against apoptosis [16]. Study on the focal ischemic stroke mouse model showed that intranasal Wnt-3a stimulated neurogenesis in subventricular zone [18]. Additionally, intranasal Wnt-3a could regulate autophagic, apoptotic, and regenerative pathways in traumatic brain injury (TBI) mice [19].

It is a consensus that neuroinflammation interacts with and causally influences several pathophysiological processes, including neuronal apoptosis and regeneration, from acute to chronic stage after ischemic stroke. However, the effect of Wnt-3a on ischemic stroke-induced brain inflammation has never been investigated. In the present study, we hypothesized that intranasal Wnt-3a could attenuate M1 microglia/macrophage and A1 astrocyte polarization, and promote M2 microglia/macrophage and A2 astrocyte polarization at 72 h after ischemic stroke. We tested our hypothesis in a transient middle cerebral artery occlusion (tMCAO) mouse model.

2. Materials and methods

2.1. Animals

We purchased male, adult C57BL/6 mice (12–14 weeks old,

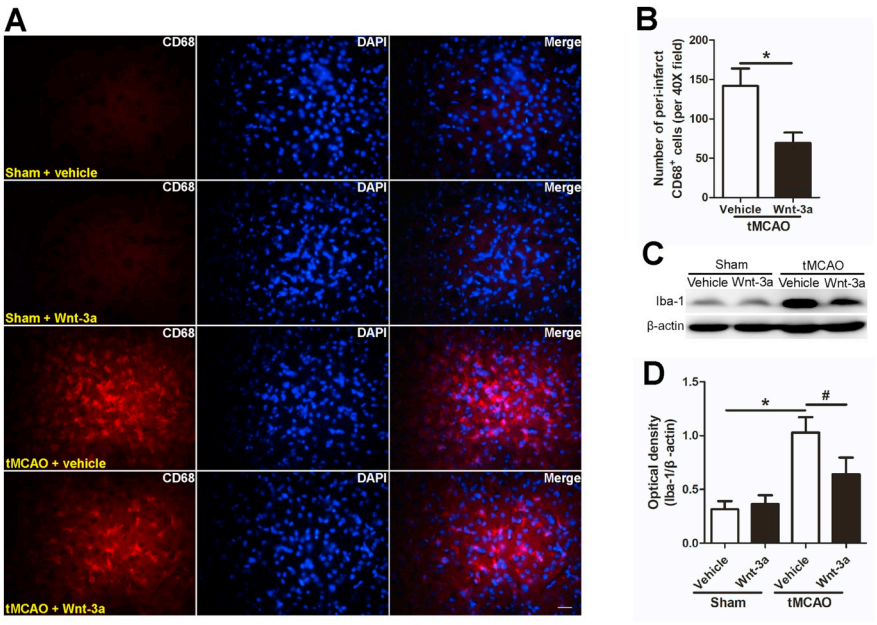

Fig. 3. Intranasal Wnt-3a affects the activation of microglia/macrophages at 72 h after brain ischemia. (A) Immunofluorescence staining of CD68-positive cells in each group. Scale bar = 25 μm. (B) Representative quantification showing that intranasal Wnt-3a notably reduced the number of peri-infarct CD68-positive cells ($^{*}p < 0.05$, $n = 8$/group). (C) Western blot analysis of Iba-1. (D) Quantification of Iba-1 protein level in each group ($^{*}p < 0.05$ vs. Sham + vehicle group, $^{\#}p < 0.05$ vs. tMCAO + Wnt-3a group, $n = 8$/group). Data are shown as mean ± SD.

25–30 g) from the Animal Experimental Center of Zhengzhou University. This study was performed following the recommendations of the Guidelines on the Care and Use of Animals for Scientific Purpose (National Advisory Committee for Laboratory Animal Research), and all protocols were approved by the Animal Care and Use Committee of the Fifth Affiliated Hospital of Zhengzhou University. All efforts were made to minimize the number of mice used and their suffering.

2.2. tMCAO model

We performed tMCAO model of ischemic stroke in this study based on our established surgical procedure [1,20,21]. Briefly, we left a 6.0 monofilament nylon suture with silicone-coated tip in the origin of the right middle cerebral artery for 60 min before withdrawing it, and used laser Doppler flowmetry (Moor Instruments, Devon, UK) to define a successful model as an approximately 80% decrease in cerebral blood flow. We subjected sham mice to an identical operation, except that we immediately withdrew the filament after introducing it to the origin of the right middle cerebral artery.

2.3. Experimental groups

We randomly [22] assigned mice to four groups: (1) sham mice treated with vehicle (Sham + vehicle, $n = 28$), (2) sham mice treated with Wnt-3a (Sham + Wnt-3a, $n = 28$), (3) tMCAO mice treated with vehicle (tMCAO + vehicle, $n = 38$), and (4) tMCAO mice treated with Wnt-3a (tMCAO + Wnt-3a, $n = 38$).

We reconstituted the recombinant Wnt-3a (R&D Systems, Minneapolis, MN, USA) in saline containing 0.1% bovine serum albumin (BSA) according to previous study [18,19]. We intranasally delivered Wnt-3a (2 μg/kg) or vehicle control (saline containing 0.1% BSA, the volume of vehicle is the same as that of Wnt-3a solution) at the time of reperfusion or at 1 h after sham operation, and repeated once a day for the next two days [18,23,24]. Mice were daily examined for infection or illness.

2.4. Infarct volume assessment

We assessed the lesion size three days after surgery as previously described [1,25]. Briefly, the 2-mm thick coronal brain sections sliced by using a mouse brain matrix slicer (Stoelting Instruments, Wood Dale, IL, USA) were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37 °C and fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde overnight at 4 °C. The investigator blinded to treatment strategy used Image J (NIH, Bethesda, MD, USA) to determine the infarct area on the posterior surface in each brain slice by subtracting the ipsilateral hemisphere healthy area from the total contralateral hemisphere area to correct for brain swelling, and then measure the total infarct volume by linear integration of the corrected infarct areas. Infarct volume percentage was computed as: total infarct volume/contralateral hemisphere volume × 100%.

2.5. Brain water content measurement

We measured the brain water content three days after operation as previously described [1,20]. The wet weight was obtained by immediately weighing the right hemisphere. The brain samples were dried at 100 °C in an oven for 24 h to obtain the dry weights. The brain water content was calculated as: (wet weight – dry weight)/wet weight × 100%.

2.6. Neurologic deficits test

The neurologic deficits were tested in a blinded manner on days1, 3, 7 and 14 after ischemic stroke using a five-point scale described in our previous study [1]: zero-point represents no neurologic deficit, one-point represents that mouse fails to fully extent left forepaw, two-point represents that mouse circles to the contralateral side, three-point represents that mouse falls to the left, four-point represents no spontaneous walking, and five-point represents depressed level of consciousness.

