

Integrated Multi-Omics Analysis Reveals the Neuroprotective Mechanism of the Inflammation Repressor TNIP1/ABIN1 in Microglia following Ischemic Stroke

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Article

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Abstract

Cerebral ischemia-reperfusion injury (CIRI) acts as a core pathological driver of ischemic stroke. Microglia derived neuroinflammation, regulated by subpopulation heterogeneity, plays a bidirectional and opposite regulatory role in neuronal injury and post-stroke tissue repair. However, the precise molecular mechanisms regulating heterogeneous activation and functional modulation of microglia remain incompletely clarified. Here, we integrated bulk RNA-seq, single-cell RNA-seq (scRNA-seq), spatial transcriptomics, and in vivo experimental validation to systematically elucidate the central role of the *TNIP1* gene in neuroinflammation after ischemic stroke. We found that *TNIP1* was specifically upregulated in microglia post-ischemia and defined a functionally distinct *TNIP1* + microglial subpopulation. This subpopulation underwent marked expansion after stroke, selectively accumulated in the ischemic penumbra, and engaged in robust crosstalk with injured neurons via the App-Trem2 signaling pathway. Functionally, *TNIP1*, encoding the A20-binding inhibitor 1 (ABIN1) protein, served as a key endogenous negative regulator of the NF- κ B signaling pathway to suppress the expression and release of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), thereby exerting a prominent neuroprotective effect. Furthermore, in vivo validation demonstrated that Genistein-3'-sodium sulfonate (GSS), a phytoestrogen, could upregulate ABIN1 expression and effectively attenuate ischemic brain injury. Our study is the first to characterize the spatiotemporal and functional specificity of the *TNIP1* + microglial subpopulation, and to confirm that *TNIP1*/ABIN1 constitutes a critical molecular node regulating post-stroke neuroinflammation. These findings provide a novel therapeutic target and theoretical basis for the development of precision immunomodulation-based strategies for ischemic stroke treatment.

1. Introduction

Ischemic stroke is a leading global cause of mortality and long-term disability, imposing a substantial burden on public health systems worldwide(1). The core pathological event of ischemic stroke is cerebral tissue ischemia and hypoxia caused by cerebrovascular occlusion. Although timely restoration of cerebral blood flow (reperfusion) is the cornerstone of clinical therapy for salvaging the ischemic core, reperfusion itself can trigger a complex cascade of secondary injury, termed cerebral ischemia-reperfusion injury (CIRI)(2). CIRI encompasses interconnected pathological processes including oxidative stress, neuroinflammation, excitotoxicity, calcium overload, apoptosis, energy metabolism dysfunction, and autophagy(3). Among these, neuroinflammation is widely recognized as a central pathological driver of injury expansion and a key determinant of long-term neurological functional outcomes(4–6).

Microglia, the resident innate immune cells of the central nervous system (CNS), play a dual role in CIRI-induced neuroinflammation(7–9). On one hand, appropriately activated microglia phagocytose cellular debris and secrete neuroprotective factors to facilitate tissue repair and regeneration. On the other hand, excessive microglial activation leads to the massive release of proinflammatory cytokines (IL-6, TNF- α), exacerbates inflammatory cascades, impairs blood-brain barrier (BBB) integrity, and ultimately induces

neuronal death and persistent neurological deficits(10–12). Thus, dissecting the precise regulatory mechanisms of microglial function post-ischemia and identifying key molecules that modulate microglial activation to suppress detrimental inflammation while promoting reparative processes is crucial for developing novel therapeutic interventions for ischemic stroke.

With the continuous advancement of sequencing technologies, scRNA-seq has emerged as a powerful tool in biomedical research, enabling the dissection of cellular composition within ischemic brain lesions at single-cell resolution, identification of disease-specific cell types, and characterization of ischemia-associated cellular subpopulations and potential clinical molecular biomarkers(13). This provides a more precise basis for molecular subtyping of ischemic stroke. Concurrently, spatial transcriptomics (ST) technology overcomes the spatial limitations of traditional sequencing approaches, enabling in situ profiling of gene expression patterns in tissue sections and direct visualization of gene activity across distinct brain regions or pathological foci(14). By precisely mapping intercellular expression differences, ST technology yields critical insights into the regional heterogeneity of the ischemic brain microenvironment(15–17).

In this study, we employed an integrated multi-omics approach to identify *TNIP1* as a key regulatory gene in the pathological progression of ischemic stroke. Bioinformatic analysis combined with machine learning algorithms screened *TNIP1* from differentially expressed genes (DEGs), and subsequent scRNA-seq and spatial transcriptomics validated its specific upregulation in microglia post-cerebral ischemia. The *TNIP1* + microglial subpopulation exhibited significant expansion after middle cerebral artery occlusion (MCAO), acting as a central hub in the intercellular communication network and engaging in active signaling crosstalk with neurons via the App/Trem2 pathway. Functional experiments confirmed that *TNIP1* is a critical negative regulator of neuroinflammation, and upregulation of its encoded protein ABIN1 suppresses the expression of proinflammatory cytokines such as TNF- α to exert neuroprotection. Spatial analysis further demonstrated that *TNIP1* + microglia selectively accumulate in the peri-ischemic region, with a high spatial correlation to areas of neuronal loss. Collectively, these findings indicate that *TNIP1* modulates the function of a specific microglial subpopulation to play a central role in neuroinflammation and injury repair after ischemic stroke, thereby representing a potential novel therapeutic target for this disease.

However, a critical gap remains in pharmacologically targeting ABIN1 for neuroprotection. Genistein sodium sulfonate (GSS), a phytoestrogen and natural bioactive compound, has attracted extensive attention due to its robust anti-inflammatory, antioxidant, and neuroprotective properties(18–22). Accumulating evidence has shown that GSS inhibits the NF- κ B signaling pathway to alleviate inflammatory injury(10, 23). As a key endogenous negative regulator of the NF- κ B pathway, ABIN1 exerts a pivotal role in inflammatory modulation. Based on these observations, we hypothesized that GSS exerts its neuroprotective effects in ischemic stroke by upregulating ABIN1 expression, thereby enhancing the inhibition of the NF- κ B signaling pathway and attenuating microglia-mediated neuroinflammation.

Building on the multi-omics identification of ABIN1 as a key regulatory molecule in cerebral ischemia, the present study aimed to elucidate whether the neuroprotective mechanism of GSS is closely associated with the regulation of ABIN1 expression and suppression of neuroinflammation. Our findings provide important experimental evidence and translational potential for the development of ABIN1-targeted therapeutic strategies for ischemic stroke.

2. Materials and Methods

2.1 Data Acquisition

All sequencing data used in this study were downloaded from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). Bulk RNA-seq data were obtained from the GSE137482 dataset, which included brain tissue samples from 12 MCAO-treated and 12 sham-operated mice. ScRNA-seq data were derived from the GSE174574 dataset, comprising brain tissue samples from 3 MCAO and 3 sham-operated mice, all harvested at 24 h post-surgery. Spatial transcriptomics data were acquired from the GSE233813 dataset.

2.2 Bulk RNA-seq Preprocessing and Differentially Expressed Gene (DEG) Screening

The expression matrix of the GSE137482 dataset was extracted, and gene-expression correspondences were organized according to the platform annotation file of the dataset. DEGs between the MCAO and sham groups were screened using the criteria of adjusted P -value < 0.05 and $|\log_2 \text{fold change (FC)}| > 1$ (24, 25), and a volcano plot was generated for visualization. The top 20 significantly upregulated and top 20 significantly downregulated DEGs were selected for heatmap correlation analysis to characterize gene expression patterns.

2.3 Weighted Gene Co-Expression Network Analysis (WGCNA)

A weighted gene co-expression network was constructed based on the previously screened DEGs using the WGCNA method, strictly adhering to the scale-free topology criterion. First, the `pickSoftThreshold` function in the WGCNA R package was used to determine the optimal soft threshold power and adjacency matrix construction parameters. The adjacency matrix was then converted to a topological overlap matrix (TOM), and the corresponding dissimilarity was calculated for hierarchical clustering analysis. Dynamic tree cutting was performed with a minimum module size of 60 to identify gene modules with highly co-expressed characteristics. To evaluate the association between each module and the ischemic stroke phenotype, gene significance (GS) and module membership (MM) values were calculated. Modules significantly associated with the disease phenotype were identified as key modules for subsequent analysis.

2.4 Machine Learning for Screening Core Signature Genes

To further refine the key module genes identified by WGCNA and determine the core signature genes associated with ischemic stroke, two machine learning algorithms—Lasso regression and random forest—were integrated(26, 27). Lasso regression analysis was performed using the glmnet R package, and the optimal penalty parameter (λ) was determined via 10-fold cross-validation. Concurrently, a classification model was constructed based on hub DEGs using the randomForest R package. To identify optimal parameters, the out-of-bag (OOB) error rate of the model was systematically evaluated across a range of decision trees (ntree = 1 to 500) to build the final random forest model. The feature importance score (e.g., MeanDecreaseGini, MeanDecreaseAccuracy) of each candidate hub gene was calculated, and top-ranked genes were selected based on these scores. Finally, the intersection of genes screened by Lasso regression and random forest was defined as the potential signature genes of ischemic stroke.

2.5 Functional Enrichment Analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses of the identified potential signature genes were performed using the clusterProfiler R package. Statistical significance was assessed using the false discovery rate (FDR), with a screening threshold of $FDR < 0.05$. This analysis aimed to elucidate the potential biological functions of these genes in the pathophysiological mechanisms of ischemic stroke from a systems biology perspective.

