Supplementary Materials

Abbreviations

BDNF= brain-derived growth factor, CAMK2N1= calcium/calmodulin-dependent protein kinase II inhibitor 1, CBF= cerebral blood flow, DEGs= differentially expressed genes, DC= duty cycle, EMG= electromyography, GO= gene ontology, HMGB1= high mobility group protein B1, IS= ischemic stroke, LIFUS= low-intensity focused ultrasound stimulation, MCAO= middle cerebral artery occlusion, PRF= pulse repetition frequency, SD= sonication duration, UMAP= uniform manifold approximation and projection, US= ultrasound stimulation, VEGF= vascular endothelial growth factor

Methods

Group and use of mice

In sham-related groups, we used n = 6 per group for laser speckle, n = 4 per group for Golgi staining and western blot, and n = 3 per group for FISH, cresyl violet-staining, immunostaining, fiber photometry, EMG, and neurobehavioral, what's more, we used n = 8 per group to be 1 sample for scRNA seq, total is 42, which was used to show that the intensity, frequency, and duration of ultrasound used in this research did not produce significant trends in normal mice. HMGB1 inhibition and CAMK2N1 overexpression in normal mice mainly to show that gene manipulations had no effect on sham mice.

In stroke related groups, we used about n = 10-12 per group for laser speckle and neurobehavioral, n = 7 for cresyl violet-staining, n = 4 per group for immunostaining, Golgi staining and western blot, and n = 3 per group for FISH, fiber photometry, EMG and iTRAQ proteomics, what's more, we used n = 8 per group to be 1 sample for scRNA seq. At the same time, to maximize the use of mice, we performed laser speckle, fiber photometry or electromyography on days 7 and 13, or neurobehavioral on days 7 and days 14, then sacrificed the mice, which could be used for immunostaining, Golgi staining and Western blot. Total is 78. Based on the above, we used 120 mice (pre-experiments and died mice are not included) to get feasible results and corresponding significance in the experiments.

A mouse model of transient middle cerebral artery occlusion (MCAO)

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University, Shanghai, China. They were conducted in accordance with the national laws for the use of animals in research and followed the ARRIVE (Animal Research: Reporting of *in vivo* Experiments) guidelines. MCAO was performed as described following. Briefly, adult mice were anesthetized with 1%-1.5% isoflurane and 30%/ 70% oxygen/nitrous oxide. Body temperature was maintained at 37.0±0.5°C using a heating pad. The common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were separated, and a 6-0 suture (Covidien, Mansfield, MA) coated with silicon

(Heraeus Kulzer, Germany) was inserted from ECA, followed by ICA, until the opening of MCA. The success of occlusion was determined by monitoring the decrease in surface CBF to the 10% of baseline CBF using a laser Doppler flowmetry (Moor Instruments, Devon, UK). The filament was then removed 90 min after MCAO to allow blood flow restoration.

Ultrasound stimulation system

A focused ultrasound transducer (Ultrasound Neurostimulation System, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China) with center frequency (f) 500 kHz, PRF 1 kHz, SD 300 ms, and DC 50% was used across the ultrasound stimulation experiment. The design of ultrasound stimulation parameters showed in the supplementary materials and the focused ultrasound transducer was placed right above the ipsilateral hemisphere of brain.

Laser sparkle imaging examination

Mice were anesthetized with isoflurane and fixed on the stereotaxic apparatus. Seven days and 13 days after MCAO, CBF was measured before and after LIFUS by a laser speckle imaging machine (RWD, Shenzhen, China). The relevant parameters were as followed, the exposure time was 1 ms, the shooting time was 5 sec, 2 frames per sec, the magnification was 3 times, and the laser intensity was 110 mW. Two data processing methods were used. For the immediate response of ipsilateral hemisphere after ultrasound stimulation following 7 and 13 days of MCAO, CBF of ROI was calculated as $(CBF_{End}\text{-}CBF_{Start})/CBF_{Start}$ %. Absolute CBF detected before ultrasound at day 7 and day 13, which was considered as the endpoint CBF of ipsilateral hemisphere. Absolute value was changed to ratio of ipsilateral/contralateral CBF in **Fig. 4** to avoid the influence of external factors on laser speckle value, and contralateral CBF was as a control.

Atrophy volume assessment by cresyl violet staining

A series of 20 μm in thickness and 200 μm in interval brain cryosections from anterior commissure to lateral ventricle were collected. The sections were stained with 0.1% cresyl violet solution (Sigma, St Louis, MO), and then the atrophied areas were measured by subtracting the stained area in the ipsilateral hemisphere area from the contralateral hemisphere using Image *J* (NIH, Bethesda, MD) software. The brain atrophy volume was calculated with the following formula: $V = \sum h/3*[\Delta Sn+(\Delta Sn*\Delta Sn+1)^{1/2}+\Delta Sn+1]$, in which V represents volume, *h* represents the distance between the two adjacent brain sections, ΔSn and ΔSn+1 represent the area differences between the two adjacent sections. Areas were then integrated to obtain the total atrophy volume. Atrophy size assessment was performed by a blinded investigator.