2.7. Immunofluorescence

We obtained brain tissues three days after surgery for immunofluorescence analysis following our previous protocol [1,20,21]. Briefly, mice were deeply anesthetized by an intraperitoneal injection of overdosed 5% chloral hydrate, and transcardially perfused with PBS and the following 4% paraformaldehyde. The brains were carefully removed, fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated in 30% sucrose, and then sliced into 20-μm-thick coronal sections using cryoultramicrotomy (CM1100, Leica Biosystems, Germany). The sections were incubated in PBS containing 0.3% Triton X-100 (PBST),

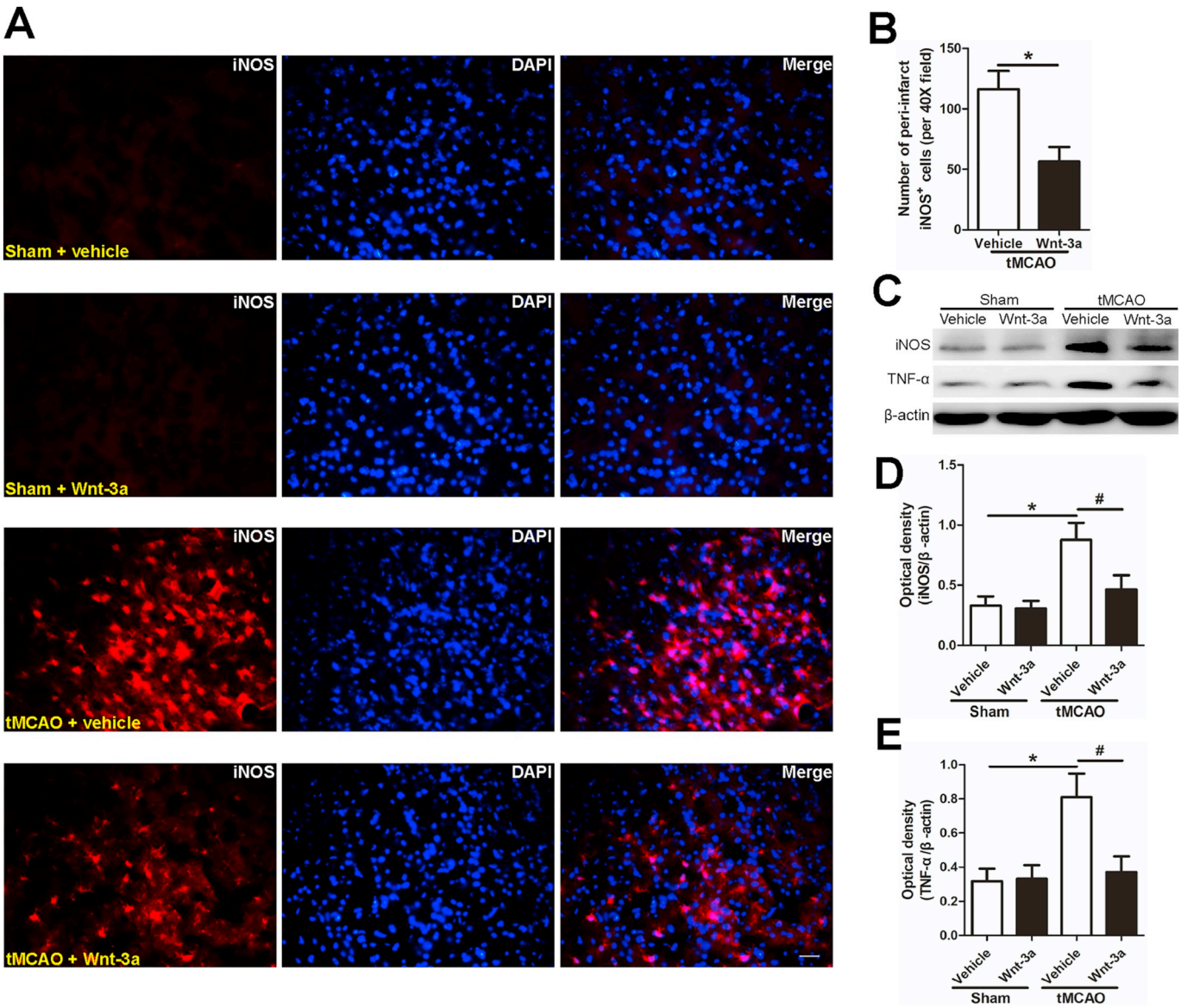

Fig. 4. Intranasal Wnt-3a reduces iNOS and TNF-α (M1 markers) expression at 72 h after stroke. (A) Immunofluorescence staining of the iNOS-positive cells in each group. Scale bar = 25 μm. (B) Quantification showing that intranasal Wnt-3a obviously decreased the number of peri-infarct iNOS-positive cells ($^{*}p < 0.05$, $n = 8$/group). (C) Western blot analysis of iNOS and TNF-α. (D) and (E) Quantification of iNOS and TNF-α protein levels in each group ($^{*}p < 0.05$ vs. Sham + vehicle group, $^{\#}p < 0.05$ vs. tMCAO + Wnt-3a group, $n = 8$/group). Data are shown as mean ± SD.

blocked in PBST containing 1% BSA, and incubated overnight at 4 °C with antibodies against CD68 (1:200, Abcam, Cambridge, MA, USA), inducible nitric oxide synthase (iNOS; 1:100, Novus Biologicals, Littleton, CO, USA), arginase 1 (Arg1; 1:200, Novus Biologicals), glial fibrillary acidic protein (GFAP; 1:1000, Abcam), C3 (1:50, Abcam) and S100A10 (1:20, R&D Systems). After being incubated with appropriate secondary antibodies, these sections were mounted with the mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI; Santa Cruz Biotech, Dallas, TX, USA). Three separate sections of each mouse and three non-overlapping 40× fields in peri-infarct areas were chose in a random and blinded manner to quantify CD68-positive microglia/macrophages, iNOS-positive cells, Arg1-positive cells, GFAP/C3-positive A1 astrocytes and GFAP/S100A10-positive A2 astrocytes. The sections were observed and quantified under a fluorescence microscope (ZEISS Scope A1, ZEISS, Germany).

2.8. Terminal deoxynucleotidyl tranferase-mediated uridine 5′-triphosphate-biotin nick end-labeling (TUNEL) assay

Brain sections were subjected to TUNEL staining according to the manufacturer's manual (Beyotime, Shanghai, China). Briefly, 20-μm-thick coronal brain sections were rinsed in PBS, incubated in 0.3% PBST for 5 min at room temperature, and then incubated in TUNEL reaction mixture (50 μl per section) for 60 min at 37 °C in the dark. After being washed in PBS and mounted with the mounting medium containing DAPI (Santa Cruz Biotech), these sections were observed under the fluorescence microscope (ZEISS). Three separate sections of each mouse and three non-overlapping 40× fields in peri-infarct regions were chose in a random and blinded manner to quantify TUNEL-positive cells.