2.6 Single-Cell RNA-seq Data Processing

ScRNA-seq data were integrated and normalized using the Seurat R package (v5.0)(28). Strict quality control (QC) was performed with the following criteria: mitochondrial gene ratio (percent.mt) $< 20\%$, number of detected genes per cell (nFeature_RNA) ranging from 200 to 8000, and erythrocyte gene expression level $< 3\%$. After QC filtering, a total of 105,849 high-quality cells were retained for subsequent analysis. Log-normalization was applied to the filtered cell data, and the top 2000 highly variable genes (HVGs) were identified using the FindVariableFeatures function. Harmony algorithm was used to correct for batch effects across different samples. Principal component analysis (PCA) was performed for dimensionality reduction based on the integrated HVG expression matrix, and the first 30 principal components (PCs) were selected for downstream analysis. Cell clustering was achieved using the FindClusters algorithm, and t-distributed stochastic neighbor embedding (t-SNE) was used for two-dimensional visualization of high-dimensional data. Finally, identified cell clusters were annotated with detailed cell types by combining known canonical cell marker genes and annotations from the CellMarker 2.0 database.

2.7 Single-Cell Trajectory Analysis

To dissect the differentiation and developmental trajectory of microglial subtypes, trajectory analysis was performed using the Monocle2 R package (v2.22.0)Trapnell C: **Reversed graph embedding resolves complex single-cell trajectories**. Genes with an average expression level ≥ 0.1 were selected, and dimensionality reduction was conducted via the DDRTree method. Cells were classified and visualized using the plot_cell_trajectory function, and pseudotime was inferred to order the cell trajectory.

2.8 Cell-Cell Communication Analysis

Intercellular interactions were inferred based on the expression levels of ligand-receptor pairs using the CellChat R package (v2.20)(29, 30). The analysis pipeline included the following steps: first, the `identifyOverExpressedGenes` and `identifyOverExpressedInteractions` functions were used to identify genes and ligand-receptor pairs specifically upregulated in each cell population. Next, the `computeCommProb` function was applied to calculate cell communication probabilities, and the `aggregateNet` function was used to construct the aggregated communication network. Finally, multiple visualization functions were used to display the cell-cell communication results from multiple dimensions.

2.9 In Silico Knockout Experiments of Target Genes

To investigate the functional consequences of perturbing the identified target genes, in silico knockout (KO) experiments were performed using the `scTenifoldKnk` R package, which implements a machine learning workflow based on scRNA-seq data(31, 32). This approach enabled the identification of downstream genes affected by virtual KO of the target genes. DEGs (adjusted $P < 0.05$) induced by virtual KO were subjected to functional enrichment analysis to elucidate perturbed biological pathways.

2.10 Spatial Transcriptomics Data Analysis

Spatial transcriptomics data were loaded using the `Load 10X_Spatial` function. Low-quality spots with < 500 detected genes or mitochondrial gene ratio $> 30\%$ were removed during QC(33–35). Data normalization and HVG screening were then performed using the `SCTransform` method. Unsupervised clustering was conducted via `FindNeighbors` and `FindClusters` based on the first 30 PCs, and uniform manifold approximation and projection (UMAP) was applied for two-dimensional visualization using the `RunUMAP` function. To further dissect the cellular composition of each spatial spot, the SPOTlight algorithm was used with annotated scRNA-seq data as the reference expression profile. This deconvolution strategy enabled the inference of cell type composition and relative proportions in each spatial spot, thus constructing a high-resolution spatial map of cellular distribution in brain tissue sections(36).

2.11 Animal Model Establishment

Male Sprague-Dawley rats (50–60 days old, 250–300 g body weight) were purchased from Hunan Shanshan Ji Te Laboratory Animal Co., Ltd. (License No.: SCXK 2016-0002). All rats were specific pathogen-free (SPF) grade and housed under controlled environmental conditions (temperature: $22 \pm 2^\circ\text{C}$, relative humidity: 60–70%) with a 12 h light/dark cycle. Rats had free access to standard rodent chow and sterile water throughout the experiment. After one week of adaptive feeding, rats were randomly divided into two groups ($n = 6$ per group) using a random number table. Fasting was performed for 12 h before surgery. Focal cerebral ischemia was induced by transient middle cerebral artery occlusion (tMCAO) using the intraluminal suture method. Briefly, rats were anesthetized with intraperitoneal injection of 0.4% sodium pentobarbital (1 mL/100 g body weight) and fixed on a surgical platform. A midline cervical incision was made, and subcutaneous tissues and muscles were bluntly dissected to expose the cervical blood vessels. A nylon monofilament suture (0.26–0.28 mm diameter;

Xinongkeji, China) was inserted into the internal carotid artery via the external carotid artery and advanced 18–20 mm to occlude the origin of the middle cerebral artery. After 120 min of occlusion, the suture was withdrawn to restore cerebral reperfusion, and the incision was sutured. Sham-operated rats underwent the same surgical procedure without suture insertion and middle cerebral artery occlusion(22, 23). The animal experimental protocol was reviewed and approved by the Animal Ethics Committee of Gannan Health Vocational College (Approval No. 2026DW004). All experimental procedures were strictly performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

2.12 Humane endpoints, animal welfare, and monitoring

The planned experimental endpoint was 24 hours after reperfusion. To prevent severe suffering, predefined, objective humane endpoints were implemented. The specific criteria for immediate euthanasia were: (1) body weight loss exceeding 20% of the pre-surgical baseline; (2) complete inability to access food or water for 12 consecutive hours; (3) severe and persistent neurological deficits, specifically the loss of righting reflex for more than 30 seconds. Animal health and behavior were monitored at least every 4 hours during the immediate postoperative recovery period and at least twice daily thereafter. No animal met these humane endpoint criteria prior to the planned endpoint, and no unanticipated deaths occurred. All animals survived until the scheduled endpoint and were euthanized via an overdose of pentobarbital sodium (150 mg/kg, i.p.). To minimize pain and distress, the following measures were taken: precise intraoperative temperature control (maintained at $36.5 \pm 0.5^{\circ}\text{C}$), provision of post-operative thermal support, maintenance of ad libitum access to standard chow and water, and the provision of softened food and water on the cage floor post-surgery to facilitate intake. All personnel involved in animal procedures and monitoring received specific training on recognizing signs of rodent pain, distress, and the application of humane endpoint criteria.

2.13. TTC staining

At 24 h post-reperfusion, rats were euthanized by rapid decapitation under deep pentobarbital anesthesia. Brains were rapidly excised, frozen at -20°C for 15 min until firm enough for slicing, and then coronally sectioned into 1.5-mm-thick slices using a rodent brain matrix. The sections were incubated in 0.5% 2,3,5-triphenyltetrazolium chloride (TTC) solution at 37°C in the dark for 15 min, and turned over once midway through incubation to ensure uniform bilateral staining. Subsequently, sections were thoroughly rinsed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 6 h. All staining procedures were performed on six rats per group. Infarct volume was quantified using ImageJ software, with edema correction applied. All image acquisition and analysis were performed by an investigator blinded to group allocation. The corrected infarct percentage was calculated according to the formula: $\text{Infarct volume (\%)} = [(\text{contralateral hemisphere area} - \text{non-infarcted ipsilateral area}) / (2 \times \text{contralateral hemisphere area})] \times 100\%$.

2.14 Western Blot Analysis

Total protein was extracted from brain tissue samples and quantified using the bicinchoninic acid (BCA) protein assay kit. Equal amounts of protein (40 µg) were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes via wet transfer. Membranes were blocked with 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST) for 2 h at room temperature, then incubated overnight at 4°C with the following primary antibodies: rabbit anti-mouse Clusterin monoclonal antibody (1:1000, ab69644, Abcam, UK), rabbit anti-mouse Gria2 polyclonal antibody (1:1500, A11316, ABclonal, China), and rabbit anti-mouse β-Tubulin polyclonal antibody (1:1500, A12289, ABclonal, China). After washing with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody for 2 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) kit (P0018S, Beyotime, China), and gray value scanning and quantitative analysis were performed using ImageJ software (NIH, USA).

2.15 Statistical Analysis

All data processing and statistical analyses were performed using R software (v4.4.3) and GraphPad Prism 10.0 (GraphPad Software, USA). Data are presented as the mean ± standard error of the mean (SEM). Differences in continuous variables between two groups were assessed using the Wilcoxon rank-sum test or unpaired Student's *t*-test. Differences among multiple groups were analyzed using one-way analysis of variance (ANOVA). Correlations between variables were evaluated using Pearson's correlation coefficient. All *P*-values were calculated using a two-tailed test, and statistical significance was defined as *P* < 0.05.

3. Results

3.1 Identification of Differentially Expressed Genes (DEGs)

The experimental workflow of this study is shown in Fig. 1. After normalization of the microarray data, a total of 1939 DEGs were identified in the GSE137482 dataset, including 1915 upregulated and 24 downregulated genes (Fig. 2A). For further visualization, a heatmap was generated to show the expression patterns of the top 20 significantly upregulated and top 20 significantly downregulated DEGs (Fig. 2B).

3.2 Construction of a Weighted Gene Co-Expression Network

WGCNA was performed to construct a gene co-expression network based on the expression profiles of 24 samples, aiming to explore the transcriptional regulatory characteristics between the MCAO and sham groups. Sample hierarchical clustering (Fig. 2C) showed no significant outliers, indicating that the transcriptional profile differences between groups were representative of the study population. In WGCNA, module eigengene-based connectivity (kME) was used to evaluate the connection strength

between genes and key modules, thus determining module membership. Genes with $kME > 0.85$ were defined as core module members, as they better represent the overall expression trends of the corresponding modules (Fig. 2D). A gene dendrogram with module color annotation (Fig. 2E) classified all genes into 14 co-expression modules, with different colored modules representing heterogeneous gene expression patterns. Module-trait association analysis (Fig. 2F) revealed that the MEyellow module was significantly positively correlated with the MCAO phenotype (correlation coefficient = 0.69, $P < 0.0001$), suggesting that it may constitute the core transcriptional program in response to cerebral ischemic injury. This module contained a total of 294 highly correlated genes.