iTRAQ proteomics analysis

Proteins were isolated from the perifocal regions of the ischemic stroke (IS) and IS ultrasound stimulation (US) groups, at least 3 independent samples for each group. Proteins were extracted then the concentrations were measured by the BCA assay. Samples were analyzed by SDS-PAGE. Trypsin was used for enzymolysis to get peptides that were labeled

with iTRAQ 8PLEX (4381663, ABSCIEX). The equally mixed labeled peptides were separated by RPLC on an 1100 HPLC System (Agilent) with an Agilent Zorbax Extend RP column (5 μm, 150 mm X 2.1 mm). Subsequently, Liquid chromatography tandem-mass spectrometry (LC-MS/MS) was performed for the identification and quantification of proteins. Samples were loaded by a capillary trap column (100 μm X 2 cm, RP-C18, Thermo Fisher, Waltham, MA) and then separated by a capillary analytical column (15 cm X 75 μm, RP-C18, Thermo Fisher) on an EASY-nLC^{TM} 1200 system (Thermo Fisher). All analyses were performed by a Q-Exactive mass spectrometer equipped with a Nanospray Flex source (Thermo Fisher). Tissue processing and data acquisition were performed by Oebiotech (Oebiotech, Shanghai, China). A $p<0.05$ and $|\log 2\text{-fold change}| > 0.58$ was set as the threshold for significantly differential expression.

Single-cell RNA sequencing

Ipsilateral target tissues from IS, IS US, sham or US, mice (n = 8/group) were rapidly and carefully collected and dissociated using adult brain dissociation kit from Miltenyi Biotec (Bergisch Gladbach, Germany). Single-cell gel beads in emulsions (GEMs) were generated by loading single-cell suspensions onto a Chromium Single-Cell Controller Instrument (10X Genomics). Approximately 12,000 cells were added to each channel. Next, reverse transcription reactions were engaged to generate barcoded full- length cDNA, and cDNA clean-up was performed with DynaBeads Myone Silane Beads (Thermo Fisher). Then cDNA was amplified by PCR and the amplified cDNA was fragmented, end-repaired, A-tailed, and ligated to an index adaptor, and then the library was amplified. Every library was sequenced on a HiSeq X Ten platform (Illumina), and 150 bp paired-end reads were generated. Tissue processing and data acquisition were then performed (Oebiotech, Shanghai, China).

scRNA seq data preprocessing and analysis

To remove the batch effects in single-cell RNA-sequencing data, the mutual nearest neighbors (MNN) was performed with the R package batchelor. To remove low-quality cells and likely multiple captures, which was a major concern in microdroplet-based experiments, we applied a criterion to filter out cells with UMI/gene numbers out of the limit of mean value±2 fold of standard deviation assuming a Gaussian distribution of each cells' UMI/gene numbers. Following visual inspection of the distribution of cells by the fraction of mitochondrial genes expressed, we further discarded low-quality cells where 10% of counts belonged to mitochondrial genes and >5% of the counts belonged to hemoglobin genes. Library size normalization was performed in the Seurat on the filtered matrix to obtain the normalized count. Top variable genes across single cells were identified using Macosko's method. To remove the batch effects in single-cell RNA sequencing data, the mutual nearest neighbors (MNN) presented was performed with the R package bachelor. Graph-based clustering was performed to cluster cells according to their gene expression profiles using the FindClusters function. Cells were visualized using a 2D Uniform Manifold Approximation and Projection (UMAP) algorithm with the Run UMAP function. FindAllMarkers function was used to identify marker genes of each cluster and identified positive markers for a given cluster compared with all other cells. Then we used the MouseRNAseqData package in

SingleR, a computational method for unbiased cell type recognition of scRNA-seq to infer the cell of origin of each of the single cells independently and identify cell types. Differentially expressed genes (DEGs) were identified using the Seurat package. A $p<0.05$ and |log 2-fold change| > 0.58 was set as the threshold for significantly differential expression. GO enrichment pathway enrichment analysis of DEGs were respectively performed using R based on the hypergeometric distribution.

Viral vector production and injection

Adeno-associated virus (AAV-PHP.eB) was packaged commercially (OBiO Technology Corp., Ltd., Shanghai, China). After purification, the viral titer was determined by real-time PCR. A total volume of 100 μl of PBS containing $3X10^{12}$ viral particles was injected by tail vein injection two weeks before MCAO. U6 and GfaABC1D promoter-driven shRNA perturbations were established in pAAV-U6/GfaABC1D -shRNA v2.0-CMV-WPRE vector, respectively. HMGB1 shRNA was designed based on the CDS sequence confirmed on Sigma (TRCN0000365912), which efficiently knocked down 95% HMGB1 expression in B16-F0 cells. Positive clones were sent for sequencing and plasmid extraction was followed then HMGB1 primer was confirmed as forward primer 5'- TGACAAGGCTCGTTATGAAAG -3' and reverse primer 5'- CTTTCATAACGAGCCTTGTCA -3'. Then sequence subcloned into the new pAAV scramble to be pAAV-U6-sh (HMGB1)-CMV-WPRE and pAAV-GfaABC1D-sh (HMGB1)-CMV-WPRE. Similarly, CAG and GfaABC1D promoter-driven Camk2N1 overexpression systems were established in the pAAV-CAG-P2A-3xFLAG-WPRE vector and pAAV-GfaABC1D-P2A-3xFLAG-WPRE vector, respectively. CAMK2N1 was designed by its CDS, primer was confirmed as forward primer 5'-CATGGTCCTGCTGGAGTTCGTG -3' and reverse primer 5'-CATAGCGTAAAAGGAGCAACA -3'. Targeted gene was designed based on the CDS sequence, then sequence subcloned into the new pAAV scramble to be pAAV-CAG-P2A-CAMK2N1-3xFLAG-WPRE and pAAV-GfaABC1D-P2A-CAMK2N1-3xFLAG-WPRE, correspondingly. In addition, we used gf to indicate GFAP in the figures.

