Inhibition of TGFβ-activated kinase 1 promotes inflammation-resolving microglial/macrophage responses and recovery after stroke in ovariectomized female mice

Yaan Liu¹, Sicheng Li¹, Rongrong Wang, Hongjian Pu, Yongfang Zhao, Qing Ye, Yejie Shi *  

Pittsburgh Institute of Brain Disorders & Recovery and Department of Neurology, University of Pittsburgh, Pittsburgh, PA 15213, United States of America

ARTICLE INFO

Keywords:  
5Z-7-Oxozeaenol  
Inflammation  
MAP3K7  
Ovariectomized mice  
TAK1  
Transient focal cerebral ischemia

ABSTRACT

TGFβ-activated kinase 1 (TAK1) is a master regulator that drives multiple cell death and proinflammatory signaling pathways, making it a promising therapeutic target to treat ischemic stroke. However, whether targeting TAK1 could improve stroke outcomes has never been tested in female subjects, hindering its potential translation into clinical use. Here we examined the therapeutic effect of 5Z-7-Oxozeaenol (OZ), a selective TAK1 inhibitor, in ovariectomized female mice after middle cerebral artery occlusion (MCAO). OZ significantly reduced neuronal cell death and axonal injury at the acute stage and mitigated neuroinflammation at the subacute stage after MCAO in ovariectomized female mice. Consistent with RNA sequencing analysis that TAK1 activation contributed to microglia/macrophage-mediated inflammatory responses in the post-stroke brain, inhibition of TAK1 with OZ caused phenotypic shift of microglia/macrophages toward an inflammation-resolving state. Furthermore, microglia/macrophage-specific TAK1 knockout (TAK1 mKO) reproduced OZ’s effects, causally confirming the role of TAK1 in determining proinflammatory microglial/macrophage responses in post-stroke females. Post-stroke treatment with OZ for 5 days effectively promoted long-term neurological recovery and the integrity of both gray matter and white matter in female mice. Together, the TAK1 inhibitor OZ elicits long-lasting improvement of stroke outcomes in female mice, at least partially through enhancing beneficial microglial/macrophage responses and inflammation resolution. Given its therapeutic efficacy on both male and female rodents, TAK1 inhibitor is worth further investigation as a valid treatment to ischemic stroke.

1. Introduction

Despite tremendous efforts in developing therapies to treat ischemic stroke in experimental models, no neuroprotective strategy has been successfully translated into clinical stroke treatment. Among the multifaceted reasons underlying this failure, a mismatch between research subjects in preclinical stroke studies and the real patient population in clinical settings may be a key explanation. Approximately 55,000 more females than males have a stroke each year (Virani et al., 2020), but less than 5% of preclinical ischemic stroke studies examined stroke females. Post-stroke treatment with OZ for 5 days effectively promoted long-term neurological recovery and the integrity of both gray matter and white matter in female mice. Together, the TAK1 inhibitor OZ elicits long-lasting improvement of stroke outcomes in female mice, at least partially through enhancing beneficial microglial/macrophage responses and inflammation resolution. Given its therapeutic efficacy on both male and female rodents, TAK1 inhibitor is worth further investigation as a valid treatment to ischemic stroke.

Abbreviations: Arg1, arginase 1; β-APP, β-amyloid precursor protein; Caspr, contactin-associated protein; CL, contralateral; DEG, differentially expressed gene; EC, external capsule; GO, gene ontology; IL, ipsilesional; IPA, Ingenuity Pathway Analysis; MAP2, microtubule-associated protein 2; MAP3K, mitogen-activated protein kinase kinase kinase; MBP, myelin basic protein; MCAO, middle cerebral artery occlusion; mKO, microglia/macrophage-specific knockout; NF200, neurofilament 200; NOR, nodes of Ranvier; OVX, ovariectomy; OZ, 5Z-7-Oxozeaenol; RNA-seq, RNA sequencing; SMI-32, non-phosphorylated neurofilament H; TAK1, TGFβ-activated kinase 1; WT, wild-type.

* Corresponding author at: Department of Neurology, University of Pittsburgh, 3500 Terrace Street, S-510 BST, Pittsburgh, PA, United States of America.

E-mail address: y.shi@pitt.edu (Y. Shi).

¹ Y.L. and S.L. contributed equally to this work.
a mitogen-activated protein kinase kinase (MAP3K) that governs multiple cell death and immune signaling pathways (Mihaly et al., 2014; Sato et al., 2005). TAK1 is rapidly activated in the brain following ischemic stroke, and inhibition of TAK1 reduces neuronal cell death and infarct volume at the acute and subacute stages (first 7 days) after stroke (Neubert et al., 2011; White et al., 2012). Another recent study by our group further extended the role of TAK1 to be a central regulator of microglia/macrophage-mediated neuroinflammation at the subacute and chronic stages after stroke, whereby TAK1 inhibition demonstrated long-lasting (up to 5 weeks) beneficial effects in stroke outcomes (Wang et al., 2020b). Although these studies identified TAK1 inhibition as a promising therapeutic approach, all existing studies on targeting TAK1 to treat ischemic stroke were based on male rodents. This caveat is especially important considering TAK1 inhibitors may exert beneficial functions through modulating microglia/macrophage-mediated inflammatory responses after stroke, as sex dimorphism in the behavior of microglia and other immune cells is well documented and could alter the efficacy of therapeutics in female subjects (Dotson and Offner, 2017; Kerr et al., 2019; Villa et al., 2018).

To fill the forementioned knowledge gap, the present study investigated the role of TAK1 in the brain after ischemic stroke using female mice. We aimed to 1) evaluate the therapeutic efficacy of TAK1 inhibitor in female mice, using clinically relevant long-term and functional parameters to facilitate its potential translation into a valid stroke treatment; and 2) investigate the mechanisms underlying TAK1 inhibitor-elicited improvement of stroke outcomes in female mice and whether there are sex differences with regards to the responses of microglia/macrophages.