3.3 Screening of Core Signature Genes via LASSO Regression and Random Forest

Two machine learning algorithms were used to identify core signature genes from the 294 genes in the MEyellow module. Lasso regression analysis identified 30 key genes (Figs. 3A-B), while random forest analysis yielded 26 key genes (Fig. 3C). The intersection of genes screened by the two algorithms identified 13 core signature genes, including *Car11*, *TNIP1*, *Elmod1*, *Dmkn*, *St8sia3*, *Grk3*, *A3galt2*, *Igsf9*, *Meak7*, *Tgfa*, *Meis1*, *Zfp521*, and *Nuak1* (Fig. 3D).

3.4 Functional Enrichment Analysis of Core Signature Genes

Functional enrichment analysis was performed on the 13 core signature genes. KEGG pathway analysis (Fig. 2E) showed that these genes were significantly enriched in pathways closely associated with cancer progression and cellular signal transduction, including glioma and other cancer-related pathways, as well as key signaling pathways such as the ErbB, Hedgehog, estrogen, and EGFR tyrosine kinase inhibitor resistance pathways. GO enrichment analysis (Fig. 2F) revealed the following functional annotations: in the biological process (BP) category, the genes were significantly enriched in glycosphingolipid/glycolipid biosynthesis and metabolism, and regulation of stem cell pluripotency; in the molecular function (MF) category, the genes were mainly associated with glycosyltransferase activity, sialyltransferase activity, UDP-galactosyltransferase activity, and D1 dopamine receptor binding; in the cellular component (CC) category, the genes were primarily localized to the basolateral plasma membrane, dendritic ends, and inhibitory synapses.

3.5 Single-Cell Transcriptomic Landscape of MCAO and Sham Brain Tissues

To investigate cellular dynamics between MCAO and sham groups, six samples (3 MCAO, 3 sham) were included in the scRNA-seq analysis. After strict QC, 105,849 high-quality cells were retained. Unsupervised clustering classified the cells into 16 subpopulations (Fig. 3A), and 11 distinct cell types were successfully annotated by combining canonical marker genes and the CellMarker 2.0 database, including astrocytes (*Aqp4*, *Slc1a3*), endothelial cells (*Cldn5*, *Flt1*), spiral ganglion neurons (*Cox8b*,

Defb11), fibroblasts (Lum, Dcn, Pdgfra), macrophages (F13a1, Ms4a7, Pf4), microglia (Tmem119, Cx3cr1, P2ry12), mural cells (Tagln, Acta2), neurons (Cmss1, Nrgn, Snap25), neutrophils (S100a8, S100a9), oligodendrocytes (Mobp, Plp1), and natural killer T (NKT) cells (Nkg7, Cd3d) (Figs. 3B-C). Intergroup comparison revealed extensive DEGs between MCAO and sham samples. Notably, *TNIP1*, a gene screened by machine learning, was significantly upregulated in microglia with prominent differential expression (Fig. 3D). Cellular composition analysis further showed that the proportions of macrophages and microglia were significantly increased in the MCAO group compared with the sham group, suggesting that these two immune cell types play key roles in the progression of ischemic stroke; concomitantly, the proportion of neurons was drastically reduced, which is consistent with the pathological feature of massive neuronal damage post-ischemia (Fig. 3E). Visualization of the expression patterns of the 13 core signature genes via FeaturePlot showed that most of these genes were highly expressed in neurons, while *TNIP1* was specifically upregulated in microglia (Fig. 3F). To systematically evaluate changes in intercellular interactions, a cell-cell communication network was constructed using CellChat. The results showed that macrophages and fibroblasts had the most extensive communication network in terms of the number of interactions; however, microglia acted as the central hub of the signal transduction network in terms of interaction strength (Fig. 3G). These findings further confirm that microglia play an indispensable role in remodeling the cellular microenvironment after ischemic stroke from the perspective of intercellular crosstalk.

3.6 Dynamic Changes and Communicative Roles of Microglial Subpopulations in MCAO

Microglia were subjected to further dimensionality reduction and clustering analysis. Based on the expression of specific key genes, microglia were subdivided into four subpopulations with distinct molecular characteristics: c0-Microglia-P2ry12, c1-Microglia-*TNIP1*, c2-Microglia-Foxq1, and c3-Microglia-Mif (Figs. 4A-B). The P2ry12 + microglial subpopulation was the predominant subtype in the sham group, while the MCAO group exhibited a marked remodeling of microglial subpopulation proportions, with a significant expansion of the *TNIP1* + microglial subpopulation (Fig. 4C). Correlation analysis and pseudotime trajectory analysis further suggested distinct developmental or state transition relationships among these subpopulations (Figs. 4D-E). FeaturePlot showed that *TNIP1* expression was significantly higher in the MCAO group than in the sham group (Fig. 4F). Cell-cell communication network analysis (Figs. 4G-H) further revealed differential signal crosstalk between distinct microglial subpopulations and neurons; in particular, the *TNIP1* + microglial subpopulation exhibited enhanced communication strength with neurons in the MCAO group and was involved in key neuron-derived signaling pathways, including App-Trem2/Tyrobp and Apoe-Trem2/Tyrobp. These results indicate that microglia are a heterogeneous population post-ischemic stroke, and the *TNIP1* + microglial subpopulation may exert a critical role in cerebral ischemia via specific ligand-receptor interaction mechanisms.

3.7 *TNIP1* Is Upregulated in the MCAO Model and Suppresses Neuroinflammatory Responses

To investigate the role of *TNIP1* in cerebral ischemic injury, DEGs between the MCAO and sham groups were analyzed in two independent datasets (GSE199066 and GSE233811) (Figs. 5A-B). The results showed that *TNIP1* was upregulated in the MCAO group compared with the sham group, and multiple inflammation-related genes (e.g., *IL-18*, *TNF-α*, *IL-6*) also exhibited significant differential expression, suggesting that *TNIP1* may be involved in the neuroinflammatory response post-cerebral ischemia. To further validate the function of *TNIP1*, virtual KO of *TNIP1* was performed in microglia from the MCAO model. The results showed that virtual KO of *TNIP1* significantly perturbed the expression of genes such as *Cstb* and *Tnf* (Figs. 6C-D). KEGG enrichment analysis of the perturbed genes revealed enrichment in multiple inflammation-related signaling pathways, including the IL-17 and TNF signaling pathways, suggesting that *TNIP1* may participate in the pathological process of cerebral ischemic injury by regulating inflammatory signaling pathways. To validate *TNIP1* expression changes in vivo, an MCAO rats model was established. 2,3,5-Triphenyltetrazolium chloride (TTC) staining showed that sham-operated brain tissue exhibited uniform red staining with no obvious infarction; the vehicle group (MCAO + solvent control) showed extensive white infarcted areas; and the GSS group (MCAO + GSS treatment) exhibited a significant reduction in white infarct volume compared with the vehicle group, indicating that GSS treatment attenuates cerebral infarct size (Fig. 5F). Western blot analysis was used to detect the expression levels of ABIN1 (the protein encoded by *TNIP1*) and the proinflammatory cytokine TNF-α in brain tissue (Figs. 5G-H). Compared with the sham group, the vehicle group showed a significant increase in TNF-α expression and a marked decrease in ABIN1 expression. Compared with the vehicle group, the GSS group exhibited significantly downregulated TNF-α expression and upregulated ABIN1 expression. These findings suggest that *TNIP1* is upregulated in cerebral ischemic injury and may act as a key regulatory molecule of the inflammatory response in the pathological process of cerebral ischemia.

3.8 Spatial Distribution Characteristics of *TNIP1* + Microglia

To further characterize the spatial distribution of *TNIP1* + microglia post-cerebral ischemia, spatial transcriptomics analysis was performed on brain tissue sections from the MCAO and sham groups. Spatial regional segmentation showed that the right ischemic region in the MCAO group underwent significant spatial structural remodeling (Figs. 6A-B). Based on SPOTlight deconvolution analysis, the spatial distribution differences of neurons and microglia between the two groups were compared. The results showed that neuronal signals were significantly reduced in the ischemic region post-MCAO, while microglia selectively accumulated and were activated in this region (Figs. 6C-D). Spatial correlation analysis further demonstrated a significant negative correlation between the spatial distribution of neurons and microglia, suggesting a reciprocal relationship between these two cell types in the ischemic microenvironment (Fig. 6E). Spatial mapping of the key gene *TNIP1* showed that its expression was

significantly upregulated in the peri-ischemic region of the MCAO group, and the region of high *TNIP1* expression was highly coincident with the microglial enrichment region (Fig. 6F). These results confirm from a spatial transcriptomic perspective that microglia accumulate and are activated in the injured region post-cerebral ischemia, accompanied by neuronal loss; *TNIP1* is specifically upregulated in microglia in the ischemic region and may play an important role in mediating microglial function in cerebral ischemia.

4. Discussion

Ischemic stroke ranks among the top causes of mortality and long-term disability globally, with its pathogenesis driven by elaborate multicellular and molecular cascades. Neuroinflammation serves as a pivotal link between acute neuronal damage and endogenous repair(4, 37–39). Microglia, the innate immune cells of the central nervous system, exert dual effects throughout stroke progression(10, 37, 40–42).Clinically applied reperfusion therapies are constrained by a narrow therapeutic window and struggle to suppress post-stroke inflammation. Hence, exploring critical molecular regulators of microglial function carries important implications for stroke clinical management.