Brain tissue collection

For immunostaining and Western blotting analysis, mice were perfused intracardially with cold sterile 1x PBS followed at 14 days of MCAO mice. The whole brain was immediately frozen in liquid nitrogen (−20°C, 10 min) then transferred to a −80°C deepfreeze. Cryosection (20 μm in thickness) from anterior commissure to hippocampus were cut and collected using a cryostat. Immunostaining was carried out by sampling one section per 200 mm, and 4 to 5 sections were collected effectively span the entire injured region of each mouse. Similarly, one section per 200 mm was collected and stored at -20°C for qPCR, eight sections per 200 mm was collected for Western blotting analysis. We collected 10 sections from 200 mm in thickness to make samples for mRNA, protein or immunostaining were all from the same mice and results could be more solid from molecular level to histological level. Then cold TRIzol reagent was added into samples for qPCR, precooled protein lysis buffer (RIPA with protease cocktail inhibitor, and phosphatase inhibitor) was used to extract protein. For sampling of iTRAQ proteomics and scRNA seq, MCAO mice were perfused

intracardially with cold sterile 1X PBS after LIFUS. The brain was rapidly placed in a cold mouse brain matrix. The brain was cut into a 2 mm-thick section before and behind the center of Willis Circle. A 2 mm punch was used to separate the target region in the ipsilateral penumbra.

Immunostaining

Brain sections were fixed by 4% paraformaldehyde (PFA, Sinopharm Chemical Reagent, China) for 10 min, after rinsed, the sections were incubated with 0.3% TritonX-100 (Sigma, St Louis, MO) for 10 min and blocked with 5% bovine serum albumin (BSA, GBICO, MA). Brain sections were then incubated with goat-anti CD31 (1:200, AF3628, R&D, Minneapolis, MN), rabbit-anti Ki67 (1:200, ab15580, Abcam, Cambridge, England) at 4°C overnight. After rinsing with PBS for 3 times, brain sections were incubated with the secondary antibodies: Alexa fluor 488-conjugated donkey anti-rabbit secondary antibody (1:400, A21206, Invitrogen, Carlsbad, CA) and Alexa fluor 555-conjugated donkey anti-goat secondary antibody (1:400, A21432, Invitrogen) for 1 hour at 37°C. For mice injected with lectin DyLight 594 (Lycopersicon esculentum (Tomato), L32471, Invitrogen), sections were co-immunostaining with Ki67 and proceeded to subsequent staining steps. Images were acquired using a confocal microscope (TCS SP5, Leica).

Western blotting analysis

Equal amounts of protein (20 μg) were loaded onto 10% (W/V) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoresed. The proteins were transferred onto PVDF Transfer Membrane, (0.45 μm, RIPA, Millipore, Burlington, Mass) and incubated with the primary antibodies (**Table S4**) at 4°C overnight. The membrane was washed in 1XTBST buffer and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mice IgG (1:5000, Invitrogen) for 1 hour at RT, and then reacted with an enhanced chemiluminescence substrate (Meilunbio, Shanghai, China). Bright-field image and chemiluminescent blots were merged using Tanon GIS software (www.Bio-tanon.com.cn). The result of chemiluminescence was semi-quantified using the Image*J* software (NIH, Bethesda, MD).

Fluorescence *in situ* hybridization

In situ hybridization was performed using one biotin-labeled riboprobe for HMGB1 (5′-GCTTTCTTCTCATAGGGCTGCTTGTCATC -3′) and 2 mix biotin-labeled riboprobes CAMK2N1 (5′- CCGTAGGGCAGCACCTCCGACAT-3′ and 5′-GGCTCAGCTTCTCGTCGCCGTA-3′). Probes were synthesized and subsequent fluorescence in situ hybridization was performed by Gene Pharma (Shanghai, China). Brain cryosections (30 μm) were reactivated in citrate buffer (RT, 15 min), treated with proteinase K (37°C, 20 min) followed by blocked (37°C, 30 min), denatured (78°C, 2 min), dehydrated using graded ethanol (RT, 8 min) and then hybridized with probe working solution (biotin-probe: SA-Cy3: PBS = 8:1:1) in hybridization buffer overnight at 37°C. Hybridized sections were washed with 0.2 x saline-sodium citrate (SSC) buffer for 15 min (wash I), with 2 x SSC at 60°C for 30 min (wash II), and washed at 37°C for 30 min (wash III). For

immunostaining, sections were incubated with anti-GFAP (1:100, ab53554, Abcam) primary antibody overnight at 4°C. After being washed with PBS, slices were incubated with a second antibody (1:400, A11055, Invitrogen). Images were visualized under a confocal microscope.