2. Materials and methods

2.1. Animals

C57BL/6 J, TAK1loxP, and Cx3cr1CreER mice were purchased from the Jackson Laboratory (Table S1). Microglia/macrophage-specific TAK1 knockout (TAK1 mKO) mice were obtained by crossing the Cx3cr1creERT2 mice (Parkhurst et al., 2013) and TAK1loxP mice (Xie et al., 2006) for two generations. The TAK1 mKO mice (genotype: Cx3cr1creERT2/wt; Map3k7flox/fluox) were viable, fertile, and normal in size and did not exhibit any gross physical or behavioral abnormalities. To induce gene deletion, TAK1 mKO mice received intraperitoneal injections of tamoxifen (75 mg/kg daily for 5 days). Hemizygous Cx3cr1CreERT2 mice (genotype: Cx3cr1CreERT2/wt; Map3k7flox/fluox) served as age- and sex-matched wild-type (WT) control mice for the TAK1 mKO mice and received the same tamoxifen treatments.

Mice were housed in a specific pathogen-free facility with regulated temperature and humidity and a 12-h light/dark cycle. Food and water were available ad libitum. All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, approved by the University of Pittsburgh Institutional Animal Care and Use Committee, and reported following the ARRIVE guidelines (Kilkenny et al., 2010). All efforts were made to minimize animal suffering and the number of animals used.

2.2. Ovariectomy

To partially reproduce the conditions in postmenopausal women, female mice were subjected to bilateral ovariectomy (OVX), a model that mimics surgical menopause. Briefly, adult female mice (10–14 weeks old, 20–28 g) were anesthetized with 1.5% isoflurane in 67%:30% N2O/O2 and were placed in a prone position on a heating pad during the sterile surgical procedures. An incision was made between the last rib and the pelvis after the skin was disinfected, followed by separation of the skin and musculature. To remove the right ovary, the skin was pulled to the right by a metal hook to expose the ovary between the last rib and the right hip. An incision was made in the musculature above the right ovary, and the ovarian fat pad was pulled out of the incision. An electric coagulator was used to seal the region below the ovary, including part of the oviduct and vessels. The sealed site was then cut by scissors to remove the entire ovary, and the musculature was sutured. Similar procedures were conducted to remove the left ovary.

2.3. Transient focal cerebral ischemia model

Transient focal cerebral ischemia was induced in female mice 3 weeks after OVX by intraluminal occlusion of the left middle cerebral artery (MCAO) for 1 h, as previously described (Shi et al., 2016). Briefly, mice were anesthetized with 1.5% isoflurane in 67%:30% N2O/O2. A 7–0 monofilament with silicon-coated tip was introduced into the common carotid artery, advanced to the origin of the MCA, and left in place for 1 h. Rectal temperature was maintained at 37 ± 0.5 °C with a heating pad during surgical procedures. Regional cerebral blood flow (rCBF) was monitored in all stroke animals using laser Doppler flowmetry. Only animals with an rCBF reduction of >70% of pre-MCAO baseline levels were included for further experimentation. Animals that died during or immediately after surgery were excluded from the studies (less than 2%). Sham-operated animals underwent the same anesthesia and surgical procedures with the exception of the MCA occlusion.

Experimental procedures were performed following criteria derived from Stroke Therapy Academic Industry Roundtable (STAIR) group guidelines for preclinical evaluation of stroke therapeutics (Fisher et al., 2009). Accordingly, experimental group assignments were randomized with a lottery-drawing box, and surgeries and all outcome assessments were performed by investigators blinded to mouse genotype and experimental group assignments.

2.4. Pharmacological inhibition of TAK1

The TAK1 inhibitor 5Z-7-Oxozeaenol (OZ) was dissolved in corn oil containing 10% DMSO and administered intraperitoneally. Based on our previous dose-response studies performed on male mice (Wang et al., 2020b), we administrated OZ at 2.5 mg/kg in 100 μL of corn oil starting at 2 h after MCAO, followed by daily administrations for a maximum of 5 days. Vehicle control mice received 100 μL corn oil containing 10% DMSO.

2.5. Neurobehavioral tests

The rotarod and Morris water maze tests were performed as we described previously (Shi et al., 2017) to assess neurological functions before and up to 35 days after MCAO. The mice that died before the endpoints were excluded from the final behavioral analyses. Methodological details for the behavioral tests are provided in Supplemental Material.

2.6. Immunohistochemistry and data analyses

Mice were deeply anesthetized and transcardially perfused with 0.9% NaCl, followed by 4% paraformaldehyde in PBS. Brains were harvested and cryoprotected in 30% sucrose in PBS, and frozen serial coronal brain sections (25-μm thick) were prepared on a sliding microtome (Microm HM 450, Thermo Scientific). Sections were blocked with 5% donkey serum in PBS for 1 h, followed by overnight incubation (4 °C) with primary antibodies (Table S1). After washing, sections were incubated for 1 h at 20 °C with donkey secondary antibodies conjugated with DyLight 488 or Cy3 fluorophores (1:1000, Jackson ImmunoResearch Laboratories). Alternate sections from each experimental condition were incubated in all solutions except the primary antibodies to assess nonspecific secondary antibody staining. To visualize apoptotic cells, TUNEL staining was performed using an In Situ Cell Death Detection Kit following manufacturer’s instructions. Sections were then
mounted and coverslipped with Fluoromount-G containing DAPI (Southern Biotech). Fluorescence images were captured with an inverted Nikon Diaphot-300 fluorescence microscope equipped with a SPOT RT slider camera and Meta Series Software 5.0 (Molecular Devices), or with an Olympus Fluoview FV1000 confocal microscope and FV10-ASW 2.0 software. Methodological details for data analysis are provided in Supplemental Material.

2.7. Flow cytometry

Single-cell suspensions were prepared from the mouse brain as we described previously (Wang et al., 2020b; Zhao et al., 2019). Briefly, mice were deeply anesthetized and transcardially perfused with 0.9% NaCl. The ipsilesional and non-injured contralesional brain hemispheres were harvested, and cell suspensions were prepared using the Neural Tissue Dissociation Kit and gentleMACS Octo Dissociator with Heaters (Miltenyi Biotec) according to the manufacturer’s instructions. Suspensions were passed through a 70 μm cell strainer, and fractionated on a 30% and 70% Percoll gradient at 500 g for 30 min. Mononuclear cells at the interface were collected, resuspended at 1 x 10^6 cells per mL, and stained with fluorochrome-conjugated antibodies (Table S1) and the appropriate isotype controls. Flow cytometry was performed using an LSR II flow cytometer driven by the FACS Diva software (BD Biosciences).