2.9. Western blot analysis

We sacrificed mice for Western blot analysis three days after the surgical procedure as previously described [1,20,26]. Extracted protein

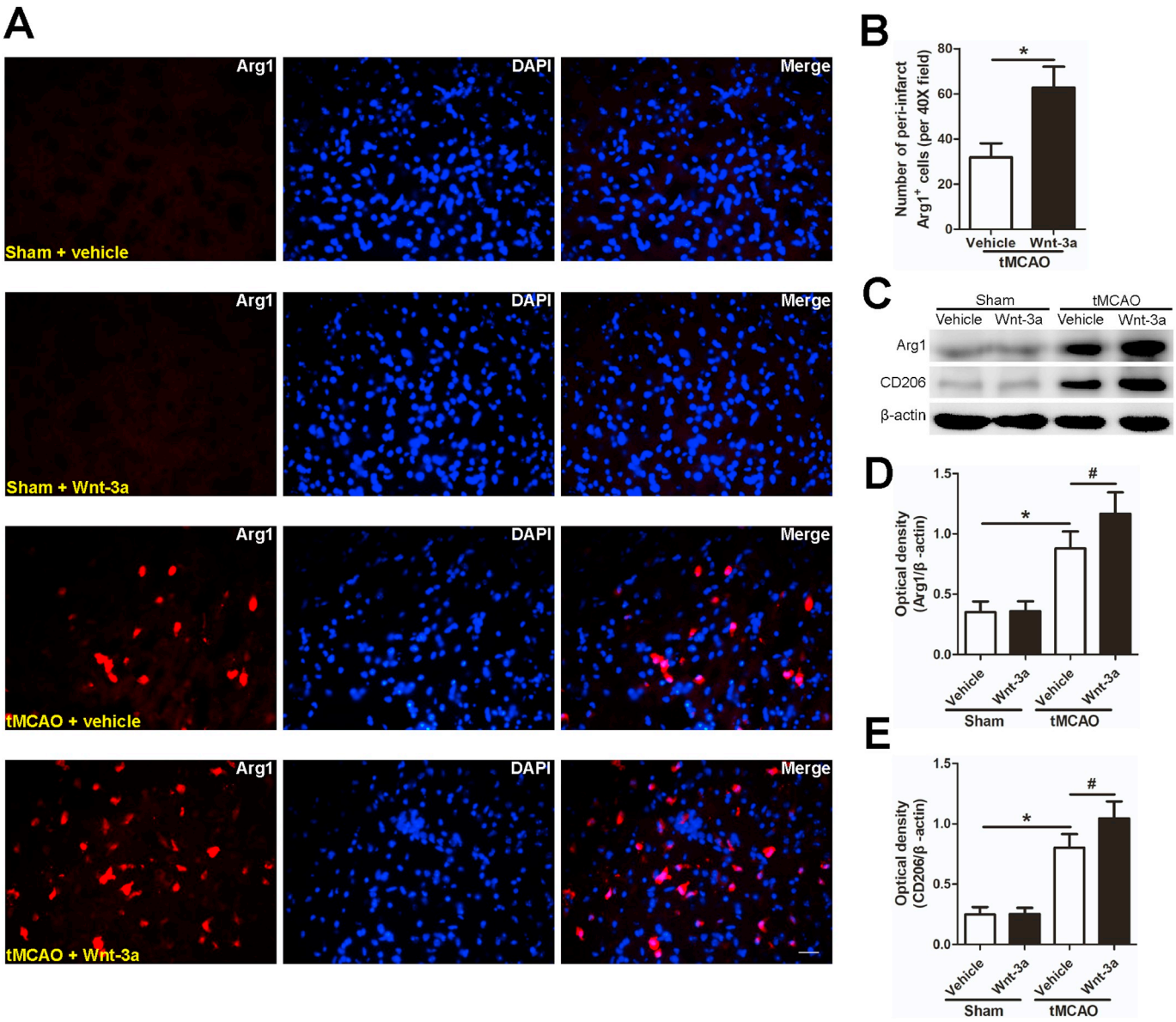

Fig. 5. Intranasal Wnt-3a elevates Arg1 and CD206 expression (M2 markers) at 72 h after stroke. (A) Immunofluorescence staining of the Arg1-positive cells in each group. Scale bar = 25 μm. (B) Quantification indicated that intranasal Wnt-3a apparently increased the number of peri-infarct Arg1-positive cells ($^{*}p < 0.05$, $n = 8$/group). (C) Western blot analysis of Arg1 and CD206. (D) and (E) Quantification of Arg1 and CD206 protein levels in each group ($^{*}p < 0.05$ vs. Sham + vehicle group, $^{\#}p < 0.05$ vs. tMCAO + Wnt-3a group, $n = 8$/group). Data are shown as mean ± SD.

from the whole ipsilateral hemisphere was separated and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After being blocked in 5% nonfat milk dissolved in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST), the membranes were incubated overnight at 4 °C with antibodies against ionized calcium-binding adapter molecule (Iba)-1 (1:500, Novus Biologicals), iNOS (1:200, Novus Biologicals), tumor necrosis factor (TNF)-α (1:500, Abcam), Arg1 (1:2000, Novus Biologicals), CD206 (Mannose receptor; 1:1000, Abcam), GFAP (1:5000, Abcam), interleukin 15 (IL15; 1:500, Abcam), IL33 (1:1000, Abcam) and β-actin (1:3000, Affinity Biosciences). Subsequently, these membranes were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase for 2 h at room temperature, and protein bands were visualized by enhanced chemiluminescence kit (Affinity Biosciences). The β-actin protein served as a loading control. The optical density of the band was quantified in a blinded manner using Gel Analysis V 2.02 software (ClinX Science Instruments, Shanghai, China).

2.10. Statistical analysis

We carried out statistical analysis with SPSS version 13.0. Results are expressed as mean ± SD. We used repeated measures ANOVA to analyze neurologic deficit score. We used Student's *t*-test or one-way ANOVA followed by LSD test to analyze changes in infarct volume, brain water content, TUNEL assay, Western blot analysis, and immunofluorescence. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effect of intranasal Wnt-3a on infarct volume, brain water content and neurologic deficits after ischemic stroke

At 72 h after ischemic stroke, we carried out TTC staining and found that intranasal Wnt-3a significantly reduced infarct volume (Fig. 1A, B). The brain water content was higher in the tMCAO + vehicle group than