In this study, an integrated multi-omics approach identified *TNIP1* as a key gene regulating neuroinflammation in ischemic stroke. *TNIP1* was specifically upregulated in microglia post-cerebral ischemia and defined a functionally distinct *TNIP1* + microglial subpopulation. This subpopulation underwent marked expansion after stroke, acted as a central hub in the intercellular communication network, and engaged in crosstalk with neurons via the App/Trem2 pathway. Functionally, *TNIP1* exerted a neuroprotective effect by encoding ABIN1 to suppress proinflammatory cytokines such as TNF- α . Spatially, this subpopulation selectively accumulated in the ischemic penumbra, with a high spatial correlation to regions of neuronal damage. These findings indicate that *TNIP1* is a core target regulating microglial function and neuroinflammation post-stroke.

TNIP1 also known as A20-binding inhibitor of NF- κ B activation 1 (ABIN1)(43, 44), is widely expressed in human tissues and has been extensively studied in inflammatory and autoimmune diseases including psoriasis(45–47), arthritis(48), systemic lupus erythematosus (SLE)(49), and systemic sclerosis(50). However, its role in cerebral ischemia remains largely unexplored. ScRNA-seq not only confirmed the specific upregulation of *TNIP1* in microglia but also, more importantly, identified a unique *TNIP1* + microglial subpopulation. This subpopulation exhibited significant expansion after stroke and was derived from the relatively quiescent P2ry12 + microglial subpopulation, suggesting that it represents an activated state in response to ischemic injury. Furthermore, spatial transcriptomics confirmed that this subpopulation selectively accumulates in the peri-ischemic region (ischemic penumbra)—a critical region determining injury plasticity and therapeutic efficacy. We found that the region of high *TNIP1* expression was highly coincident with areas of neuronal loss and microglial accumulation, strongly suggesting that *TNIP1* expression is an adaptive reprogramming of microglia in the ischemic microenvironment. Severe necrosis in the ischemic core may drive microglial differentiation toward an

extreme proinflammatory phenotype, while milder stress signals in the penumbra may induce a regulatory, protective phenotype characterized by *TNIP1* upregulation.

ABIN1 has been shown to promote the deubiquitination of upstream NF- κ B molecules and inhibit NF- κ B activity, exerting anti-inflammatory effects in various inflammatory diseases(48, 51–53). However, its role in microglia-mediated neuroinflammatory injury in cerebral ischemia has not been reported. Our cell-cell communication analysis revealed that the *TNIP1* + microglial subpopulation acts as a central hub in the intercellular communication network post-stroke, with particularly robust signal crosstalk with neurons. Ligand-receptor pair analysis identified the amyloid precursor protein (App)-Trem2/Dap12 pathway as a key bridge connecting neurons and this subpopulation. Under ischemic stress, neurons may release large amounts of soluble App fragments, which activate the Trem2 receptor on microglia to trigger downstream responses including phagocytosis of cellular debris and inflammatory regulation. The *TNIP1* + subpopulation highly expresses genes associated with the Trem2 pathway, indicating that it may be specialized in receiving and processing "danger signals" from injured neurons, thus coordinating cellular debris clearance and maintenance of microenvironmental homeostasis. At the molecular level, we confirmed that *TNIP1* is a key effector molecule mediating the function of this subpopulation. ABIN1, the protein encoded by *TNIP1*, is a classic negative regulator of the NF- κ B signaling pathway. In vivo experiments showed that upregulation of ABIN1 post-MCAO was associated with decreased TNF- α levels and reduced infarct volume; in silico KO of *TNIP1* perturbed the inflammatory network including Tnf. These findings suggest a refined feedback regulatory model: ischemic injury strongly activates the NF- κ B pathway in microglia via receptors such as TLR4 and TNF-R, driving proinflammatory responses; concurrently, induced *TNIP1*/ABIN1 forms an endogenous brake mechanism that negatively regulates excessive NF- κ B activation by inhibiting the IKK complex, thus confining the inflammatory response to a moderate, beneficial range and preventing "collateral damage" to surviving neurons in the peri-ischemic region.

Based on the above mechanisms, we validated the potential neuroprotective effect of the phytoestrogen GSS. The results showed that GSS treatment significantly reduced cerebral infarct volume in MCAO rats, accompanied by upregulated ABIN1 protein levels and downregulated TNF- α expression in the ischemic penumbra. These findings link ABIN1—the key target identified by multi-omics—to the neuroprotective effect of GSS, suggesting that GSS may enhance the inhibition of the NF- κ B pathway by upregulating ABIN1, thereby attenuating microglia-mediated neuroinflammation. This provides novel clues for the development of GSS as a lead compound targeting this pathway for ischemic stroke therapy.

Despite the comprehensive insights gained from our integrated multi-omics approach, this study has several limitations. The current findings primarily suggest the function of *TNIP1* through correlation analysis, expression profile association, and phenotypic changes after pharmacological intervention. Future studies are warranted to establish a strict causal relationship in vivo using microglia-specific conditional *TNIP1* knockout mice, to directly verify whether *TNIP1* deletion exacerbates post-ischemic neuroinflammation, increases infarct volume, and worsens neurological deficits. Additionally, the specific

molecular mechanisms by which GSS upregulates ABIN1 expression remain to be elucidated, and further studies are needed to explore the upstream signaling pathways mediating this effect.

In summary, this multi-omics combined in vivo study delineates the protective function of penumbra-resident *TNIP1*⁺ microglia and confirms ABIN1 as a feasible therapeutic target for ischemic stroke. GSS alleviates CIRI through elevating ABIN1 expression to constrain NF-κB-dependent neuroinflammation. The discovery of this spatially localized functional microglial subset facilitates the development of novel precision immunomodulatory strategies for stroke clinical conversion.

Abbreviations

ABIN1 A20-binding inhibitor of NF-κB activation 1

CIRI Cerebral ischemia-reperfusion injury

DEGs Differential expressed genes

GO Gene Ontology

GSS Genistein-3'-sodium sulfonate

GEO Gene Expression Omnibus

HVGs Highly variable genes

LASSO Least Absolute Shrinkage and Selection Operator

PCA Principal component analysis

ScRNA-seq Single-cell RNA sequencing

ST Spatial transcriptomics

tMCAO Transient middle-cerebral artery occlusion

Declarations

Data availability

The datasets utilised in this study, GSE137482, GSE174574, GSE199066 and GSE233811, were procured from the GEO database. All data and codes necessary for the analyses are available upon request. Please contact the corresponding author for further information.

CRedit authorship contribution statement

Jiali Xie: Writing - original draft, Project administration, Data curation, Conceptualization. Chunyan Zhou: Methodology, Investigation. Jing Yao: Data curation, Methodology. Jiaying Zhu: Writing - review & editing, Methodology, Formal analysis, Investigation, Funding acquisition.

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Ethics Approval and Consent to Participate

All animal procedures were performed following the guidelines of the China Council on Animal Care and were approved by the Gannan Health Vocational College (Ganzhou China, ethical approval No. 2026DW004).

Consent for Publication

All authors read and approved the final manuscript for publication.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Feigin, V. L. et al. World Stroke Organization: Global Stroke Fact Sheet 2025. *Int. J. Stroke*. **20** (2), 132–144 (2025).
2. Xu, A., Zhang, H., Zhang, Y., Wu, J. & Huang, Z. Ischemic stroke and intervention strategies based on the timeline of stroke progression: Review and prospects. *Acta Pharm. Sin B*. **15** (9), 4543–4581 (2025).
3. Zhang, M. et al. Ischemia-reperfusion injury: molecular mechanisms and therapeutic targets. *Signal. Transduct. Target. Ther.* **9** (1), 12 (2024).
4. García-Domínguez, M. & Neuroinflammation Mechanisms, Dual Roles, and Therapeutic Strategies in Neurological Disorders. *Curr. Issues Mol. Biol.* ;**47**(6). (2025).
5. Xu, W., Huang, Y. & Zhou, R. NLRP3 inflammasome in neuroinflammation and central nervous system diseases. *Cell. Mol. Immunol.* **22** (4), 341–355 (2025).
6. Shafi, A., Akmal, M., Sethi, A. & Chauhdary, Z. A mechanistic insight of neuro-inflammation signaling pathways and implication in neurodegenerative disorders. *Inflammopharmacology* **34** (1), 309–318 (2026).