Golgi-Cox staining

Golgi-Cox staining were strictly followed by the manufacturer's instructions (FD Rapid GolgiStain™ Kit, MD). Brains were quickly removed and rinsed, and then immersed in a 1:1 mixture of solutions A and B (containing mercuric chloride, potassium dichromate and potassium chromate) for 2 weeks at RT in dark. Brains were then transferred to solution C and kept in the dark for 4 days. Solution C was replaced after the first 24 hours. Brains were rapidly frozen in isopentane and kept at −80°C until sectioning. Cryosectioning was performed on a sliding microtome (Leica). Coronal sections of 100 μm thickness were cut and transferred to microscope slides (Lab Scientific) onto small drops of solution C and allowed to dry at RT overnight. Serial sections were stained with a mixture of D and E solutions for 10 min, then dehydrated in a series of graded ethanol, cleared in xylene, and cover slipped with neutral resin. Single-plane images were taken under a light field using a confocal microscope (Leica).

Neurobehavioral tests

Neurobehavioral tests were performed before and 1, 3, 7, and 14 days after MCAO by an investigator blinded to the experimental design and treatment.

Modified neurologic severity score (mNSS)

mNSS included a combination of motor, reflex, and balance tests. Severity scores ranged from 0 to 14, with 0 being normal and higher scores indicating more severe damage.

Tail suspension test

The tail suspension test was to raise the tail of a mouse to observe its deviation from the vertical midline. Each observation was repeated 20 times at 1 min interval, and the shift to the right/total was recorded.

Grid-walking test

The elevated grid walking apparatus was manufactured using wire mesh with a grid area of 32 cm/20 cm/50 cm (length/width/height). Each mouse was individually placed on the grid and allowed to walk freely for 5 min. A step was considered a stepping error (foot fault) if it did not provide support and the foot went through the grid hole. The total number of foot failures in right limb, as well as the total number of steps taken were counted. The ratio of foot faults was calculated as (contralateral faults/ total steps) *100%.

Rotarod test

The Rotarod test evaluates motor coordination function and balance. Mice were trained for 3 consecutive days before surgery. The Rotarod was accelerated from 5 to 40 rpm. for 300 sec as preoperative baseline. Following surgery, three trials were performed for each mouse.

Motor performance was assessed and the latency before falling off the accelerating rotating rod was recorded with a maximum of 300 sec.

Electromyogram recording

We recorded signals from electromyography using a commercialized system RZ2 BioAmp Processor (Tucker-Davis Technologies, TDT, Alachua County, FL). Connecting the RZ2 Interface to PC and a PZ PreaLmplifier to amplify signals, these electrical signals were translated into numerical values automatically. We implanted 5 fibers together in one mouse, two fibers implanted in the left hind limb, one of them connected to the positive pole, another connected to the negative pole, both two were in the same coordinates. Similarly, two fibers implanted in the right hind limb were connected in the same way, and the last fiber was connected to a backside grounding pole. Bandpass filtered (1017 Hz) amplitude of the electromyogram was collected automatically and visualized and completed the digital transformation by Synapse Software, which means after that data could be exported, processed and quantified by using custom MATLAB (MathWorks) scripts. Then electromyogram data were recorded continuously (300 sec were the total record duration, the former 60 sec were for baseline, the middle 240 sec were for ultrasound stimulation, and the last 60 sec were for post-record) in overall 5 min. For the statistical charts, average EMG amplitude values were calculated as (EMG duration - EMG baseline)/EMG baseline. EMG baseline was the mean of the amplitude signals from 1 sec to 60 sec. The heat map was plotted using a self-developed R program to display the variance of amplitude in 3 individual mice from each group.

Fiber photometry recording

We used a commercialized system (R810 dual-color multi-channel fiber photometry recording system, RWD) to detected calcium signals from neurons with LIFUS. Two weeks before MCAO, a total volume of 200 nl of PBS containing 5 x 10^9 pAAV-CMV-GCaMP6s-P2A-nls-dTomato (AAV2/9) viral particles were injected stereotactically at a rate of 50 nl/min at 1.7 mm lateral to the bregma and 3.5 mm under the dura using a micro-infusion pump (WPI, city, FL). The needle was left in place for 5 min to avoid reflux. After withdrawal to 3.4 mm ventral to the bregma, repeat the steps above and wait for 10 min to avoid reflux for the second time. The scalp was then sutured. Then mice were still anesthetized using isoflurane and fixed on a stereotaxic frame, optical fibers (200 mm O.D., 0.22 NA, RWD) were implanted into the exact place of 200 mm above viral vector injection coordinates, 3.5 mm under the dura is the exact coordinate to allow for optogenetic manipulation of neuron calcium florescence detect. The fiber was secured to the skull with bone screws and dental cement for every mouse. Mice were allowed to recover for 2 weeks after implantation, then performed MCAO. Mice were habituated for 10 min after connection to a laser source. A laser at wavelengths of 470 nm (blue) was applied and controlled with an intelligent optogenetic system to excite the GCamp6s fluorescent protein, laser at wavelengths of 410 nm was used as the reference, which effectively removed the motion artifact interference and obtained the real fluorescence signal in GCaMP6s-expressing mice.