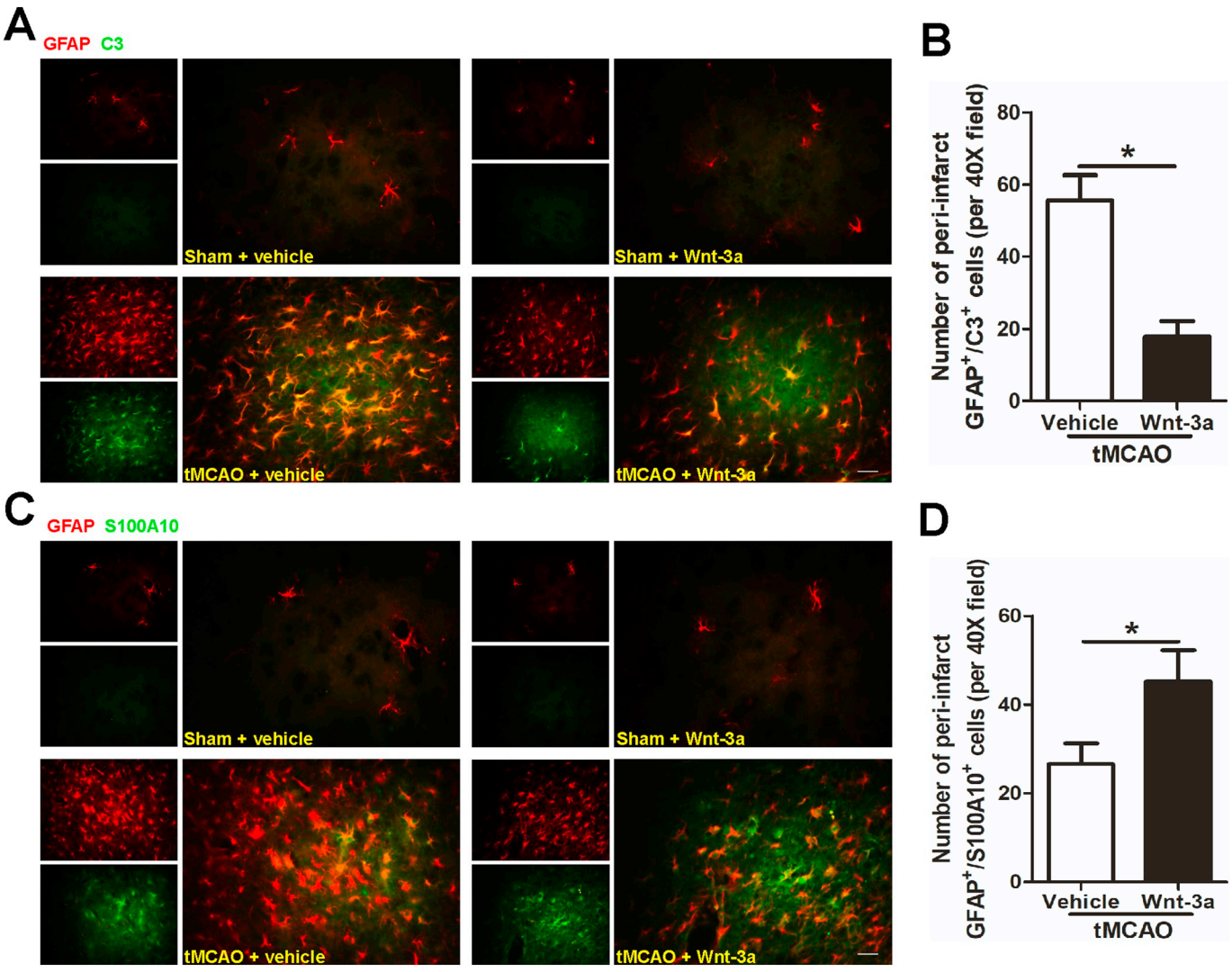

Fig. 6. Effects of intranasal Wnt-3a on A1 and A2 reactive astrocytes at 72 h post-stroke. (A) and (C) Double immunofluorescence staining showed that GFAP/C3-positive A1 astrocytes and GFAP/S100A10-positive A2 astrocytes were present in peri-infarct areas. Scale bar = 25 μm. (B) and (D) Representative quantification showing that intranasal Wnt-3a significantly decreased the number of peri-infarct GFAP/C3-positive cells and increased the number of peri-infarct GFAP/S100A10-positive cells. ($^{*}p < 0.05$, $n = 8$/group). Data are shown as mean ± SD.

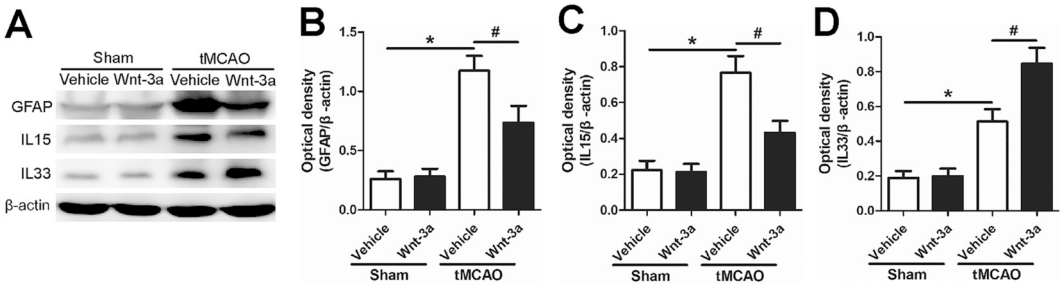

Fig. 7. Determination of GFAP, IL 15 and IL33 protein levels at 72 h post-surgery. (A) Western blot analysis of GFAP, IL 15 and IL33. (B, C and D) Quantification of these three protein levels in each group ($^{*}p < 0.05$ vs. Sham + vehicle group, $^{\#}p < 0.05$ vs. tMCAO + Wnt-3a group, $n = 8$/group). Data are shown as mean ± SD.

that in the Sham + vehicle group, but it was significantly lower in the tMCAO + Wnt-3a group than that in the tMCAO + vehicle group. No difference was apparent between the vehicle-treated and Wnt-3a-treated sham mice (Fig. 1C). Moreover, intranasal Wnt-3a significantly decreased stroke-induced elevation of neurologic deficit scores on days 3, 7, and 14 after cerebral infarction (Fig. 1D).

3.2. Intranasal Wnt-3a suppresses apoptosis after ischemic stroke

We performed TUNEL staining to identify the effect of intranasal Wnt-3a on peri-infarct apoptosis at 72 h after ischemic insult. We found that the apoptotic cells were largely induced in the tMCAO + vehicle group. However, the Wnt-3a-treated tMCAO mice had significantly fewer number of apoptotic cells than did the vehicle-treated tMCAO

mice. No change was notably different between the vehicle-treated and Wnt-3a-treated sham mice (Fig. 2A, B).

3.3. Intranasal Wnt-3a attenuates the activation of microglia/macrophages after ischemic stroke

In comparison with the vehicle-treated tMCAO mice, the Wnt-3a-treated mice had significantly fewer peri-infarct CD68-positive microglia/macrophages at 72 h after stroke (Fig. 3A, B). Furthermore, the protein level of Iba-1 measured by Western blot was apparently higher in the tMCAO + vehicle group than that in the Sham + vehicle group, but it was significantly decreased in the tMCAO + Wnt-3a group. We did not detect significant change of Iba-1 expression between the Sham + vehicle and Sham + Wnt-3a groups (Fig. 3C, D).

3.4. Intranasal Wnt-3a decreases the expression of iNOS and TNF-α (markers of M1 microglia/macrophages), and increases the expression of Arg1 and CD206 (markers of M2 microglia/macrophages) after ischemic stroke

We found that peri-infarct iNOS-positive cells were obviously fewer in the tMCAO + Wnt-3a group than that in the tMCAO + vehicle group at 72 h after cerebral infarction (Fig. 4A, B). Further investigation of protein levels showed that the expression of iNOS and TNF-α was significantly higher in the tMCAO mice treated with vehicle than that in the sham mice which received vehicle. The tMCAO mice treated with intranasal Wnt-3a had significantly lower levels of iNOS and TNF-α than did the tMCAO mice which received vehicle. Neither of the two protein levels differed significantly between the Sham + vehicle and Sham + Wnt-3a groups (Fig. 4C-E).