Flow cytometry data were analyzed using the FlowJo software to quantify positively stained cells. Furthermore, individual immune cells were plotted using viSNE (Amir et al., 2013) to map high-dimensional cytometry data onto two dimensions based on the t-distributed Stochastic Neighbor Embedding (t-SNE) algorithm (Van der Maaten and Hinton, 2008). Briefly, immune cells were gated as CD45-positive in FlowJo, and the relative intensity values of each fluorophore were exported and used to generate viSNE plots in a MATLAB-based tool CYT (Amir et al., 2013). Data were transformed using hyperbolic arcsin with a cofactor of 150, and 5000 cells were selected from each group. viSNE plots were generated with 500 iterations, and subpopulations of cells were identified according to their expression of prototypic markers.

2.8. Proteomic array analysis

Protein was extracted from the ipsilesional brain hemisphere 5 days after MCAO or from the corresponding hemisphere of sham-operated animals as described previously (Mao et al., 2019). The content of 40 inflammatory factors was measured using a RayBiotech Mouse Inflammation Array kit according to manufacturer’s instructions. The concentrations of various factors were normalized to levels of non-stroke control brains and data expressed as fold changes.

2.9. Bioinformatics analyses

RNA sequencing (RNA-seq) data on young adult (3 months old) and aged (18 months old) female C57BL/6 mice brains under physiological condition and 3 days after permanent MCAO were obtained from the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (GSE137482) (Androvic et al., 2020). Read counts were downloaded from the GEO and subsequently analyzed using R/Bioconductor R Core Team (2020) as we described previously (Wang et al., 2020a; Zhang et al., 2019). The R package DESeq2 (Love et al., 2014) was used to normalize the counts and to perform differential expression analysis. Differentially expressed genes (DEGs) were defined as genes with a log2(fold change) > 1 or < -1, and with a false discovery rate (FDR) adjusted P-value < 0.05 (Benjamini-Hochberg method). For principal component analysis (PCA), variance-stabilizing transformation was performed on normalized counts for each sample. Heatmaps were generated using the R package pheatmap.

Functional enrichment analysis was performed using an online tool Metascape (Zhou et al., 2019). All genes in the mouse genome were used as the enrichment background. Metascape returns a list of significantly overrepresented (P < 0.01) ontology terms with a minimum count of 3, and an enrichment factor (the ratio between the observed counts and the counts expected by chance) larger than 1.5. Terms were grouped into clusters based on their membership similarities, and the term with the smallest P-value in each cluster was selected to represent that cluster. Cell-specific pattern genes were enriched based on the PaGenBase database (Pan et al., 2013).

DEGs identified by DESeq2 were submitted to Ingenuity Pathway Analysis (IPA) for pathway analysis (Kramer et al., 2014) using the Ingenuity Knowledge Base (Qiagen Bioinformatics). The fold change and FDR adjusted P-value for each gene were used to perform the core analysis. The Upstream Regulator analysis was used to identify master upstream regulators that can explain the observed gene expression changes in the dataset. An upstream regulator was predicted to be strongly activated or inhibited if its activation z-score was larger than 2 or smaller than -2, respectively. The cutoff of P-value was set at 0.01.

2.10. Statistical analyses

High-throughput sequencing data were analyzed as described above. Other datasets are presented as mean ± standard deviation (SD) unless otherwise stated. Individual data points are plotted where applicable. Statistical comparison between two groups was accomplished by the student’s t-test (parametric) or Mann-Whitney U test (nonparametric). Differences in means among multiple groups were analyzed by one or two-way ANOVA followed by the Bonferroni/Dunn post hoc correction. A P value less than 0.05 was deemed statistically significant, and all testing was two-tailed. All statistics are summarized in Table S2.

3. Results

3.1. Genome-wide transcriptomic profiling predicts TAK1 to be a master regulator of brain inflammatory responses after ischemic stroke in female mice

The role of TAK1 in the brain of female subjects after ischemic stroke is so far unknown. We first explored stroke-induced transcriptomic changes in female mouse brains and determined whether TAK1 plays a critical role, using whole-brain RNA-seq data available at the GEO (GSE137482). In this dataset, young adult (3-month-old) and aged (18-month-old) female C57BL/6 mice were subjected to focal cerebral ischemia induced by unilateral permanent MCAO, and bulk RNA-seq was performed on the brain tissue after 3 days (Fig. 1A). Compared to control homeostatic brains in age-matched female mice, ischemic stroke induced robust transcriptomic changes in the brain in both young and aged female mice (Fig. S1A and S1B). There were 2031 and 2847 DEGs in young and aged female mice, respectively, the majority of which were upregulated after stroke (Fig. 1B). From a functional angle, gene ontology (GO) enrichment analysis revealed that many inflammation-related biological processes were among the top 15 significantly overrepresented GO terms by these upregulated DEGs, suggesting strongly induced inflammatory responses in the post-stroke brain (Fig. 1C and Fig. S1C). On the other hand, cell-specific pattern gene enrichment analysis identified that macrophages and microglia were the 2 most significantly overrepresented (i.e., with the smallest F-values) cell types by these upregulated DEGs (Fig. 1D and Fig. S1D).

We further examined the expression and activation status of TAK1 in female mouse brains after ischemic stroke. Although the expression levels of TAK1 mRNA were similar between stroke and control groups (Fig. 1E), TAK1 as a master MAP3K may still be activated and regulate multiple downstream targets. Therefore, we performed Upstream Regulator analysis in IPA, which can predict the activation/inhibition status of potential master regulators based on the expression of all DEGs in the dataset (Kramer et al., 2014). Based on cutoff values of activation z-score > 2 and P < 0.01, TAK1 was predicated by IPA to be strongly
Activated in both young (z-score = 2.845, $P = 1.8 \times 10^{-9}$) and aged (z-score = 4.055, $P = 1.5 \times 10^{-14}$) female mouse brains (Fig. 1F).