Fiber photometry record analysis

The normalized DF/F values and traces were visualized by the R810 dual-color multi-channel optical fiber recording system itself and it can export data to be further processed using custom MATLAB (MathWorks) scripts to draw averaged Ca^{2+} traces. For the statistical charts, average DF/F (%) values were calculated as (Fduration-Fbaseline)/Fbaseline. Fiber photometry was recorded (300 sec was the total record duration, the former 60 sec was for baseline, the middle 240 sec was for ultrasound stimulation, and the last 60 sec for post-record) in overall 5 min, Fbaseline was the mean of the GCaMP6m signals from 1 sec to 60 sec, and F duration was the mean of GCaMP6m signals for 5 min record displayed on the statistical charts. The heatmap was plotted using a self-developed R program to display the variance of average DF/F (%) in 3 individual mice from each group. The GCaMP6s fluorescence was bandpass filtered automatically and fluorescence intensity and change were converted to a digital signal to be recorded and quantified automatically by fiber photometry recording system (RWD).

Image acquirement

Quantitative analysis of the acquired images was performed using LAS AF Lite for quantification of cell numbers and Image *J* software for quantification of fluorescence intensity. Four images of the penumbra surrounding the injury core in the ipsilateral hemisphere were taken under a confocal microscope (experimental protocol). For co-localization assays, the number of positive cells was counted in LAS AF Lite software. For dendritic spine quantification, representative brain coronal sections (100 μm thick) of ipsilateral perifocal region were imaged under confocal microscope, x 20 and x 120 images were taken at a single panel.

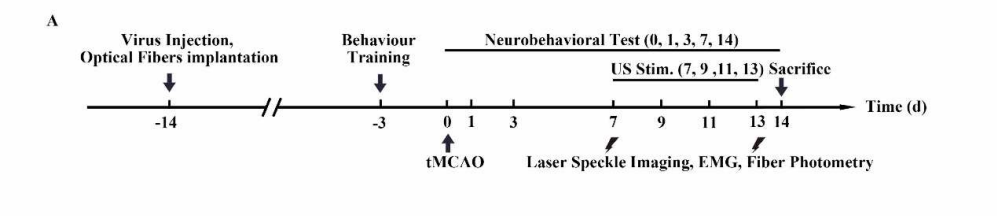
Statistical analysis

Data analysis was performed with IBM SPSS Statistics 24 at a confidence of 95%, and figures were generated from GraphPad Prism 9. All values were presented as mean ± SD. Animals used (N) was indicated in the figure legends. Normality of continuous variables was assessed by the Kolmogorov-Smirnov test if $n \geq 30$ and the Shapiro-Wilk test if $n < 30$. To assess the difference between two independent groups, an unpaired two-tailed test was used for normally distributed variables. One-way or two-way analysis of variance followed by Dunnett's or Tukey's multiple comparisons were used for more than two groups. Results were presented as means ±SD. A $p< 0.05$ was considered statistically significant.