7. Prinz, M., Masuda, T., Wheeler, M. A. & Quintana, F. J. Microglia and Central Nervous System-Associated Macrophages-From Origin to Disease Modulation. *Annu. Rev. Immunol.* **39**, 251–277 (2021).
8. Jurcau, A. & Simion, A. Neuroinflammation in Cerebral Ischemia and Ischemia/Reperfusion Injuries: From Pathophysiology to Therapeutic Strategies. *Int. J. Mol. Sci.* ;**23**(1). (2021).
9. Colonna, M. & Butovsky, O. Microglia Function in the Central Nervous System During Health and Neurodegeneration. *Annu. Rev. Immunol.* **35**, 441–468 (2017).
10. Liu, C. et al. Genistein-3'-sodium sulfonate Attenuates Neuroinflammation in Stroke Rats by Down-Regulating Microglial M1 Polarization through $\alpha 7$ nAChR-NF- κ B Signaling Pathway. *Int. J. Biol. Sci.* **17** (4), 1088–1100 (2021).
11. Li, J., Dai, X., Zhou, L., Li, X. & Pan, D. Edaravone Plays Protective Effects on LPS-Induced Microglia by Switching M1/M2 Phenotypes and Regulating NLRP3 Inflammasome Activation. *Front. Pharmacol.* **12**, 691773 (2021).
12. Liu, X. et al. Bone marrow mesenchymal stem cell-derived exosomes attenuate cerebral ischemia-reperfusion injury-induced neuroinflammation and pyroptosis by modulating microglia M1/M2 phenotypes. *Exp. Neurol.* **341**, 113700 (2021).
13. Woo, H. & Eyun, S. I. Applications and techniques of single-cell RNA sequencing across diverse species. *Brief. Bioinform* ;**26**(4). (2025).
14. Song, W., Wang, D., Li, J. & Zhang, R. Spatial transcriptomics: integrating platforms and computational approaches for clinical insights. *Biotechnol. Adv.* **87**, 108791 (2026).
15. Zhang, L. et al. Clinical and translational values of spatial transcriptomics. *Signal. Transduct. Target. Ther.* **7** (1), 111 (2022).
16. Zhang, Y., Li, T., Wang, G. & Ma, Y. Advancements in Single-Cell RNA Sequencing and Spatial Transcriptomics for Central Nervous System Disease. *Cell. Mol. Neurobiol.* **44** (1), 65 (2024).
17. Longo, S. K., Guo, M. G., Ji, A. L. & Khavari, P. A. Integrating single-cell and spatial transcriptomics to elucidate intercellular tissue dynamics. *Nat. Rev. Genet.* **22** (10), 627–644 (2021).
18. Li, L. et al. Neuroprotective effects of genistein-3'-sodium sulfonate on focal cerebral ischemia in rats. *Neurosci. Lett.* **646**, 43–48 (2017).
19. Shuang, L. et al. Genistein-3'-sodium sulfonate enhances neurological function in neonatal rats with hypoxia-ischemia during the recovery period. *Heliyon* **10** (18), e37696 (2024).
20. Xie, T. et al. Insight into the Neuroprotective Effect of Genistein-3'-Sodium Sulfonate Against Neonatal Hypoxic-Ischaemic Brain Injury in Rats by Bioinformatics. *Mol. Neurobiol.* **60** (2), 807–819 (2023).
21. Yu, Y. et al. Genistein-3'-sodium sulfonate suppresses NLRP3-mediated cell pyroptosis after cerebral ischemia. *Metab. Brain Dis.* **40** (1), 99 (2025).
22. Xie, J. et al. Genistein-3'-sodium sulfonate ameliorates cerebral ischemia injuries by blocking neuroinflammation through the $\alpha 7$ nAChR-JAK2/STAT3 signaling pathway in rats. *Phytomedicine* **93**,

153745 (2021).

23. Liu, R. et al. Genistein-3'-sodium sulfonate promotes brain functional rehabilitation in ischemic stroke rats by regulating astrocytes polarization through NF- κ B signaling pathway. *Chem. Biol. Interact.* **400**, 111159 (2024).
24. Ritchie, M. E. et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* **43** (7), e47 (2015).
25. Rutter, L., Moran Lauter, A. N., Graham, M. A. & Cook, D. Visualization methods for differential expression analysis. *BMC Bioinform.* **20** (1), 458 (2019).
26. Hu, J. & Szymczak, S. A review on longitudinal data analysis with random forest. *Brief. Bioinform.* **24**(2). (2023).
27. Wang, Q. et al. Nomogram established on account of Lasso-Cox regression for predicting recurrence in patients with early-stage hepatocellular carcinoma. *Front. Immunol.* **13**, 1019638 (2022).
28. Hao, Y. et al. Dictionary learning for integrative, multimodal and scalable single-cell analysis. *Nat. Biotechnol.* **42** (2), 293–304 (2024).
29. Jin, S., Plikus, M. V. & Nie, Q. CellChat for systematic analysis of cell-cell communication from single-cell transcriptomics. *Nat. Protoc.* **20** (1), 180–219 (2025).
30. Rao, Y. et al. Single-Cell Transcriptome Profiling Identifies Phagocytosis-Related Dual-Feature Cells in A Model of Acute Otitis Media in Rats. *Front. Immunol.* **12**, 760954 (2021).
31. Osorio, D. et al. scTenifoldKnk: An efficient virtual knockout tool for gene function predictions via single-cell gene regulatory network perturbation. *Patterns (N Y)*. **3** (3), 100434 (2022).
32. Yang, B., Bao, W., Chen, B. & Song, D. Single_cell_GRN: gene regulatory network identification based on supervised learning method and Single-cell RNA-seq data. *BioData Min.* **15** (1), 13 (2022).
33. Guo, Z. Z. et al. Mapping biology in space: from spatial transcriptomics platforms to analytical tools and databases. *Sci. Bull. (Beijing)*. **71** (4), 921–945 (2026).
34. Li, S. et al. Artificial Intelligence Revolution in Transcriptomics: From Single Cells to Spatial Atlases. *Adv. Sci. (Weinh)*. **13** (5), e18949 (2026).
35. Qing, J., Wu, J., Li, Y. & Wu, J. SCNT: an R package for data analysis and visualization of single-cell and spatial transcriptomics. *BMC Bioinform.* **26** (1), 184 (2025).
36. Elosua-Bayes, M., Nieto, P., Mereu, E., Gut, I. & Heyn, H. SPOTlight: seeded NMF regression to deconvolute spatial transcriptomics spots with single-cell transcriptomes. *Nucleic Acids Res.* **49** (9), e50 (2021).
37. Ajoalabady, A. et al. Dual role of microglia in neuroinflammation and neurodegenerative diseases. *Neurobiol. Dis.* **216**, 107133 (2025).
38. Anwar, M. M., Pérez-Martínez, L. & Pedraza-Alva, G. Exploring the Significance of Microglial Phenotypes and Morphological Diversity in Neuroinflammation and Neurodegenerative Diseases: From Mechanisms to Potential Therapeutic Targets. *Immunol. Invest.* **53** (6), 891–946 (2024).

39. Dendrou, C. A., McVean, G. & Fugger, L. Neuroinflammation - using big data to inform clinical practice. *Nat. Rev. Neurol.* **12** (12), 685–698 (2016).
40. Shimizu, T. & Prinz, M. Microglia across evolution: from conserved origins to functional divergence. *Cell. Mol. Immunol.* **22** (12), 1533–1548 (2025).
41. Tang, Y. & Le, W. Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. *Mol. Neurobiol.* **53** (2), 1181–1194 (2016).
42. Kwon, H. S. & Koh, S. H. Neuroinflammation in neurodegenerative disorders: the roles of microglia and astrocytes. *Transl Neurodegener.* **9** (1), 42 (2020).
43. Zhou, J. et al. A20-binding inhibitor of NF- κ B (ABIN1) controls Toll-like receptor-mediated CCAAT/enhancer-binding protein β activation and protects from inflammatory disease. *Proc. Natl. Acad. Sci. U S A.* **108** (44), E998–1006 (2011).
44. Hong, J. Y., Lin, S. C., Kuo, B. J. & Lo, Y. C. Structural and Biochemical Basis for Higher-Order Assembly between A20-Binding Inhibitor of NF- κ B 1 (ABIN1) and M1-Linked Ubiquitins. *J. Mol. Biol.* **433** (18), 167116 (2021).
45. Chen, Y. et al. Downregulation of TNIP1 Expression Leads to Increased Proliferation of Human Keratinocytes and Severer Psoriasis-Like Conditions in an Imiquimod-Induced Mouse Model of Dermatitis. *PLoS One.* **10** (6), e0127957 (2015).
46. Ippagunta, S. K. et al. Keratinocytes contribute intrinsically to psoriasis upon loss of Tnip1 function. *Proc. Natl. Acad. Sci. U S A.* **113** (41), E6162–e71 (2016).
47. Shamilov, R. & Aneskievich, B. J. TNIP1 in Autoimmune Diseases: Regulation of Toll-like Receptor Signaling. *J. Immunol. Res.* **2018**, 3491269 (2018).
48. Peng, K., Li, Y., Lu, C. & Hu, S. ABIN-1 protects chondrocytes from lipopolysaccharide-induced inflammatory injury through the inactivation of NF- κ B signalling. *Clin. Exp. Pharmacol. Physiol.* **47** (7), 1212–1220 (2020).
49. Pasula, S. et al. Role of Systemic Lupus Erythematosus Risk Variants With Opposing Functional Effects as a Driver of Hypomorphic Expression of TNIP1 and Other Genes Within a Three-Dimensional Chromatin Network. *Arthritis Rheumatol.* **72** (5), 780–790 (2020).
50. Bossini-Castillo, L. et al. Confirmation of TNIP1 but not RHOB and PSORS1C1 as systemic sclerosis risk factors in a large independent replication study. *Ann. Rheum. Dis.* **72** (4), 602–607 (2013).
51. Shinkawa, Y. et al. ABIN1 is a signal-induced autophagy receptor that attenuates NF- κ B activation by recognizing linear ubiquitin chains. *FEBS Lett.* **596** (9), 1147–1164 (2022).
52. Verstrepen, L., Carpentier, I., Verhelst, K., Beyaert, R. & ABINs A20 binding inhibitors of NF-kappa B and apoptosis signaling. *Biochem. Pharmacol.* **78** (2), 105–114 (2009).
53. Verstrepen, L., Carpentier, I. & Beyaert, R. The biology of A20-binding inhibitors of NF-kappaB activation (ABINs). *Adv. Exp. Med. Biol.* **809**, 13–31 (2014).