Consistent with this prediction and the GO enrichment analysis, multiple TAK1-targeted molecules were upregulated after stroke, including proinflammatory cytokines TNF-α and IL-1β, which contributed to the activation of microglia and macrophages and inflammatory response (Fig. 1G). Collectively, these data suggest that TAK1 is activated after ischemic stroke and contributes to microglia/macrophage-mediated...
3.2. Inhibition of TAK1 reduces acute brain injury after ischemic stroke in female mice

TAK1 has long been considered as a master molecule that activates multiple cell death pathways at the acute stage after ischemic stroke (Naito et al., 2020; Neubert et al., 2011; White et al., 2012). However, whether targeting TAK1 could effectively improve short-term stroke outcome has not been tested in female subjects. We therefore examined the effects of a potent and selective TAK1 inhibitor OZ (Ninomiya-Tsuji et al., 2003) against ischemic brain injury in 3-month-old adult female mice, using a transient MCAO model. Based on our previous dose-response and time-window studies in male mice (Wang et al., 2020b), we administered OZ at a dosage of 2.5 mg/kg beginning at 2 h after MCAO and repeated daily for a maximum of 5 days. To eliminate potential neuroprotective effects from gonadal hormones (Manwani and McCullough, 2011) and better represent the aged female stroke patients, we performed ovariectomy prior to MCAO, a model of surgical menopause that depletes endogenous estrogen and partially reproduces the conditions in postmenopausal women. The results showed that OZ significantly reduced infarct volume 3 days after MCAO in O VXed female mice (Fig. 2A and Fig. S2A).

On a microscopic scale, OZ effectively reduced neuronal apoptosis in the peri-infarct cortex and striatum 3 days after MCAO, as assessed by NeuN/TUNEL double-labeling (Fig. 2B). We further examined axonal injury 3 days after MCAO using double-label immunostaining of β-amyloid precursor protein (β-APP) and neurofilament 200 (NF200; Figure 2Ca). Accumulation of β-APP—an established indicator of axonal damage—was frequently observed in the peri-infarct white matter such as classic axonal bulb and varicosities (arrowhead) after stroke. 

**Fig. 2.** Inhibition of TAK1 ameliorates acute brain injury after ischemic stroke in female mice. Ovariectomized female mice were subjected to 1-h MCAO and received treatment with the TAK1 inhibitor OZ. Brain injury was assessed 3 days after MCAO. (A) Infarct volume was measured on six microtubule-associated protein 2 (MAP2; green)-immunostained coronal brain sections encompassing the MCA territory. (B) Neuronal apoptosis was evaluated by TUNEL/NeuN double-staining (a). Dashed line: infarct border. Apoptotic neurons (TUNEL + NeuN + cells) were counted in the inner border zone (rectangles) in the cortex (CTX) and striatum (STR) (b). (C) Axonal damage was assessed by β-APP/NF200 double-immunostaining in the white matter-enriched external capsule (EC) and striatum in the ipsilesional (IL) and non-injured contralesional (CL) hemispheres (a). β-APP immunosignal in the shape of classic axonal bulb and varicosities (arrowhead) was observed after stroke, suggesting axonal damage (b). Areas immunopositive for β-APP and NF200 were quantified in the ipsilesional EC and striatum (c). n = 7 mice/group. *P < 0.05, **P < 0.01 OZ vs. vehicle. ns, no significant difference.
as the external capsule (EC) and striatum, displaying a classic shape of axonal bulb and varicosities (Figure 2Cb). Although OZ did not alter the levels of NF200, it significantly alleviated the accumulation of $\beta$-APP in the peri-infarct EC and striatum, suggesting less axonal damage (Figure 2Cc). In summary, these data indicate that post-stroke inhibition of TAK1 effectively ameliorates acute ischemic brain injury in female mice.

3.3. Inhibition of TAK1 promotes inflammation-resolving microglial/macrophage responses after ischemic stroke in female mice

Our RNA-seq analysis predicted TAK1 to contribute to neuro-inflammation in post-stroke female mice (Fig. 1D); we therefore examined brain inflammatory profiles at the subacute stage (3–5 days) after MCAO. The inflammation burden of the post-stroke brain originates from both the activation of resident microglia and infiltrating blood immune cells (Figure 3Aa and Fig. S3). Flow cytometry analysis showed
that stroke-induced brain invasion of peripheral immune cells was largely reduced 3 days after MCAO upon OZ treatment (Figure 3Ab). There were significantly less T cells, neutrophils, and CD11b+CD45high macrophages and activated microglia in OZ-treated mice compared to vehicle control (Fig. 3B). The relative portion of CD11b+CD45high cells, mostly quiescent microglia, was therefore increased in OZ-treated mice (Fig. 3B). As a result of both reduced immune cell infiltration and less activation of microglia, production of various inflammatory cytokines was alleviated by OZ treatment 5 days after MCAO (Fig. 3C and Fig. S4). Among a panel of 40 inflammation markers measured in the brain using an antibody array, 20 were significantly elevated in vehicle-treated mice compared to non-stroke controls and 10 of them were downregulated by OZ treatment (Fig. 3C).

Immunodepression and infections are leading causes of death and a major concern in stroke patients. Given the strong anti-inflammatory effects of OZ, we assessed whether OZ treatment exacerbated the disruption of immune homeostasis after stroke. OZ-treated mice had less bodyweight loss in the first 3 days after MCAO than vehicle-treated mice (Fig. S2B), indicating improved general condition. We also did not notice any sign of exacerbated peripheral immune status, as reflected by similar blood cell counts (Fig. S2C) and comparable spleen shrinkage between OZ- and vehicle-treated female mice 3 days after MCAO (Fig. S2D). These data suggest that short-term inhibition of TAK1 with OZ offered good safety and did not negatively impact systemic immune function after stroke in female mice.

Brain resident microglia and monocyte-derived macrophages are major players of inflammatory responses in the post-stroke brain. Based on our RNA-seq prediction that TAK1 may dictate neuroinflammation through regulating microglial/macrophage activation (Fig. 1D), we examined the functional status of microglia/macrophages 3 days after MCAO using established proinflammatory and pro-resolving phenotypic markers CD16/32 and arginase 1 (Arg1; Fig. 3D). Resting microglia in the non-injured contralesional hemisphere expressed negligible levels of CD16/32 and Arg1, whereas activated microglia/macrophages in the ipsilesional peri-infarct area upregulated both CD16/32 and Arg1 (Fig. 3Da). OZ treatment significantly reduced the number of CD16/32+ proinflammatory microglia/macrophages but increased the number of Arg1+ inflammation-resolving microglia/macrophages in the peri-infarct cortex and striatum (Figure 3Db), suggesting an overall phenotypic switch of microglia/macrophages toward an inflammation-resolving state upon TAK1 inhibition. Since Iba1 labels both microglia and macrophages, we performed additional immunostaining using a recently identified microglia-specific marker TMEM119 and a relatively macrophage-specific marker F4/80 (Fig. S5). The results demonstrated that both TMEM119+ microglia and F4/80+ macrophages displayed functional heterogeneity after stroke and had CD16/32+ and Arg1+ subpopulations (Fig. S5). In summary, these data suggest that OZ mitigates brain inflammation after ischemic stroke in female mice, possibly via boosting inflammation-resolving responses of both microglia and macrophages.