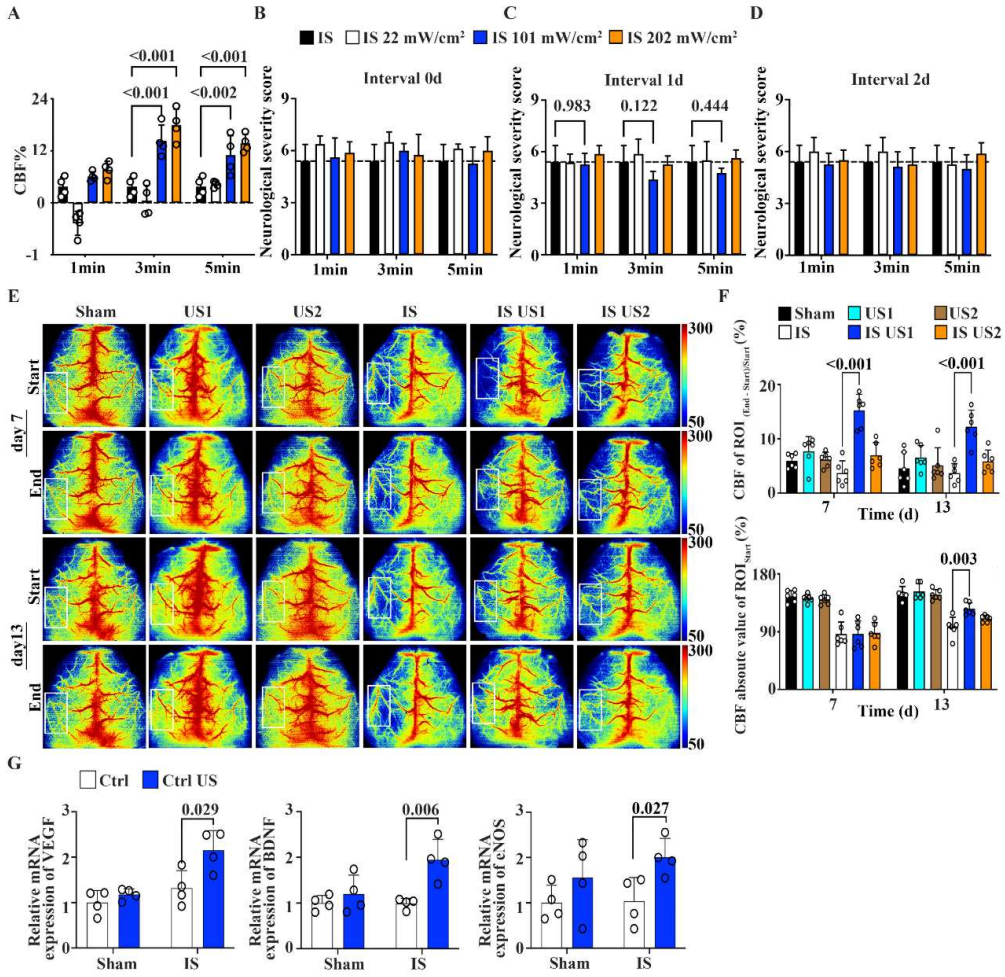
Data and materials availability

The datasets produced in this study are available in the following databases: scRNA-seq: BioProject accession is PRJNA882071 (https://submit.ncbi.nlm.nih.gov/subs/bioproject/SUB12080901/overview), BioSample accession are SAMN30928884 and SAMN30928936 (https://www.ncbi.nlm.nih.gov/biosample/30928936). Proteomics: iProX dataset identifier PXD036927, the corresponding password is: A6IZ. (https://www.iprox.cn/page/PSV023.html;?url=1681800664925SwUt) .

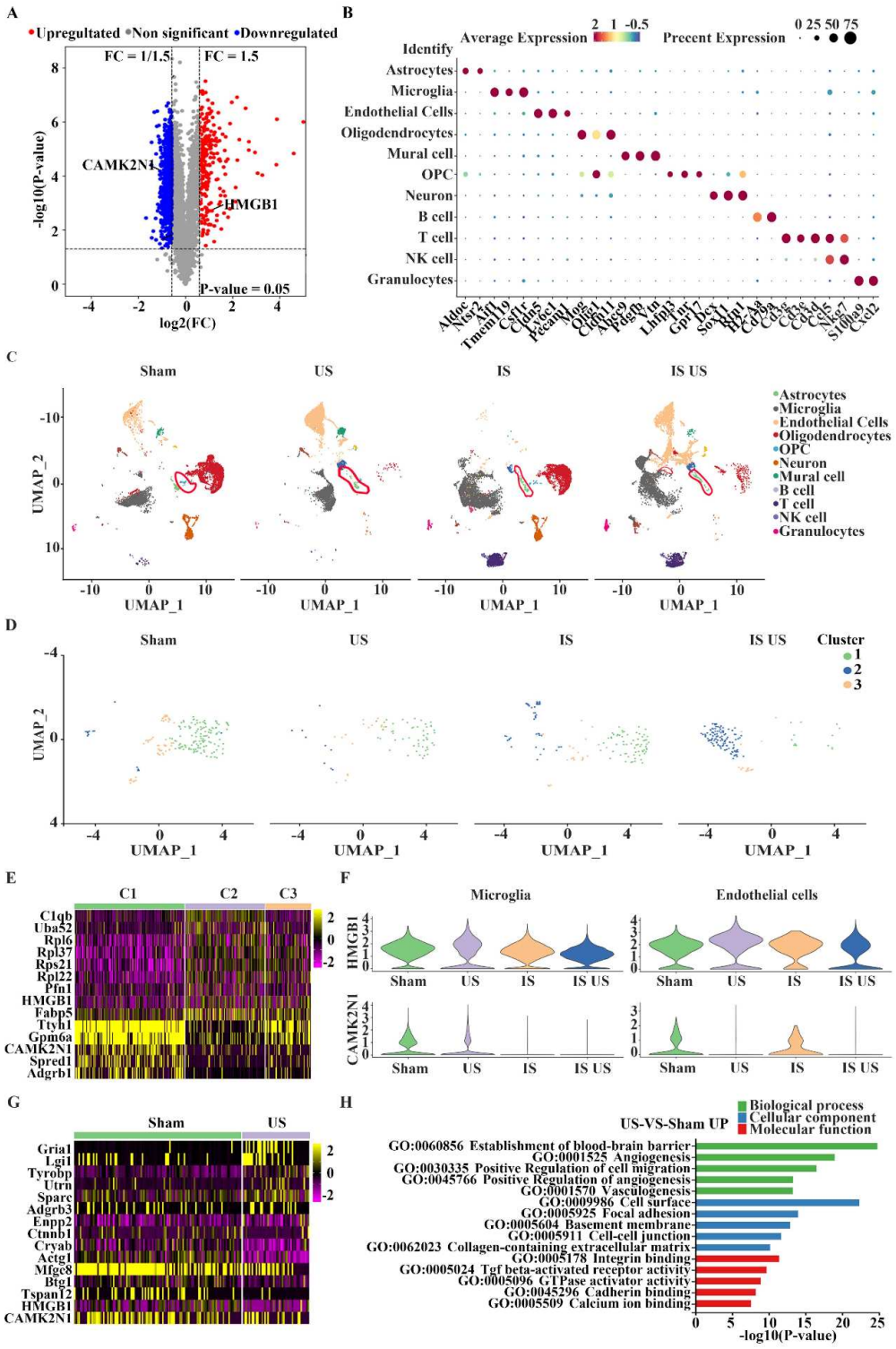
Supplementary Figures



Supplementary Figure 1. Experimental scheme. Scheme of experimental timepoints included virus injection, optical fibers implantation, ultrasound stimulation, laser speckle, EMG, fiber photometry records, neurobehavioral tests and a series of immunohistochemistry after animal sacrifice.