Immunoflurorescence staining showed that the Wnt-3a-administered tMCAO mice had significantly greater number of peri-infarct Arg1-positive cells than did the vehicle-administered tMCAO mice at 72 h after surgery (Fig. 5A, B). Western blot analysis further confirmed that the expression of Arg1 and CD206 was significantly higher in the vehicle-treated tMCAO mice than that in the vehicle-treated sham mice. The Arg1 and CD206 protein levels were notably higher in the tMCAO + Wnt-3a group than those in the tMCAO + vehicle group. Intranasal Wnt-3a did not apparently change the two protein levels in sham mice (Fig. 5C-E).

3.5. Intranasal Wnt-3a decreases the number of A1 astrocytes, and increases the number of A2 astrocytes after ischemic stroke

We investigated the presence of A1 astrocytes (labeled by GFAP/C3) and A2 astrocytes (labeled by GFAP/S100A10) in peri-infarct regions at 72 h post-stroke, and subsequently assessed the influence of intranasal Wnt-3a on the number of peri-infarct GFAP/C3-positive cells and GFAP/S100A10-positive cells by conducting immunoflurorescence staining. We found that GFAP/C3-positive cells and GFAP/S100A10-positive cells were obviously induced in tMCAO + vehicle group (Fig. 6A and C). The Wnt-3a-treated tMCAO mice had significant fewer GFAP/C3-positive cells than did the vehicle-treated tMCAO mice (Fig. 6A, B). The number of GFAP/S100A10-positive cells in the tMCAO + Wnt-3a group was significant greater than that in the tMCAO + vehicle group (Fig. 6C, D).

3.6. Intranasal Wnt-3a reduces the GFAP and IL 15 protein levels, and elevates the IL33 protein level after ischemic stroke

We performed Western blot analysis to evaluate the effect of intranasal Wnt-3a on the protein levels of GFAP, IL 15 and IL33, and found that these three protein levels were significantly higher in the tMCAO + vehicle group than those in the Sham + vehicle group at 72 h after brain ischemia. Furthermore, the Wnt-3a-delivered mice had significantly lower levels of GFAP and IL 15, and higher expression of IL33 than did the vehicle-delivered mice. However, none of these protein levels was apparently different between the Sham + vehicle and Sham + Wnt-3a groups (Fig. 7A-D).

4. Discussion

In this study, we investigated the effect of intranasal Wnt-3a on ischemic stroke-induced reactive microglia/macrophages and astrocytes using the tMCAO mouse model. We found that intranasal Wnt-3a contributed to better outcomes after ischemic stroke. Further, intranasal Wnt-3a decreased the number of peri-infarct apoptotic cells and CD68-positive microglia/macrophages, reduced the ipsilateral protein levels of iNOS and TNF-α (markers of M1 microglia/macrophages), and upregulated the expression of Arg1 and CD206 (markers of M2 microglia/macrophages). Lastly, intranasal Wnt-3a promoted A2 astrocyte (labeled by GFAP/S100A10) polarization, reduced the expression of GFAP and IL15, and elevated the expression of IL33. Our study suggests that intranasal Wnt-3a might alleviate ischemic brain injury, at least partly through regulating reactive microglia/macrophages and astrocytes.

Wnt-3a is an endogenous glycolipoprotein which stimulates the canonical Wnt/β-catenin signaling pathway by reducing GSK-3β activity and stabilizing β-catenin [17,27]. Since Wnt/β-catenin signaling pathway participates in many brain pathologies and targets multiple cells, such as neuron, neural stem cell, and glial cell, its role in central nervous system (CNS) has been acquiring close attention [15]. Studies have characterized that Wnt-3a mainly binds the Frizzled-1 receptor, which needs low-density lipid receptor-related protein 5/6 functioning as co-receptor, and subsequently recruits the phosphoprotein dishevelled and Axin, leading to the suppression of GSK-3β-stimulated phosphorylation and degradation of β-catenin. Stabilized β-catenin translocates into the nucleus and interacts with the T-cell specific transcription factor and the lymphoid enhancer-binding factor, and then promotes transcription of Wnt target genes [15,16,28].

Intranasal delivery has been generally considered as a non-invasive and clinically relevant approach for drug administration to the central nervous system, including intranasal application of exogenous recombinant Wnt-3a [24,29–31]. Studies showed the beneficial effects of intranasal Wnt-3a on neuronal apoptosis, autophagy, blood-brain barrier dysfunction, and neurogenesis in models of MCAO, focal ischemic stroke and TBI [16,18,19]. However, the influence of intranasal Wnt-3a on reactive microglia/macrophages and astrocytes after brain ischemia remains unknown. In this study, we treated tMCAO or sham mice with a dose of 2 μg/kg/d intranasal Wnt-3a according to literature which showed neuroprotective roles without side effects of this treatment strategy [18,19]. We found that intranasal Wnt-3a significantly decreased infarct volume and promoted neurologic recovery after ischemic stroke, consistent with the previous studies showing therapeutic potential of intranasal Wnt-3a [16,18,19]. In addition, we showed that intranasal Wnt-3a notably reduced stroke-induced cerebral edema and the number of peri-infarct apoptotic cells. Brain edema mainly results from BBB disruption that leads to the increase of cerebral vascular permeability and is the major cause of neurologic dysfunction and mortality during acute cerebral infarction [1,32,33]. Our findings suggest that intranasal Wnt-3a might improve BBB integrity at 72 h post-stroke. Although intranasal Wnt-3a has been showed to promote BBB stabilization at 48 h post-TBI [19], further investigation into the effect of intranasal Wnt-3a on the dynamic of BBB breakdown after ischemic stroke and the underlying mechanism of action should be conducted. Recent studies has demonstrated that rapid matrix metalloproteinase (MMP)-9, not MMP-2, activation at pericyte somata before capillary leakage contributes to BBB disruption in the capillary bed during acute cerebral infarction [34]. We hypothesize that intranasal Wnt-3a might promote pericyte survival and reduce the release of MMP-9 during acute ischemic stroke, which needs careful investigation.

Acute cerebral infarction initiates rapid activation of the resident microglia and the invading macrophages [35,36], and leads to subsequent polarization of reactive microglia/macrophages towards toxic M1 and protective M2 phenotypes [3,37]. Accumulating evidence suggests that M2 microglia/macrophages transiently increase and then switch to M1 microglia/macrophages in the first 3 days after ischemic stroke [38,39]. Therefore, we chose 72 h post-ischemia as the time point of observation in this study. We found that intranasal Wnt-3a significantly reduced the peri-infarct CD68-positive, which is mainly expressed in reactive microglia/macrophages [40], cell number. Furthermore, our results showed that intranasal Wnt-3a reduced Iba-1, iNOS and TNF-α protein levels, and increased Arg1 and CD206 protein levels. Since iNOS and TNF-α are well defined markers of M1 microglia/macrophages, and Arg1 and CD206 are generally considered as markers of M2 microglia/macrophages [3,41,42], our results indicates that Wnt-3a may reverse the switch of M2 microglia/macrophages to M1 at 72 h after ischemic stroke. Although previous study showed that Wnt-3a derived the pro-inflammatory transformation in primary mouse microglia cells [43,44], we did not observed significantly elevated expression of pro-inflammatory iNOS and TNF-α in intranasal Wnt-3a-treated sham mice, possibly because the in vivo environment has more complicated cell-cell communication than the in vitro environment with single cell population. These findings suggest that intranasal Wnt-3a could balance stroke-induced reactive microglia/macrophages into neuroprotective polarization.