3.4. Microglia/macrophage-targeted deletion of TAK1 reproduces the beneficial effects of TAK1 inhibitor in improving stroke outcomes in female mice

To test whether loss-of-function of TAK1 in microglia/macrophages could causally lead to an inflammation-resolving phenotype after stroke in female mice, we employed a previously characterized (Wang et al., 2020b) tamoxifen-inducible conditional TAK1 knockout model whereby TAK1 is selectively deleted in CX3CR1+ microglia/macrophages (Fig. 4A; TAK1 mKO). Female TAK1 mKO mice and age-matched WT controls were OVXed and subjected to 1-h MCAO, followed by the assessment of microglial/macrophage phenotype in the peri-infarct cortex and striatum 3 days later (Fig. 4B). TAK1 mKO had similar effects as OZ treatment in enhancing inflammation-resolving responses of microglia/macrophages, manifested by a decline in CD16/32+ proinflammatory microglia/macrophages and an increase in Arg1+ pro-resolving cells compared to WT controls (Fig. 4B). Since microgli/macrophage-mediated inflammatory responses have profound and long-term impact on brain injury after stroke, we extended the outcome assessment to 35 days after MCAO and evaluated stroke-induced neurological deficits in female TAK1 mKO mice using two established behavioral tests. TAK1 mKO significantly improved the performance of female mice in the rotarod test 7–35 days after MCAO (Fig. 4Ca; n = 9–10/group, P = 0.014 by two-way repeated measures ANOVA). We did not detect significant differences in the rotarod performance between TAK1 mKO and WT female mice until 21 days after MCAO (Figure 4Cb), suggesting that the beneficial effects of TAK1 mKO transpired at the delayed phase after stroke. In the Morris water maze, stroke-induced cognitive deficits in spatial learning were also improved in female TAK1 mKO mice compared to WT mice 22–26 days after MCAO (Figure 4Da; n = 7–9/group, P = 0.042). No prominent spatial memory deficits were detected between MCAO and sham groups after MCAO (Figure 4Db). Chronic brain tissue loss measured 35 days after MCAO did not differ between female TAK1 mKO and WT mice (Fig. 4E).

Together, these data suggested that microglia/macrophage-targeted TAK1 deletion is sufficient to reproduce the phenotype-switching effect of OZ on microglia/macrophages and confer long-term outcome improvement after stroke in female mice.

3.5. TAK1 inhibitor 5Z-7-Oxozeaenol promotes long-term recovery of neurological functions after ischemic stroke in female mice

Given that the beneficial effects of TAK1 mKO lasted for 35 days after MCAO, we assessed whether the TAK1 inhibitor OZ could also improve long-term neurological functions after stroke in female mice (Fig. 5A). Similar to TAK1 mKO mice, OZ-treated female mice demonstrated significantly better performance in the rotarod test than vehicle-treated mice for 35 days after MCAO (Figure 5Ba; n = 10–12/group, P = 0.001 by two-way repeated measures ANOVA). Interestingly, OZ-elicted improvement in sensorimotor functions occurred as early as 5 days after MCAO (Figure 5Bb), in contrast to the delayed improvement observed in female TAK1 mKO mice (Figure 4Cb). The effect of OZ to ameliorate cognitive deficits was modest as tested by the Morris water maze. Although there was no significant difference between OZ- and vehicle-treated MCAO mice over the entire course of 5 days in the spatial learning test (Fig. 5Ca; n = 11–12/group), MCAO-induced deficits were significant in vehicle groups (P = 0.001) but not in OZ-treated mice. OZ-treated mice also demonstrated a trend toward learning improvement on Day 26 compared to vehicle control mice (Figure 5C; P = 0.07). Neuronal tissue loss measured 35 days after MCAO was also reduced in OZ-treated female mice (Fig. 5D).

3.6. Inhibition of TAK1 improves long-term integrity of gray matter and white matter after ischemic stroke in female mice

Not only did OZ promote the recovery of neurological functions, it also improved the long-term integrity of both gray matter and white matter after stroke in female mice. Significantly more NeuN+ viable neurons were observed in OZ-treated mouse brains than vehicle controls in both the peri-infarct cortex and striatum (Figure 6A). We also evaluated white matter integrity in brain sections double-immunostained with contactin-associated protein (Caspr) and sodium channel Nav1.6 to label the nodes of Ranvier (NORs; Figure 6Ba) (Dai et al., 2020). Thirty-five days after MCAO, the typical NOR morphology was disrupted in the ipsilesional EC, represented by the reduced NOR numbers and shorter paranodal Caspr signal (Figure 6Bb). OZ-treated mice had significantly more intact NORs and larger paranode lengths compared to vehicle controls 35 days after MCAO (Figure 6Bc), suggesting improved white matter integrity. In parallel, we assessed myelin injury 35 days after MCAO by double-immunostaining of myelin basic protein (MBP) and non-phosphorylated neurofilament H (SMI-32), a marker for
Fig. 4. Selective knockout of TAK1 in microglia/macrophages promotes inflammation-resolving microglial/macrophage responses and improves long-term outcomes after ischemic stroke in female mice.