Figures

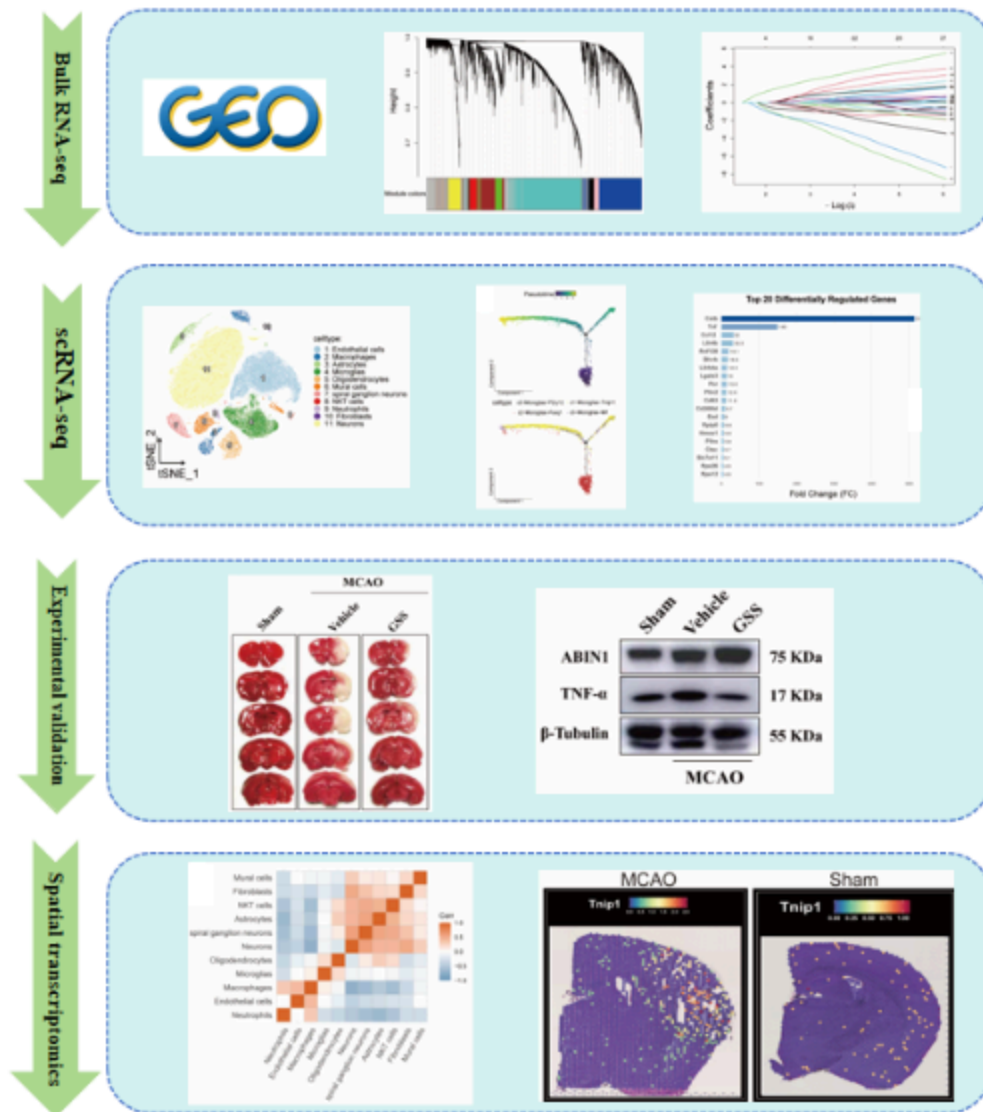


Figure 1

Flowchart depicting the study design.

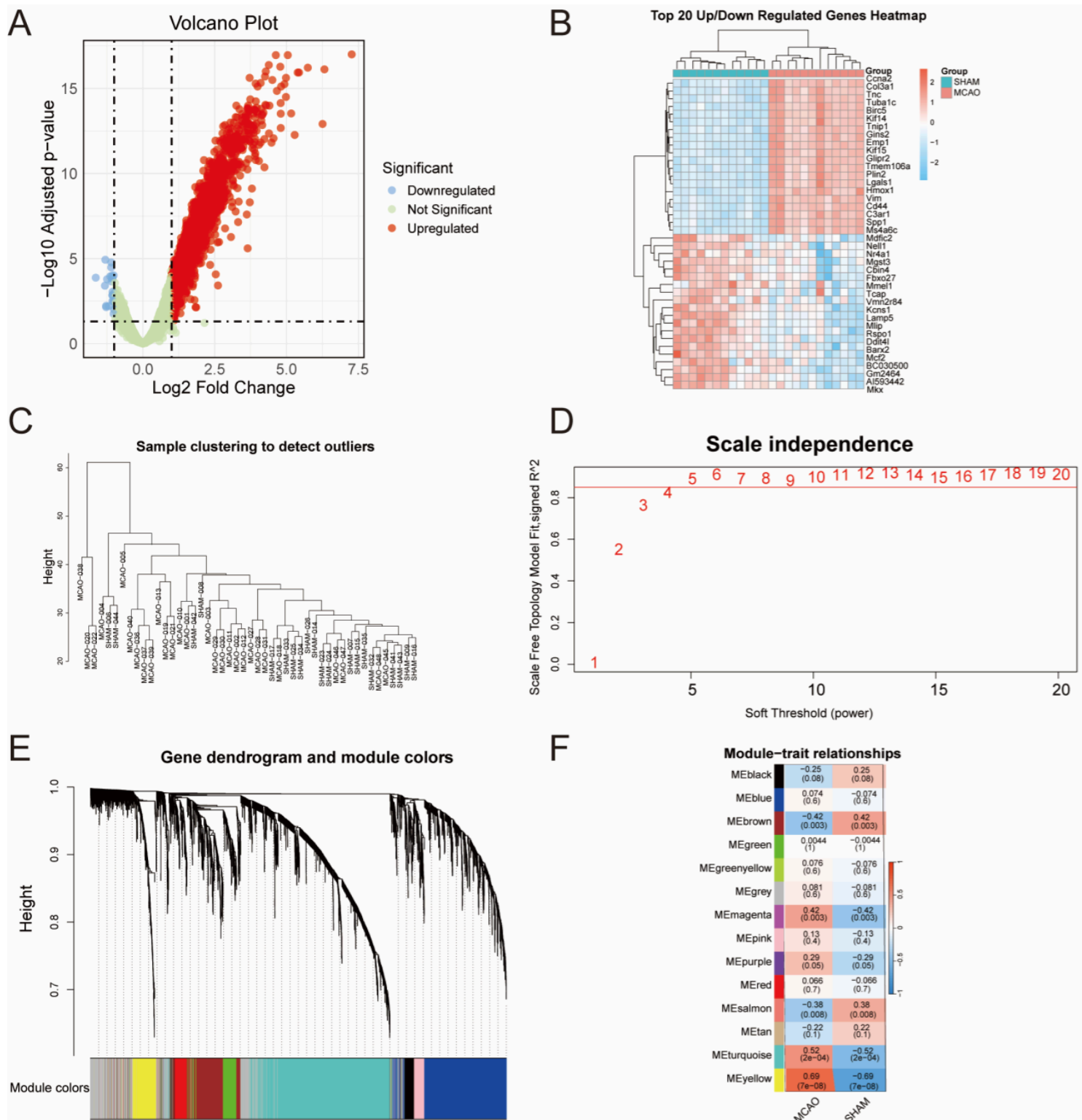


Figure 2

DEG identification and WGCNA analysis of bulk transcriptome data.

(A) Volcano plot for DEG distribution between MCAO and sham groups. (B) Expression heatmap of top 40 representative DEGs. (C) Sample clustering confirmed no outlier samples. (D) Soft threshold screening for scale-free network construction (power = 5). (E) Gene dendrogram with color-coded co-

expression modules. (F) Module-MCAO/sham correlation heatmap; yellow module was the key stroke-associated module with the highest phenotype correlation.

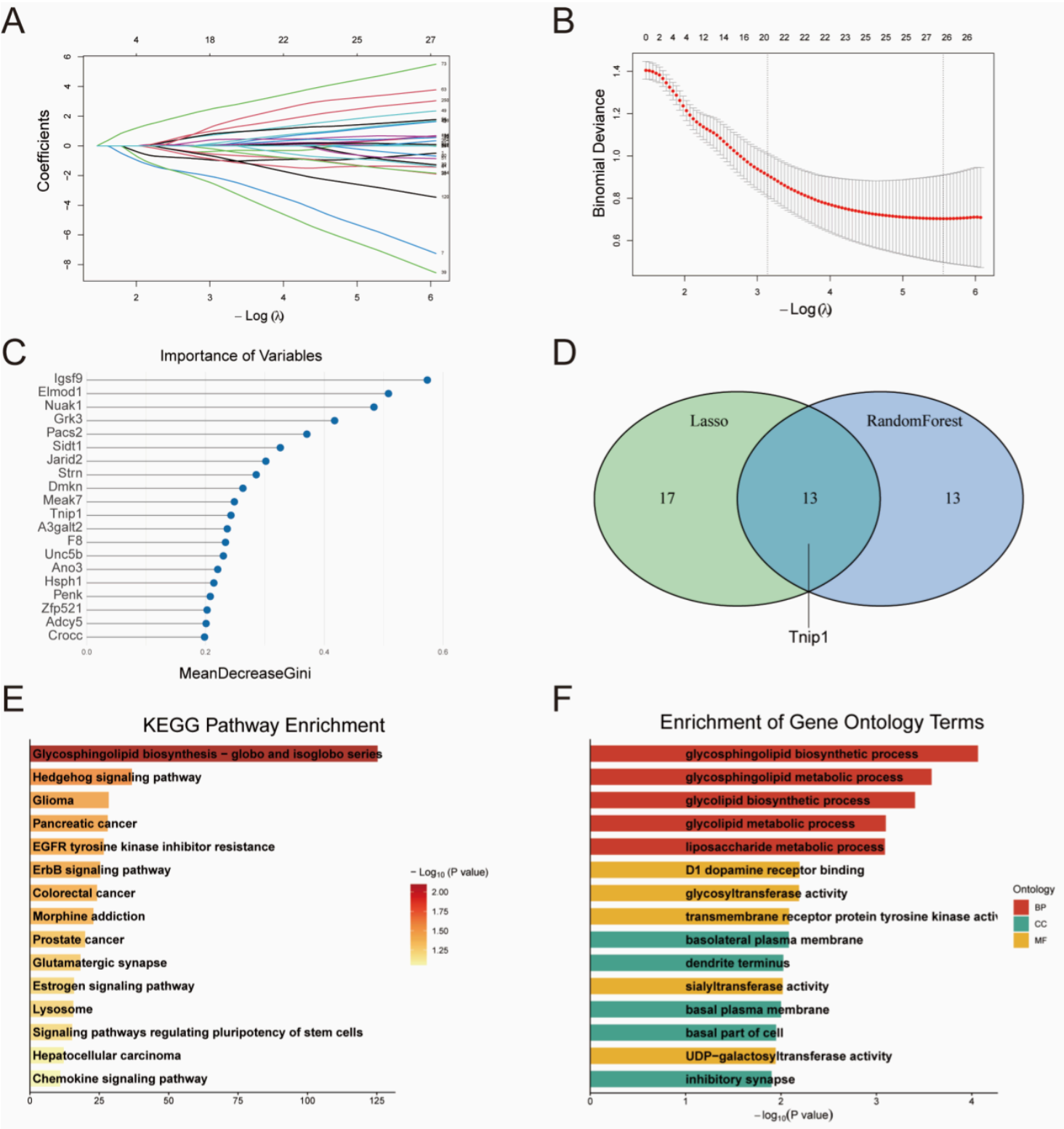


Figure 3

Hub gene identification by machine learning and subsequent functional enrichment.