Astrocytes are the most abundant cells in the mammalian CNS that perform many functions important to maintain normal brain function [45]. Although studies have generally revealed that ischemic stroke strongly induces the activation of astrocytes [2,46], the influence of reactive astrocytes on the recovery of neurological function after ischemic stroke remains controversial. Recent studies showed the reactive astrocyte polarization, called A1 (neurotoxic phenotype) and A2 (neuroprotective phenotype) astrocytes, in some animal models including Parkinson's disease, spinal cord injury, Alzheimer's disease and aging [47–51]. However, the reactive astrocyte polarization after ischemic stroke and the effect of intranasal Wnt-3a on it are still not well defined. In the present study, we found that A1 and A2 astrocytes existed in the peri-infarct zones after ischemic stroke. Intranasal Wnt-3a could decrease the number of A1 astrocytes and increase the number of A2 astrocytes. Recent study uncovered the crucial effect of reactive microglia on astrocyte polarization [4]. Although we didn't show whether intranasal Wnt-3a directly targeted stroke-induced reactive astrocytes, our findings at least suggest a kind of indirectly regulation. We further showed that intranasal Wnt-3a reduced the expression of GFAP and IL15, and elevated the protein level of IL33. Previous studies have identified that reactive astrocytes are specific or major source of IL15 and IL33 in the CNS [52–55]. IL15 could aggravate brain damage after stroke by inducing neurotoxic glial activation and neuroinflammation [56,57]. IL33 could attenuate the ischemic brain injury after stroke through promoting beneficial microglial responses [58,59]. Considering that A1 and A2 astrocytes derived toxic and protective factors remain largely unclear, we hypothesize that IL15 might be one of the A1 astrocytes derived neurotoxic factors, and IL33 might be one of the A2 astrocytes derived neuroprotective factors, which needs further careful investigation to make a convincing conclusion. Our present findings suggest that intranasal Wnt-3a could facilitate neuroprotective responses of astrocytes after ischemic stroke.

There are some limitations in our present study. We didn't investigate the mechanistic basis for the action of Wnt-3a, while we believed that identification of the mechanism by which Wnt-3a directly or indirectly modulated the activation and polarization of microglia/macrophages and astrocytes, such as the possible interaction between Wnt-3a/GSK-3β/β-catenin pathway and nuclear factor erythroid-2 related factor 2 (Nrf2), signal transducers and activators of transcription 3 (STAT3) and nuclear factor κB (NFκB) pathways, could provide better comprehension of intranasal Wnt-3a promoting functional recovery after ischemic stroke. Additionally, we didn't assess the expression of GSK-3β and β-catenin, because previous study verified that intranasal Wnt-3a could inhibit GSK-3β activity and increase the β-catenin protein level. Although we mainly explored the effect of intranasal Wnt-3a on the reactive microglia/macrophages and astrocytes three days after ischemic stroke, it would be interesting to investigate the long-term influence of intranasal Wnt-3a on the stroke-induced neuroinflammation.

5. Conclusion

In conclusion, we confirmed that intranasal Wnt-3a protects against ischemic brain injury and this beneficial action might be at least partly mediated by modulating the activation and polarization of microglia/macrophages and astrocytes. Our findings could further support the assumption that Wnt-3a may potentially be considered as a therapeutic agent for ischemic stroke.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

References

[1] J. Wang, D. Zhang, X. Fu, L. Yu, Z. Lu, Y. Gao, X. Liu, J. Man, S. Li, N. Li, X. Chen, M. Hong, Q. Yang, J. Wang, Carbon monoxide-releasing molecule-3 protects against ischemic stroke by suppressing neuroinflammation and alleviating blood-brain barrier disruption, J. Neuroinflammation 15 (1) (2018) 188.
[2] Z. Liu, M. Chopp, Astrocytes, therapeutic targets for neuroprotection and neurorestoration in ischemic stroke, Prog. Neurobiol. 144 (2016) 103–120.
[3] Y. Ma, J. Wang, Y. Wang, G.Y. Yang, The biphasic function of microglia in ischemic stroke, Prog. Neurobiol. 157 (2017) 247–272.
[4] S.A. Liddelow, K.A. Guttenplan, L.E. Clarke, F.C. Bennett, C.J. Bohlen, L. Schirmer, M.L. Bennett, A.E. Munch, W.S. Chung, T.C. Peterson, D.K. Wilton, A. Frouin, B.A. Napier, N. Panicker, M. Kumar, M.S. Buckwalter, D.H. Rowitch, V.L. Dawson, T.M. Dawson, B. Stevens, B.A. Barres, Neurotoxic reactive astrocytes are induced by activated microglia, Nature 541 (7638) (2017) 481–487.
[5] S.A. Liddelow, B.A. Barres, Reactive astrocytes: production, function, and therapeutic potential, Immunity 46 (6) (2017) 957–967.
[6] L.E. Clarke, S.A. Liddelow, C. Chakraborty, A.E. Munch, M. Heiman, B.A. Barres, Normal aging induces A1-like astrocyte reactivity, Proc. Natl. Acad. Sci. U. S. A. 115 (8) (2018) E1896–e1905.
[7] S.P. Yun, T.I. Kam, N. Panicker, S. Kim, Y. Oh, J.S. Park, S.H. Kwon, et al., Block of A1 astrocyte conversion by microglia is neuroprotective in models of Parkinson's disease, Nat. Med. 24 (7) (2018) 931–938.
[8] J. Duan, J. Cui, Z. Yang, C. Guo, J. Cao, M. Xi, Y. Weng, Y. Yin, Y. Wang, G. Wei, B. Qiao, A. Wen, Neuroprotective effect of Apelin 13 on ischemic stroke by activating AMPK/GSK-3beta/Nrf2 signaling, J. Neuroinflammation 16 (1) (2019) 24.
[9] S. Ramagiri, R. Taliyan, Remote limb ischemic post conditioning during early reperfusion alleviates cerebral ischemic reperfusion injury via GSK-3beta/CREB/BDNF pathway, Eur. J. Pharmacol. 803 (2017) 84–93.
[10] B. D'Angelo, C.J. Ek, Y. Sun, C. Zhu, M. Sandberg, C. Mallard, GSK3beta inhibition protects the immature brain from hypoxic-ischaemic insult via reduced STAT3 signalling, Neuropharmacology 101 (2016) 13–23.
[11] W. Wang, M. Li, Y. Wang, Q. Li, G. Deng, J. Wan, Q. Yang, Q. Chen, J. Wang, GSK-3beta inhibitor TWS119 attenuates rtPA-induced hemorrhagic transformation and activates the Wnt/beta-catenin signaling pathway after acute ischemic stroke in rats, Mol. Neurobiol. 53 (10) (2016) 7028–7036.
[12] A.K. Rana, D. Singh, Targeting glycogen synthase kinase-3 for oxidative stress and neuroinflammation: opportunities, challenges and future directions for cerebral stroke management, Neuropharmacology 139 (2018) 124–136.
[13] S. Huang, E. Turlova, F. Li, M.H. Bao, V. Szeto, R. Wong, A. Abussaud, H. Wang, S. Zhu, X. Gao, Y. Mori, Z.P. Feng, H.S. Sun, Transient receptor potential melastatin 2 channels (TRPM2) mediate neonatal hypoxic-ischemic brain injury in mice, Exp. Neurol. 296 (2017) 32–40.
[14] A. Cuadrado, S. Kugler, I. Lastres-Becker, Pharmacological targeting of GSK-3 and NRF2 provides neuroprotection in a preclinical model of tauopathy, Redox Biol. 14 (2018) 522–534.
[15] C. Lambert, P. Cisternas, N.C. Inestrosa, Role of Wnt signaling in central nervous system injury, Mol. Neurobiol. 53 (4) (2016) 2297–2311.
[16] N. Matei, J. Camara, D. McBride, R. Camara, N. Xu, J. Tang, J.H. Zhang, Intranasal wnt3a attenuates neuronal apoptosis through Frz1/PIWIL1a/FOXM1 pathway in MCAO rats, J. Neurosci. 38 (30) (2018) 6787–6801.