(A) Generation of tamoxifen-induced, microglia/macrophage-specific TAK1 knockout (TAK1 mKO) mice by crossing the Cx3cr1<sup>CreER</sup> mice with TAK1<sup>LoxP</sup> mice harboring LoxP-flanked exon 1 of the Map3k7 gene. (B-E) OVXed female TAK1 mKO mice and WT control mice were subjected to 1-h MCAO. (B) Microglial/macrophage phenotype was assessed by immunofluorescence staining of CD16/32 and Arg1, double-labeled with Iba1 3 days after MCAO. Iba1<sup>+</sup>CD16/32<sup>+</sup> cells and Iba1<sup>+</sup>Arg1<sup>+</sup> cells were counted in the peri-infarct cortex and striatum. n = 4–7 mice/group. (C) Sensorimotor deficits were evaluated up to 35 days after MCAO by the rotarod test. In a, data on Days 7–35 were analyzed. Individual data points on Days 21, 28, and 35 are presented in b. (D) Cognitive deficits were evaluated 22–27 days after MCAO by the Morris water maze. Spatial learning and memory were assessed by the escape latency (a) and the time spent in the target quadrant (b). All mice had similar locomotor functions, reflected by comparable swim speeds (c). (E) Brain tissue atrophy was measured 35 days after MCAO on MAP2-immunostained coronal sections. Dashed line shows the relative area of the contralesional hemisphere to illustrate by comparison the area of ipsilateral neuronal tissue loss. n = 5 (sham) or 7–10 (MCAO) mice/group. ***P < 0.001, ****P < 0.0001 MCAO vs. sham. *P < 0.05, **P < 0.01, ***P < 0.001 TAK1 mKO vs. WT.
Neural atrophy was measured 35 days after MCAO on MAP2-immunostained coronal sections. The exact mechanism of cell death involved is beyond the scope of our study, indicating that neuronal death in OZ-treated female mice compared to male mice. The multiple cell death pathways at the acute stage. Although stroke-induced cell death mechanisms can be sexually dimorphic (Manwani and McCullough, 2011), we observed similar reduction of acute cell death and enhancing inflammation-resolving microglial/macrophage responses at the subacute stage after ischemic stroke.

Fig. 5. Short-term treatment with the TAK1 inhibitor OZ improves long-term outcomes after ischemic stroke in female mice.

(A) Experimental timeline. Female mice were subjected to 1-h MCAO or sham operation 3 weeks after OVX. The TAK1 inhibitor OZ or vehicle control was administered beginning at 2 h after MCAO and then daily for 5 days. (B) Sensorimotor functions were assessed in OZ- or vehicle-treated female mice before (Pre) and up to 35 days after MCAO or sham operation by the rotarod test (a). Individual data points on Days 5, 7, and 10 are presented in b. (C) Cognitive functions were assessed 22–27 days after MCAO by the Morris water maze. Spatial learning and memory were assessed by the escape latency (a) and the time spent in the target quadrant (b), respectively. All mice had similar locomotor functions, reflected by comparable swim speeds (c). n = 7–8 (sham) or 10–12 (MCAO) mice/group. (D) Tissue atrophy was measured 35 days after MCAO on MAP2-immunostained coronal sections. n = 9 mice per group. *p < 0.01, **p < 0.001 MCAO vs. sham. *p < 0.05, **p < 0.01 MCAO+OZ vs. MCAO+Veh. ns, no significant difference among groups.

4. Discussion

The present study is the first to investigate TAK1 as a valid therapeutic target for ischemic stroke in female subjects, and found that 1) the selective TAK1 inhibitor OZ demonstrated good safety and efficacy on female mice, and improved short-term and long-term stroke outcomes evaluated using clinically relevant functional parameters; and 2) OZ exerted beneficial effects in female mice via reducing acute neuronal death and enhancing inflammation-resolving microglial/macrophage responses at the subacute stage after ischemic stroke.

Targeting TAK1 to treat ischemic stroke is a promising approach according to previous male rodent-based studies from multiple groups (Naito et al., 2020; Neubert et al., 2011; Wang et al., 2020a; White et al., 2012), but had never been tested on female subjects. Earlier studies on TAK1 in ischemic stroke largely focused on its central role in activating multiple cell death pathways at the acute stage. Although stroke-induced cell death mechanisms can be sexually dimorphic (Manwani and McCullough, 2011), we observed similar reduction of acute neuronal death in OZ-treated female mice compared to male mice. The exact mechanism of cell death involved is beyond the scope of our study, but previous reports suggest that apoptosis, necrosis, and necroptosis may all be affected by TAK1 after stroke (Naito et al., 2020; Neubert et al., 2011).

While reducing neuronal death may account for the decrease in acute brain injury upon OZ treatment, the main reason underlying sustained outcome improvement in OZ-treated female mice may be its mitigation of neuroinflammation at the subacute stage after stroke. Unbiased RNA-seq analysis predicted that TAK1 was activated and governed post-stroke inflammatory responses in the brain of both young adult and aged female mice. One limitation of these data, however, is that the young female mice were gonadally intact and the stages of the estrous cycle were not taken into consideration in the study design, which could affect brain injury and inflammatory processes. Nevertheless, similar conclusions were obtained from data collected from aged female mice, which better represent the stroke patient population in clinical settings. In line with these RNA-seq analyses, inhibition of TAK1 reduced brain inflammatory burden after stroke, reflected by less invasion of peripheral immune cells and less production of inflammatory cytokines. Although similar anti-inflammatory effects of OZ had been observed in male mice in our previous study (Wang et al., 2020a), some sex differences have been noted. OZ displayed selective effects over macrophage subsets in male mice, reducing only the Ly6C+ proinflammatory macrophages without changing the Ly6C+ patrolling macrophages (Wang et al., 2020a). In contrast, OZ reduced both subpopulations in female mouse brains after MCAO (Fig. 3). Such sex dimorphism in immune cell profiles may affect long-term outcomes, as part of the macrophage population may be important for brain repair and regeneration (Wang et al., 2020a). Nevertheless, our long-term functional and immunohistochemical assessments showed an overall improved stroke outcome in OZ-treated female mice.
Microglia and macrophages are key players of inflammatory responses in the post-stroke brain, and their functional heterogeneity (proinflammatory versus inflammation-resolving) are well documented (Hu et al., 2015; Lee et al., 2019). Although OZ targeted all types of cells, our use of TAK1 mKO mice showed that TAK1 causally determined the proinflammatory state of microglia/macrophages. It is worth noting that TAK1 inhibition seemed to fine-tune the proinflammatory versus pro-resolving functions of microglia/macrophages instead of indiscriminately suppressing all functions. Some activities of microglia/macrophages in the post-stroke brain, e.g., phagocytic clearance of dead cells and debris (efferocytosis), are critical for the resolution of local inflammation and facilitate regenerative/remodeling responses of the brain. To this end, Arg1⁺ microglia/macrophages were significantly increased upon OZ treatment, likely representing a subset of efferocytic cells that may benefit tissue repair (Cai et al., 2019). Whether the phenotype-shifting effect of TAK1 inhibition/deletion was achieved through microglia or monocyte-derived macrophages was not distinguished in this study, but immunohistochemistry using TMEM119 and F4/80 suggested that both cell types were involved. Future gain- or loss-of-function studies using microglia or macrophage-specific promoters (e.g., Tmem119, Sall1, Ccr2) may offer further insights on which specific myeloid compartment is involved. A difference between OZ-treated and TAK1 mKO female mice was the early onset of behavioral improvement upon OZ treatment, in contrast to the relatively delayed improvement in TAK1 mKO mice (Fig. 4 and Fig. 5). This was likely due to the early neuroprotective effects of OZ on neurons. With dual and bi-phasic
beneficial effects on neurons and microglia/macrophages, TAK1 inhibitor was capable of improving both short-term and long-term stroke outcomes.