(A-B) LASSO regression coefficient curve and deviance plot for optimal λ determination. (C) Gene importance ranking from random forest based on MeanDecreaseGini index. (D) Venn plot showing shared 13 overlapping genes between LASSO and random forest outcomes, including TNIP1. (E-F) Top enriched KEGG pathways and GO functional terms of intersecting hub genes. KEGG pathway enrichment analysis was performed based on the KEGG database (Kanehisa Laboratories, <https://www.kegg.jp/>). Publication permission (Ref: 262373) has been obtained from Kanehisa Laboratories.

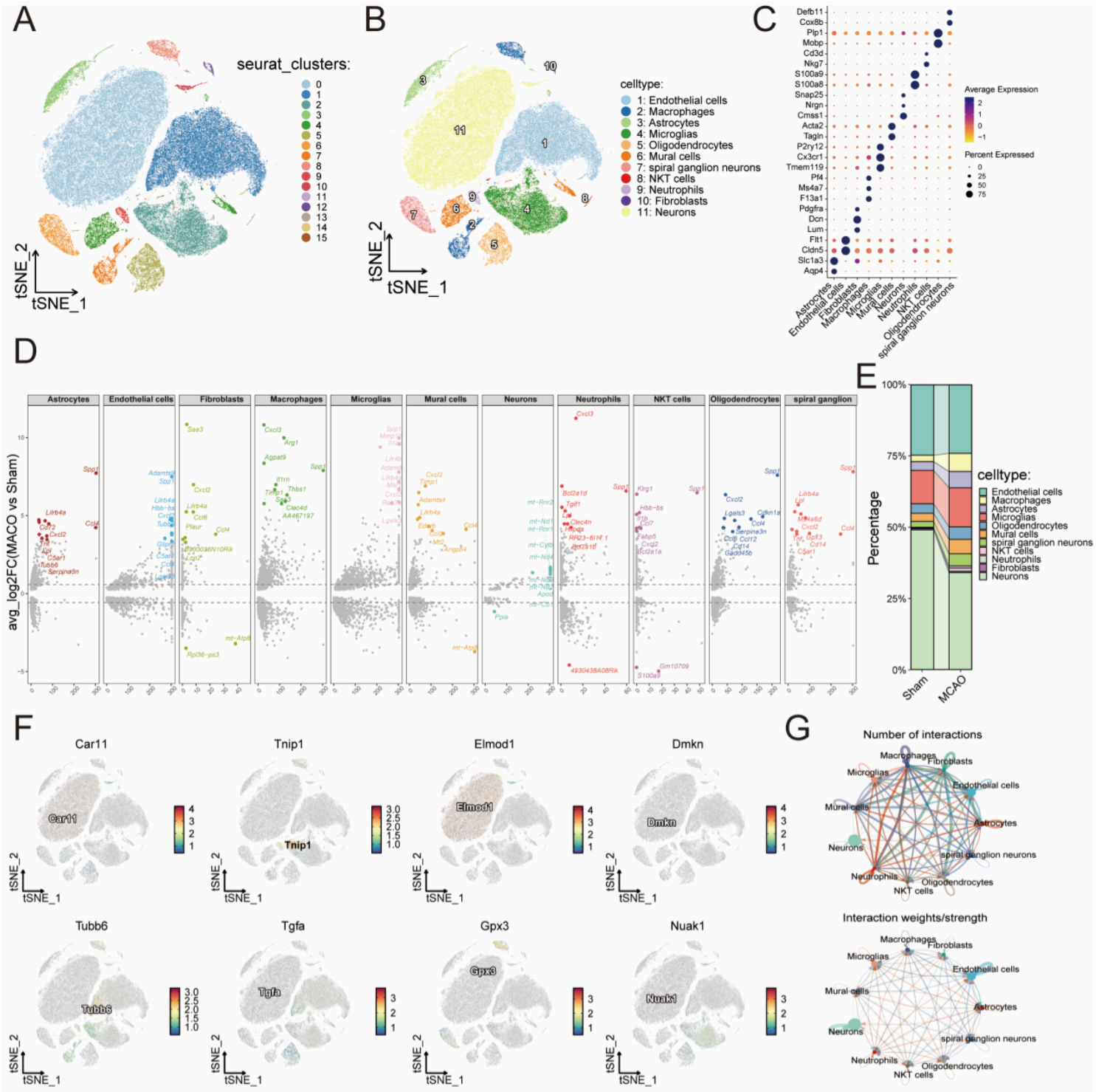


Figure 4

Single-cell transcriptome characterization of ischemic brain.

(A-B) t-SNE plots showing unsupervised cell clustering and subsequent cell-type annotation. (C) Dot plot of cell-type-specific marker gene expression. (D) Split violin plots depicting transcriptional alteration between MCAO and sham per cell subtype. (E) Bar graph of cellular proportion changes upon cerebral ischemia. (F) Feature plots of eight core hub genes, highlighting microglia-specific enrichment of *TNIP1*. (G) Cell-cell interaction network demonstrating microglia-centered intercellular communication after stroke.

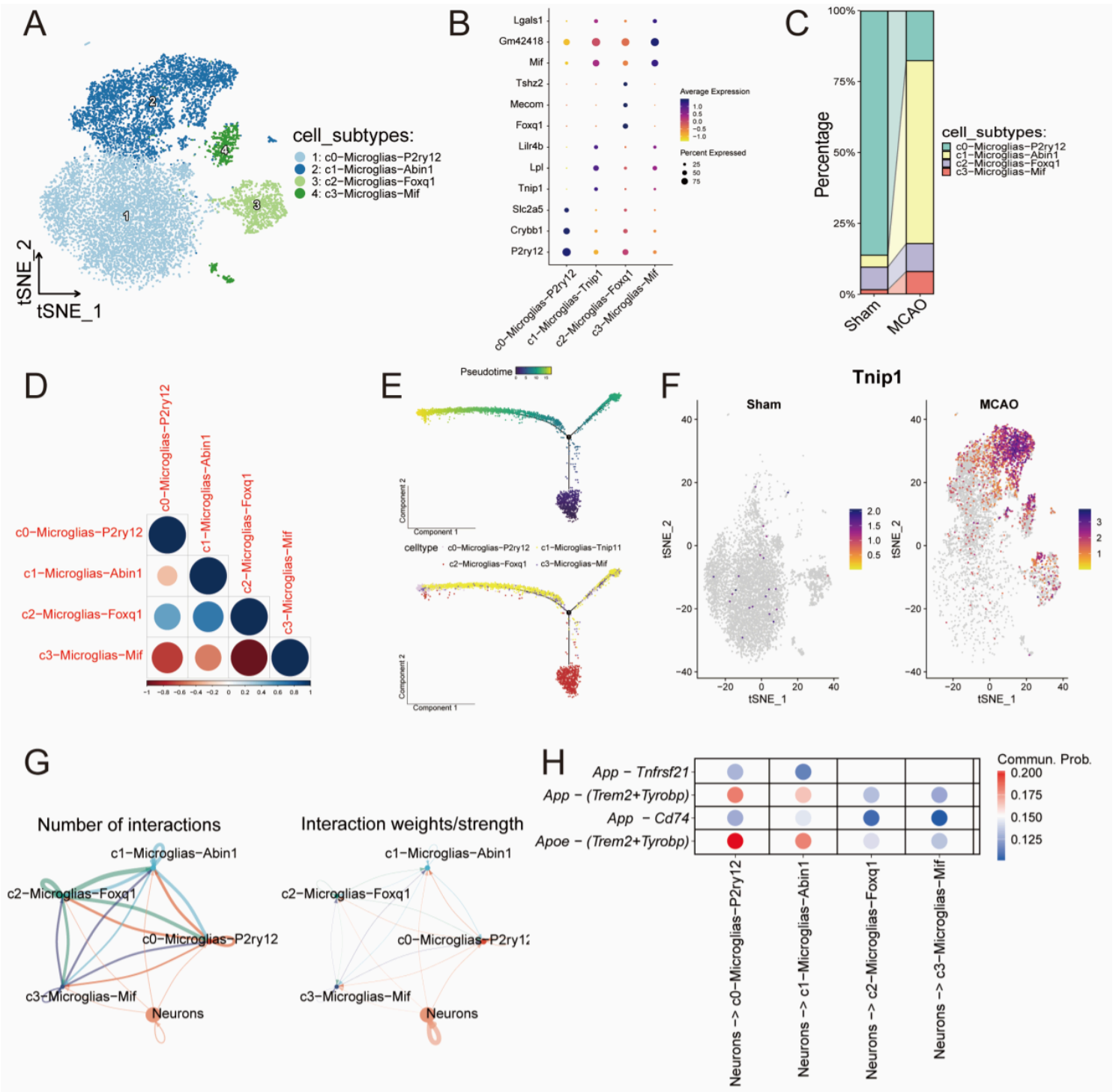


Figure 5

Dynamic Changes and Communicative Roles of Microglial Subpopulations post MCAO.