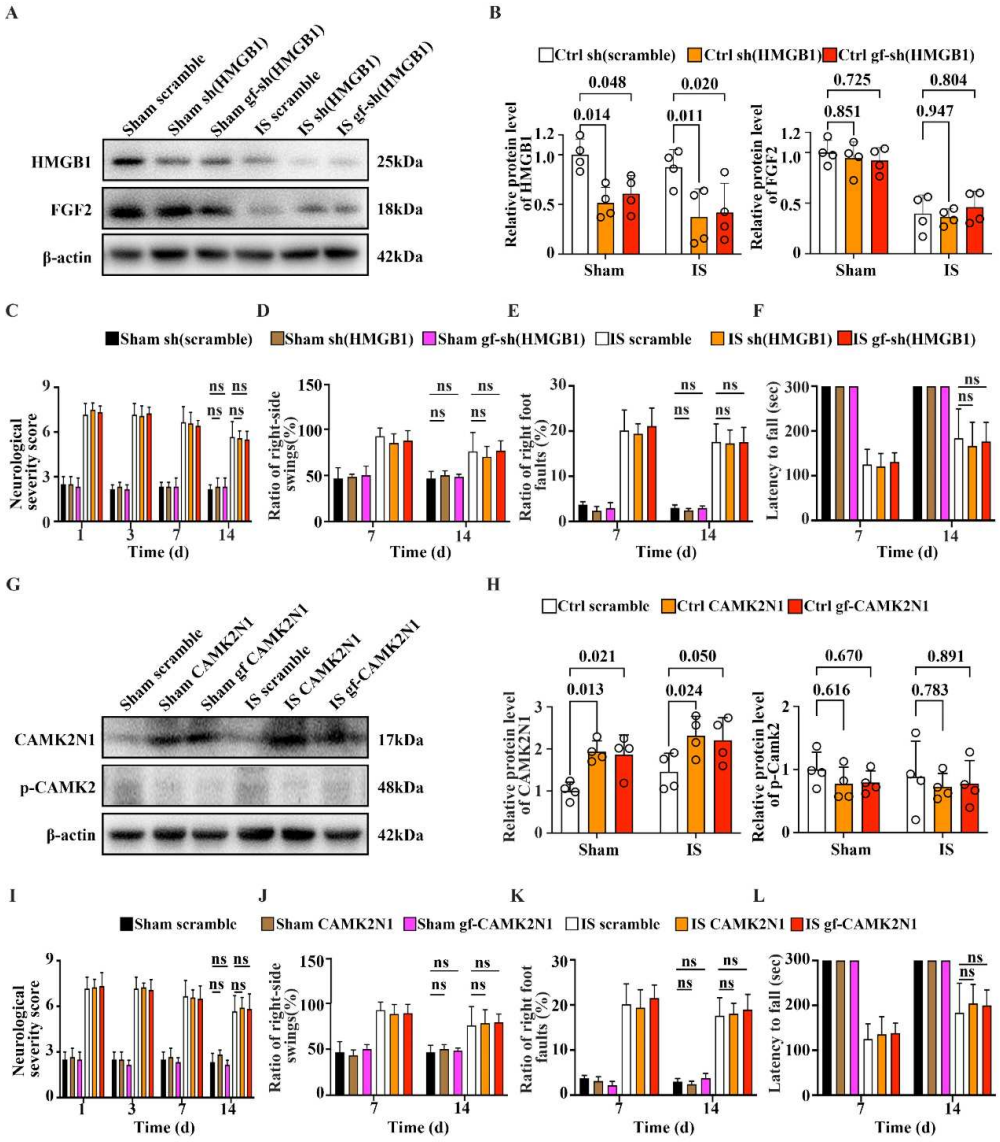


Supplementary Figure 2. LIFUS promoted angiogenesis and neurobehavioral outcomes in MCAO mice. (**A**) Laser speckle imaging showed immediate change of CBF when 22, 101, 201 mW/cm2-sound pressure was tested on the MCAO mouse brain with 1, 3, 5 min respectively, (n = 4 mice/group). (**B-D**) mNSS records for neurobehavioral outcomes in each group at 14 days of MCAO, (n = 4 mice/group). (**E**) Representative laser speckle images of CBF of ROI after immediate, 7 days and 13 days of MCAO in the mouse brain. The start row showed CBF absolute value at 7 days and 13 days after MCAO respectively. The end row showed immediate CBF recording followed by ultrasound at 7 days and 13 days respectively. (**F**) Semi-quantification of the CBF, (n = 6 mice/group). (**G**) qPCR results for mRNA level of VEGF, BDNF and eNOS in ipsilateral hemisphere of the mouse brain, (n = 4 mice/group). US1, 2 = mice treated with different dose of ultrasound. IS = ischemic stroke mice, IS US = ischemic stroke mice treated with US. Data are mean ± SD.