Immunodepression and infection is a major concern in stroke patients, and caution should be taken when developing anti-inflammatory approaches to treat ischemic stroke. In our experimental design, we employed a short-term treatment regimen whereby OZ was administered for a maximum of 5 days after MCAO. On the one hand, this short-term treatment regimen would not interfere with beneficial immune functions at the chronic stage after ischemic stroke. On the other hand, an early overshoot of immune responses disrupts immune homeostasis and can lead to long-term immunodepression (Liesz et al., 2015; Öffner et al., 2006). Effective mitigation of early inflammatory responses may shut down the immune overshoot and depletion of immune cells. Consistent with this notion, our current OZ treatments did not exacerbate stroke-induced immunodepression, as measured by blood cell count and spleen shrinkage, demonstrating good safety.

An important reason underlying the unsuccessful translation of stroke therapeutics into clinical use is the insufficient consideration of female subjects in preclinical research. This study used OVXed female mice to partially mimic the systemic condition of postmenopausal women. We also used long-term and functions parameters to assess stroke outcomes, which is the gold standard in clinical stroke settings. Together with previous studies, our study shows that TAK1 inhibitor demonstrate good efficacy and safety on both male and female subjects, and is a promising agent for further translational investigations. There remain some limitations in our models to be tackled in future studies. Firstly, OVX as a model of surgical menopause is different from natural menopause. Although OVX eliminates the effect of gonadal hormones, it cannot fully recapitulate the conditions in the aged females. Furthermore, aging itself alters microglia behavior and brain inflammatory profiles (Hu et al., 2019; Jiang et al., 2020; Shi et al., 2020). Secondly, we used a transient MCAO model which involved post-ischemic reperfusion, whereas a large portion of stroke patients do not have reperfusion. Therefore, future studies are warranted to verify the safety and efficacy of TAK1 inhibitor on aged male and female subjects, preferably using two different stroke models with and without reperfusion.

**Declarations of Competing Interest**

None.

**CReditT author statement.**

**Yaan Liu**: Investigation, Formal analysis, Writing - Review & Editing, Visualization.

**Sicheng Li**: Investigation, Formal analysis.

**Rongrong Wang**: Investigation, Formal analysis.

**Hongjian Pu**: Investigation, Formal analysis.

**Yongfang Zhao**: Resources.

**Qing Ye**: Investigation.

**Yejie Shi**: Conceptualization, Software, Resources, Formal analysis, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

**Acknowledgments**

This work was supported by the Competitive Medical Research Fund of the University of Pittsburgh Medical Center, the American Heart Association grant 17SDG33630130, and the Pittsburgh Institute of Brain Disorders & Recovery startup funds (to Y.S.). This work was not supported by any United States federal grants, in part or in its entirety. RNA-seq data analysis was performed using IPA software licensed through the Molecular Biology Information Service of the Health Sciences Library, University of Pittsburgh.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2021.105257.

**References**

Allakay, N.I., Murphy, S.J., Traystman, R.J., Hurn, P.D., Miller, V.M., 2000. Neuroprotective effects of female gonadal steroids in reproductively senescent female rats. Stroke 31, 161–168.

Amir, E.D., Davis, K.I., Tadmor, D.M., Simonds, E.F., Levine, J.H., Bendall, S.C., Shenfeld, D.K., Krishnamurthy, S., Nolan, G.P., Peér, D., 2013. viSNE enables visualization of high-dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. Nat. Biotechnol. 31, 545–552.

Androvic, P., Kirdajova, D., Tureckova, J., Zucha, D., Rohlova, E., Abaffy, P., Kriska, J., Valny, M., Androsova, M., Kubista, M., Valibrhac, L., 2020. Decoding the transcriptional response to ischemic stroke in young and aged mouse brain. Cell Rep. 11, 107777.

Cai, W., Dai, X., Chen, J., Zhao, J., Xu, M., Zhang, L., Yang, B., Zhang, W., Rocha, M., Nakao, T., Kofler, J., Shi, Y., Steffel, R.A., Hu, X., Chen, J., 2019. STAT5/Arg1 promotes microglia/macrophage effectorcytosis and inflammation resolution in stroke mice. JCI Insight 4, 131355.

Dai, X., Chen, J., Xu, F., Zhao, J., Cai, W., Sun, Z., Hitchins, T.K., Foley, L.M., Leak, R.K., Chen, J., Hu, X., 2020. TAK1 inhibition generates 5Z-7-olegondrocyte lineage cells and improves white matter integrity after cerebral ischemia. J. Cereb. Blood Flow Metab. 40, 639–655.

Dotson, A.L., Öffner, H., 2017. Sex differences in the immune response to experimental stroke: implications for translational research. J. Neurosci. Res. 95, 437–446.

Fisher, M., Feuerstein, G., Howells, D.W., Hurn, P.D., Kent, T.A., Savitz, S.I., Lo, E.H., STAIR Group, 2009. Update of the stroke therapy academic industry roundtable preclinical recommendations. Stroke 40, 2244–2250.

Huang, M.Y., Lin, Y.V., Zhang, R.J., Lu, D.L., Lu, Z.Q., Cai, W., 2019. Update of inflammation activation in microglia/macrophage in aging and aging-related disease. CNS Neurosci Ther 25, 1299–1307.

Hu, X., Leak, R.K., Shi, Y., Suengae, J., Gao, Y., Zheng, P., Chen, J., 2015. Microglial and macrophage polarization-plus prospects for brain repair. Nat. Rev. Neuroil. 11, 56–64.

Jiang, L., Ma, H., Xu, F., Xie, D., Su, W., Xu, J., Sun, Z., Liu, S., Luo, J., Shi, Y., Leak, R.K., Wechsler, L.R., Chen, J., Hu, X., 2020. Transcriptomic and functional studies reveal unbalanced chemotactic and angiostimulatory properties of aged microglia during stroke recovery. J. Cereb. Blood Flow Metab. 40, 581–597.