(A) t-SNE plot showing four microglial subclusters. (B) Marker gene dot plot for subcluster identification. (C) Bar chart of subtype proportion changes between sham and MCAO. (D) Correlation heatmap of signature genes across four microglia subsets.(E) Pseudotime trajectory of microglial differentiation. (F) *TNIP1* expression feature plots in sham versus MCAO microglia. (G-H) CellChat-derived network and ligand-receptor probability heatmap for neuron-to-microglia communication.

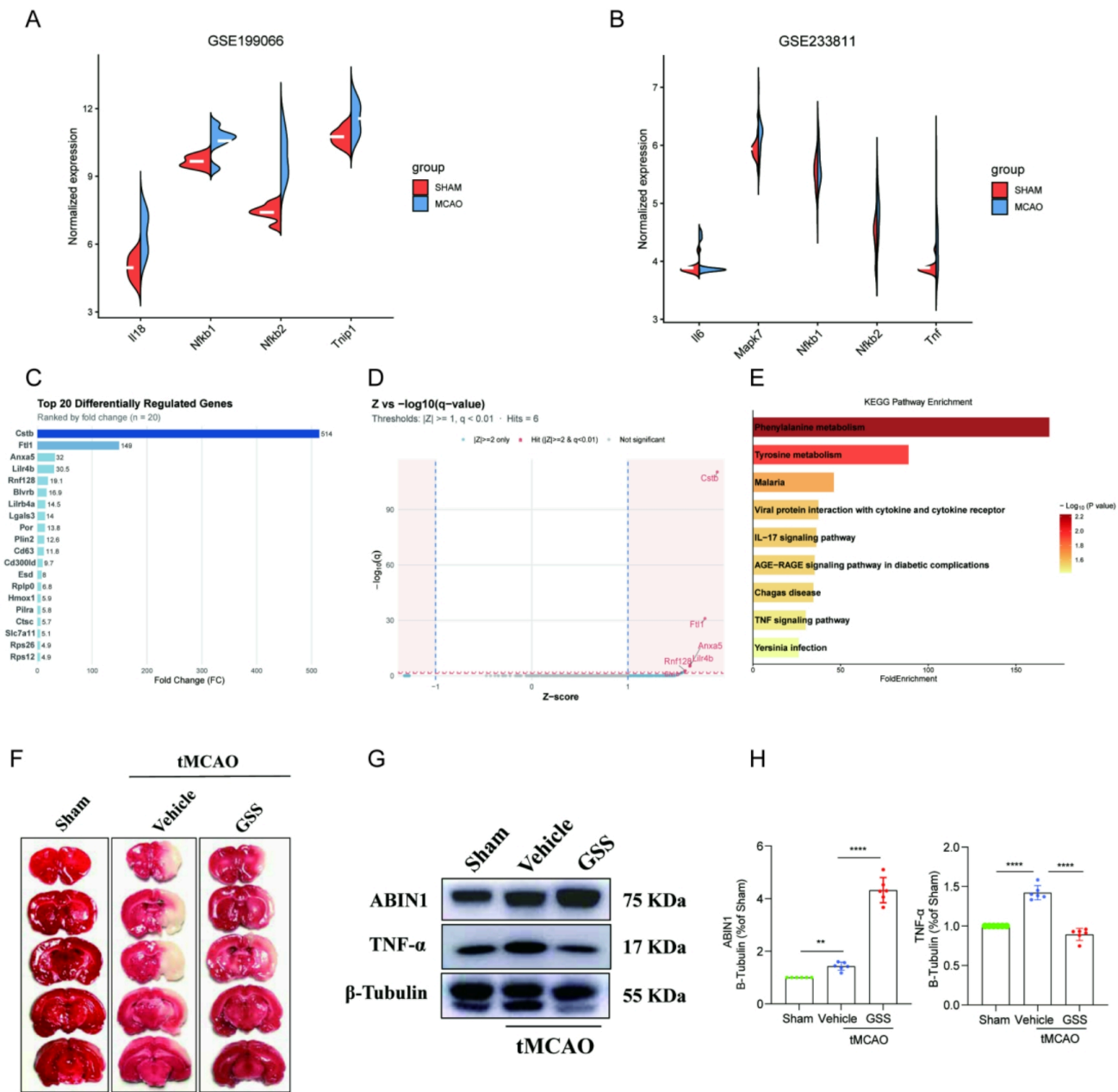


Figure 6

Dataset validation & in vivo verification of GSS on ABIN1/TNF-α axis.

(A-B) Violin plots validated elevated *TNIP1* and pro-inflammatory transcripts in two independent MCAO public datasets. (C-E) Transcriptome and pathway enrichment after *TNIP1* knockout confirmed the regulatory role of *TNIP1* in inflammatory signaling. (F-I) TTC staining and western blot verified that GSS upregulates ABIN1, inhibits TNF-α and alleviates cerebral infarction in MCAO rats. Results are expressed as means ± S.E.M. Data was analyzed by one-way ANOVA followed by Tukey's multiple comparisons test as post-hoc test. ** $P < 0.01$, *** $P < 0.001$.

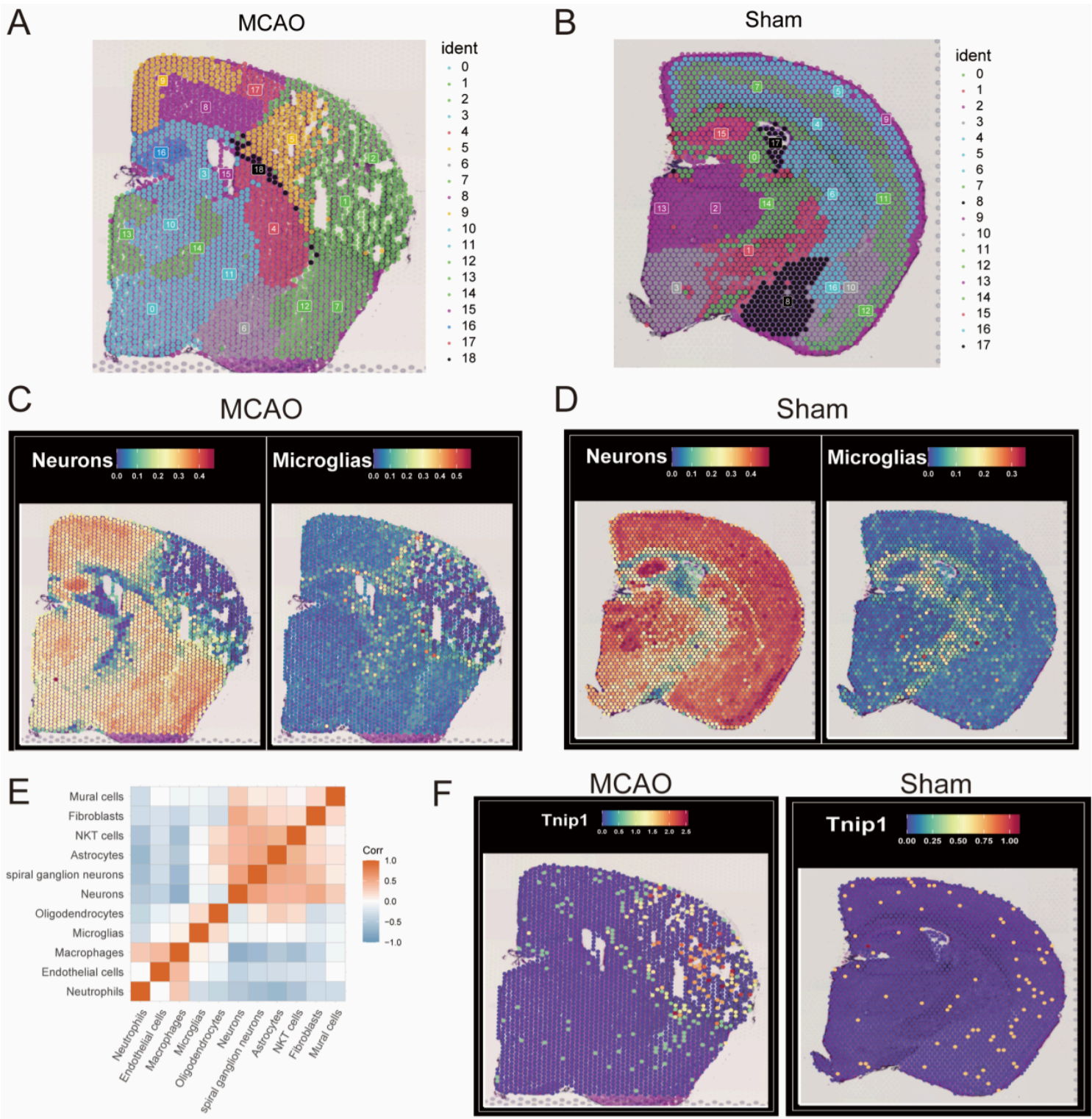


Figure 7

Spatial transcriptome comparison between MCAO and Sham cerebral tissue.

(A-B) Spatial cluster segmentation of brain slices in MCAO and Sham groups. (C-D) Spatial distribution of neurons and microglia across brain sections. (E) Intercellular transcriptional correlation matrix of all brain cell subtypes. (F) Spatial expression landscape of *TNIP1*, showing ischemia-induced upregulation predominantly within microglia-rich regions.

Supplementary Files

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

- [SupplementaryFigureS1.pdf](#)
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