[17] Y. Zhao, S.L. Gibb, J. Zhao, A.N. Moore, M.J. Hylin, T. Menge, H. Xue, G. Baimukanova, D. Potter, E.M. Johnson, J.B. Holcomb, C.S. Cox Jr., P.K. Dash, S. Pati, Wnt3a, a protein secreted by mesenchymal stem cells is neuroprotective and promotes neurocognitive recovery following traumatic brain injury, Stem Cells 34 (5) (2016) 1263–1272.

[18] Z.Z. Wei, J.Y. Zhang, T.M. Taylor, X. Gu, Y. Zhao, L. Wei, Neuroprotective and regenerative roles of intranasal Wnt-3a administration after focal ischemic stroke in mice, J. Cereb. Blood Flow Metab. 38 (3) (2018) 404–421.

[19] J.Y. Zhang, J.H. Lee, X. Gu, Z.Z. Wei, M.J. Harris, S.P. Yu, L. Wei, Intranasally delivered Wnt3a improves functional recovery after traumatic brain injury by modulating autophagic, apoptotic, and regenerative pathways in the mouse brain, J. Neurotrauma 35 (5) (2018) 802–813.

[20] J. Wang, Z. Lu, X. Fu, D. Zhang, L. Yu, N. Li, Y. Gao, X. Liu, C. Yin, J. Ke, L. Li, M. Zhai, S. Wu, J. Fan, L. Lv, J. Liu, X. Chen, Q. Yang, J. Wang, Alpha-7 nicotinic receptor signaling pathway participates in the neurogenesis induced by ChAT-positive neurons in the subventricular zone, Transl. Stroke Res. 8 (2017) 484–493.

[21] J. Wang, X. Fu, D. Zhang, L. Yu, Z. Lu, Y. Gao, X. Liu, J. Man, S. Li, N. Li, M. Wang, X. Liu, X. Chen, W. Zang, Q. Yang, J. Wang, Effects of crenolanib, a nonselective inhibitor of PDGFR, in a mouse model of transient middle cerebral artery occlusion, Neuroscience 364 (2017) 202–211.

[22] T. Cheng, W. Wang, Q. Li, X. Han, J. Xing, C. Qi, X. Lan, J. Wan, A. Potts, F. Guan, J. Wang, Cerebroprotection of flavanol (−)-epicatechin after traumatic brain injury via Nrf2-dependent and -independent pathways, Free Radic. Biol. Med. 92 (2016) 15–28.

[23] L.R. Hanson, J.M. Fine, A.L. Svitak, K.A. Faltesek, Intranasal administration of CNS therapeutics to awake mice, J. Vis. Exp. (74) (2013), https://doi.org/10.3791/4440.

[24] T.P. Crowe, M.H.W. Greenlee, A.G. Kanthasamy, W.H. Hsu, Mechanism of intranasal drug delivery directly to the brain, Life Sci. 195 (2018) 44–52.

[25] J. Wang, X. Liu, H. Lu, C. Jiang, X. Cui, L. Yu, X. Fu, Q. Li, J. Wang, CXCR4(+)CD45(−) BMMNC subpopulation is superior to unfractionated BMMNCs for protection after ischemic stroke in mice, Brain Behav. Immun. 45 (2015) 98–108.

[26] J. Wang, X. Fu, D. Zhang, L. Yu, N. Li, Z. Lu, Y. Gao, M. Wang, X. Liu, C. Zhou, W. Han, B. Yan, J. Wang, ChAT-positive neurons participate in subventricular zone neurogenesis after middle cerebral artery occlusion in mice, Behav. Brain Res. 316 (2017) 145–151.

[27] R. Nusse, H. Clevers, Wnt/beta-catenin signaling, disease, and emerging therapeutic modalities, Cell 169 (6) (2017) 985–999.

[28] M.B. Kilander, C. Halleskog, G. Schulte, Recombinant WNTs differentially activate beta-catenin-dependent and -independent signalling in mouse microglia-like cells, Acta Physiol. 203 (3) (2011) 363–372.

[29] F. Erdo, L.A. Bors, D. Farkas, A. Bajza, S. Gizurarson, Evaluation of intranasal delivery route of drug administration for brain targeting, Brain Res. Bull. 143 (2018) 155–170.

[30] S. Ganger, K. Schindowski, Tailoring formulations for intranasal nose-to-brain delivery: a review on architecture, physico-chemical characteristics and mucociliary clearance of the nasal olfactory mucosa, Pharmaceutics 10 (3) (2018) 116.

[31] V. Bourganis, O. Kammona, A. Alexopoulos, C. Kiparissides, Recent advances in carrier mediated nose-to-brain delivery of pharmaceutics, Eur. J. Pharm. Biopharm. 128 (2018) 337–362.

[32] X. Jiang, A.V. Andjelkovic, L. Zhu, T. Yang, M.V.L. Bennett, J. Chen, R.F. Keep, Y. Shi, Blood-brain barrier dysfunction and recovery after ischemic stroke, Prog. Neurobiol. 163-164 (2018) 144–171.

[33] W. Wang, M. Li, Q. Chen, J. Wang, Hemorrhagic transformation after tissue plasminogen activator reperfusion therapy for ischemic stroke: mechanisms, models, and biomarkers, Mol. Neurobiol. 52 (3) (2015) 1572–1579.

[34] R.G. Underly, M. Levy, D.A. Hartmann, R.I. Grant, A.N. Watson, A.Y. Shih, Pericytes as inducers of rapid, matrix metalloproteinase-9-dependent capillary damage during ischemia, J. Neurosci. 37 (1) (2017) 129–140.