Kerr, N., Dietrich, D.W., Bramlett, H.M., Raval, A.P., 2019. Sexually dimorphic microglia and ischemic stroke. CNS Neurosci Ther 25, 1308–1317.

Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., Altman, D.G., 2010. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 8, e1000412.

Kim, T., Chellaboina, B., Chokkalla, A.K., Vemuganti, R., 2019. Age and sex differences in the pathophysiology of acute CNS injury. Neurochem. Int. 127, 22–28.

Kramer, A., Green, J., Pollard Jr., J., Tegendrech, S., 2014. Causal analysis approaches in ingenious pathway analysis. Bioinformatics 30, 530–535.

Liu, J., Hamanská, G., Lo, E.H., Arai, K., 2019. Heterogeneity of microglia and their differential roles in white matter pathology. CNS Neurosci Ther 25, 1290–1298.

Liesz, A., Dalpke, A., Mracsko, E., Antoine, D.J., Roth, S., Zhou, W., Yang, H., Na, S.Y., Akhisaroglu, M., Fleming, T., Eigenbrod, T., Navroth, P.P., Tracey, K.J., Volkman, R., 2015. DAMP signaling is a key pathway inducing immune modulation after brain injury. J. Neurosci. 35, 583–596.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.

Marwani, B., McCallough, L.D., 2011. Sexual dimorphism in ischemic stroke: lessons from the laboratory. Womens Health (Lond) 7, 319–328.

Marwani, B., Liu, F., Scantron, V., Hammond, M.D., Sansing, L.H., McCallough, L.D., 2013. Differential effects of aging and sex on stroke induced inflammation across the lifespan. Exp. Neurol. 249, 120–131.

Mao, L., Yang, T., Lin, X., Sun, Y., Zhao, J., Zhang, W., Gao, Y., Sun, B., Zhang, F., 2019. Protective effects of sulforaphane in experimental vascular cognitive impairment: contribution of the Nrf2 pathway. J. Cereb. Blood Flow Metab. 39, 352–366.

Mihaly, S.R., Ninomiya-Tsuji, J., Moroioka, S., 2014. TAK1 control of cell death. Cell Death Differ. 21, 1667–1676.

Naito, M.G., Xu, A., Damin, P., Lee, J., Wang, H., Li, W., Keliher, M., Pasparakis, M., Yuan, J., 2020. Sequential activation of necroptosis and apoptosis cooperates to mediate vascular and neural pathology in stroke. Proc. Natl. Acad. Sci. U. S. A. 117, 4950–4970.

Neubert, M., Bidder, D.A., Bargjotan, P., Akira, S., Schwanger, M., 2011. Acute neuroprotection of TAK1 protects against neuronal death in cerebral ischemia. Cell Death Differ. 18, 1521–1530.

Niinomiya-Tsuji, J., Kajino, T., Ono, K., Ohtomo, T., Matsumoto, M., Shina, M., Mihiara, M., Tsuchiya, M., Matsumoto, K., 2003. A retinoic acid lactone, 5Z-7-oxo-7-oxo, prevents inflammation by inhibiting the catalytic activity of TAKI MAPK kinase kinase. J. Biol. Chem. 278, 18485–18490.

Öffner, H., Subramanian, S., Parker, S.M., Afratoullis, M.E., Vandenbergab, A., Hurn, P., 2012. Experimental stroke induces massive, rapid activation of the peripheral immune system. J. Cereb. Blood Flow Metab. 32, 564–665.

Y. Liu et al. Neurobiology of Disease 151 (2021) 105257
Pan, J.B., Hu, S.C., Shi, D., Cai, M.C., Li, Y.B., Zou, Q., Ji, Z.L., 2013. PaGenBase: a pattern gene database for the global and dynamic understanding of gene function. PLoS One 8, e80747.

Parkhurst, C.N., Yang, G., Ninan, I., Savas, J.N., Yates 3rd, J.R., Lafaille, J.J., Hempstead, B.L., Littman, D.R., Gan, W.B., 2013. Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. Cell 155, 1596–1609.

Pu, H., Shi, Y., Zhang, L., Lu, Z., Ye, Q., Leak, R.K., Xu, F., Ma, S., Mu, H., Wei, Z., Xu, N., Xia, Y., Hu, X., Hitchens, T.K., Bennett, M.V., Chen, J., 2015. Protease-independent action of tissue plasminogen activator in brain plasticity and neurological recovery after ischemic stroke. Proc. Natl. Acad. Sci. U. S. A. 116, 9115–9124.

R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Sato, S., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Matsumoto, K., Takeuchi, O., Akira, S., 2005. Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat. Immunol. 6, 1087–1095.

Shi, L., Rocha, M., Leak, R.K., Zhao, J., Bhatia, T.N., Mu, H., Wei, Z., Yu, F., Weiner, S.L., Jovin, T.G., Chen, J., 2018. A new era for stroke therapy: integrating neurovascular protection with optimal reperfusion. J. Cereb. Blood Flow Metab. 38, 2073–2091.

Shi, L., Rocha, M., Zhang, W., Jiang, M., Li, S., Ye, Q., Hassan, S.H., Liu, L., Adir, M.N., Xu, J., Luo, J., Hu, X., Wechsler, L.R., Chen, J., Shi, Y., 2020. Genome-wide transcriptomic analysis of microglia reveals impaired responses in aged mice after cerebral ischemia. J. Cereb. Blood Flow Metab. 40, 549–566.

Shi, Y., Zhang, L., Pu, H., Mao, L., Hu, X., Jiang, X., Xu, N., Stetler, R.A., Zhang, F., Liu, X., Leak, R.K., Kepp, R.F., Ji, X., Chen, J., 2016. Rapid endothelial cytoskeletal reorganization enables early blood-brain barrier disruption and long-term ischemic reperfusion brain injury. Nat. Commun. 7, 10523.

Shi, Y., Jiang, X., Zhang, L., Pu, H., Hu, X., Zhang, W., Cai, W., Gao, Y., Leak, R.K., Kepp, R.F., Bennett, M.V., Chen, J., 2017. Endothelium-targeted overexpression of heat shock protein 27 ameliorates blood-brain barrier disruption after ischemic brain injury. Proc. Natl. Acad. Sci. U. S. A. 114, E1243–E1252.

Siegel, C., Turtzo, C., McCullough, L.D., 2010. Sex differences in cerebral ischemia: possible molecular mechanisms. J. Neurosci. Res. 88, 2765–2774.