[35] X.Y. Xiong, L. Liu, Q.W. Yang, Functions and mechanisms of microglia/macrophages in neuroinflammation and neurogenesis after stroke, Prog. Neurobiol. 142 (2016) 23–44.

[36] K.L. Lambertsen, B. Finsen, B.H. Clausen, Post-stroke inflammation-target or tool for therapy? Acta Neuropathol. 137 (5) (2019) 693–714.

[37] J. Tsuyama, A. Nakamura, H. Ooboshi, A. Yoshimura, T. Shichita, Pivotal role of innate myeloid cells in cerebral post-ischemic sterile inflammation, Semin. Immunopathol. 40 (2018) 523–538.

[38] Y. Wang, Y. Huang, Y. Xu, W. Ruan, H. Wang, Y. Zhang, J.M. Saavedra, L. Zhang, Z. Huang, T. Pang, A dual AMPK/Nrf2 activator reduces brain inflammation after stroke by enhancing microglia M2 polarization, Antioxid. Redox Signal. 28 (2) (2018) 141–163.

[39] M. Kanazawa, I. Ninomiya, M. Hatakeyama, T. Takahashi, T. Shimohata, Microglia and monocytes/macrophages polarization reveal novel therapeutic mechanism against stroke, Int. J. Mol. Sci. 18 (10) (2017) 2135.

[40] D.A.E. Hendrickx, C.G. van Eden, K.G. Schuurman, J. Hamann, I. Huitinga, Staining of HLA-DR, Iba1 and CD68 in human microglia reveals partially overlapping expression depending on cellular morphology and pathology, J. Neuroimmunol. 309 (2017) 12–22.

[41] X. Hu, R.K. Leak, Y. Shi, J. Suenaga, Y. Gao, P. Zheng, J. Chen, Microglial and macrophage polarization-new prospects for brain repair, Nat. Rev. Neurol. 11 (1) (2015) 56–64.

[42] X. Lan, X. Han, Q. Li, Q.W. Yang, J. Wang, Modulators of microglial activation and polarization after intracerebral haemorrhage, Nat. Rev. Neurol. 13 (7) (2017) 420–433.

[43] C. Halleskog, J. Mulder, J. Dahlstrom, K. Mackie, T. Hortobagyi, H. Tanila, L. Kumar Puli, K. Farber, T. Harkany, G. Schulte, WNT signaling in activated microglia is proinflammatory, Glia 59 (1) (2011) 119–131.

[44] C. Halleskog, G. Schulte, WNT-3A and WNT-5A counteract lipopolysaccharide-induced pro-inflammatory changes in mouse primary microglia, J. Neurochem. 125 (6) (2013) 803–808.

[45] A.R. Filous, J. Silver, Targeting astrocytes in CNS injury and disease: a translational research approach, Prog. Neurobiol. 144 (2016) 173–187.

[46] S. Koizumi, Y. Hirayama, Y.M. Morizawa, New roles of reactive astrocytes in the brain; an organizer of cerebral ischemia, Neurochem. Int. 119 (2018) 107–114.

[47] M. Neal, J. Luo, D.S. Harischandra, R. Gordon, S. Sarkar, H. Jin, V. Anantharam, L. Desaubry, A. Kanthasamy, A. Kanthasamy, Prokineticin-2 promotes chemotaxis and alternative A2 reactivity of astrocytes, Glia 66 (10) (2018) 2137–2157.

[48] A. Fujita, H. Yamaguchi, R. Yamasaki, Y. Cui, Y. Matsuoka, K.I. Yamada, J.I. Kira, Connexin 30 deficiency attenuates A2 astrocyte responses and induces severe neurodegeneration in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride Parkinson's disease animal model, J. Neuroinflammation 15 (1) (2018) 227.

[49] W. Liu, Y. Wang, F. Gong, Y. Rong, Y. Luo, P. Tang, Z. Zhou, Z. Zhou, T. Xu, T. Jiang, S. Yang, G. Yin, J. Chen, J. Fan, W. Cai, Exosomes derived from bone mesenchymal stem cells repair traumatic spinal cord injury by suppressing the activation of A1 neurotoxic reactive astrocytes, J. Neurotrauma 36 (3) (2019) 469–484.

[50] X. Xu, A. Zhang, Y. Zhu, W. He, W. Di, Y. Fang, X. Shi, MFG-E8 reverses microglial-induced neurotoxic astrocyte (A1) via NF-kappaB and PI3K-Akt pathways, J. Cell. Physiol. 234 (1) (2018) 904–914.

[51] E.J. Goetzl, J.B. Schwartz, E.L. Abner, G.A. Jicha, D. Kapogiannis, High complement levels in astrocyte-derived exosomes of Alzheimer disease, Ann. Neurol. 83 (3) (2018) 544–552.

[52] D. Gomez-Nicola, B. Valle-Argos, D.W. Pita-Thomas, M. Nieto-Sampedro, Interleukin 15 expression in the CNS: blockade of its activity prevents glial activation after an inflammatory injury, Glia 56 (5) (2008) 494–505.

[53] Z. Li, J. Han, H. Ren, C.G. Ma, F.D. Shi, Q. Liu, M. Li, Astrocytic interleukin-15 reduces pathology of neuromyelitis optica in mice, Front. Immunol. 9 (2018) 523.

[54] G. Wicher, U. Wallenquist, Y. Lei, M. Enoksson, X. Li, B. Fuchs, S. Abu Hamdeh, N. Marklund, L. Hillered, G. Nilsson, K. Forsberg-Nilsson, Interleukin-33 promotes recruitment of microglia/macrophages in response to traumatic brain injury, J. Neurotrauma 34 (22) (2017) 3173–3182.

[55] I.D. Vainchtein, G. Chin, et al., Astrocyte-derived interleukin-33 promotes microglial synapse engulfment and neural circuit development, Science 359 (6381) (2018) 1269–1273.

[56] M. Li, Z. Li, Y. Yao, W.N. Jin, K. Wood, Q. Liu, F.D. Shi, J. Hao, Astrocyte-derived interleukin-15 exacerbates ischemic brain injury via propagation of cellular immunity, Proc. Natl. Acad. Sci. U. S. A. 114 (3) (2017) E396–e405.

[57] G.A. Lee, T.N. Lin, C.Y. Chen, S.Y. Mau, W.Z. Huang, Y.C. Kao, R.Y. Ma, N.S. Liao, Interleukin 15 blockade protects the brain from cerebral ischemia-reperfusion injury, Brain Behav. Immun. 73 (2018) 562–570.

[58] Q. Luo, Y. Fan, L. Lin, J. Wei, Z. Li, Y. Li, S. Nakae, W. Lin, Q. Chen, Interleukin-33 protects ischemic brain injury by regulating specific microglial activities, Neuroscience 385 (2018) 75–89.

[59] Y. Yang, H. Liu, H. Zhang, Q. Ye, J. Wang, B. Yang, L. Mao, W. Zhu, R.K. Leak, B. Xiao, B. Lu, J. Chen, X. Hu, ST2/IL-33-dependent microglial response limits acute ischemic brain injury, J. Neurosci. 37 (18) (2017) 4692–4704.