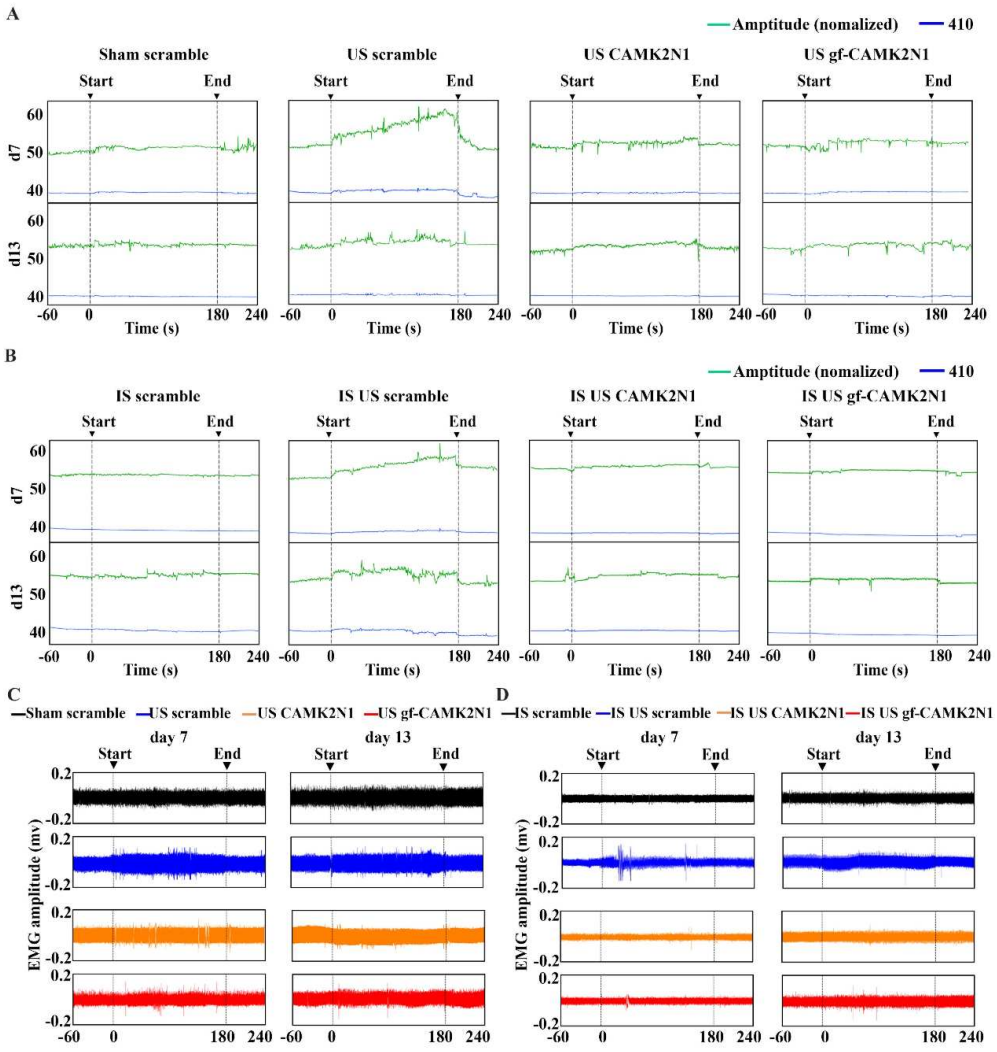


Supplementary Figure 3. Differential expressed genes and expression profiles in iTRAQ proteomics and scRNA-seq analysis. (**A**) Volcano Plot demonstrated fold change of protein level in IS US group compared to the IS group. (**B**) Dot plot of marker genes for different cell

clusters by scRNA seq. (**C**) Expression profiles of three subclusters in astrocytes by grouped groups were shown and colored based on gene expression patterns. (**D**) Secondary profile of 3 subclusters in astrocytes. (**E**) Heatmap showed differential expressed genes in three subclusters of astrocytes. (**F**) Violin plots represented the expression distributions of HMGB1 and CAMK2N1 by grouped groups in different brain cell types, exemplified by microglia and endothelial cells. (**G**) Heatmap showed the differential expressed genes in astrocytes of US group compared to the sham group. (**H**) Bar chart of GO terms showed enriched pathways in the US group compared to the sham group.



Supplementary Figure 4. Gene manipulations on sham and ischemic stroke mice did not cause further damage after LIFUS treatment. (**A**) Western blotting and (**B**) quantification of HMGB1, FGF2 normalized to actin in ipsilateral mouse brain of 6 different groups, (n = 6 mice/group). (**C**) mNSS, (**D**) tail suspension, (**E**) grid walking and (**F**) rotarod test showed that neurobehavioral outcomes of different groups, (n = 6 mice/group). (**G**) Western blotting and (**H**) quantification of CAMK2N1, p-CAMK2 normalized to actin in ipsilateral mouse brain of 6 different groups, (n = 4 mice/group). (**I**) mNSS, (**J**) tail suspension, (**K**) grid walking, and (**L**) rotarod test showed the neurobehavioral outcomes of different groups, (n = 6 mice/group). Data are mean ± SD.



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365 **Supplementary Figure 5. Fiber photometry records showed calcium waveforms of one**
366 **representative mouse in the eight groups.** (**A**) and (**B**) Original waveform was excited by
367 470 nm (green) and 410nm (blue) were detected in striatum perifocal region in 8 different
368 groups at day 7 and 13 after MCAO. (**C**) and (**D**) Original waveform was detected in left hind
369 leg of 8 different groups at day 7 and 13 after MCAO.
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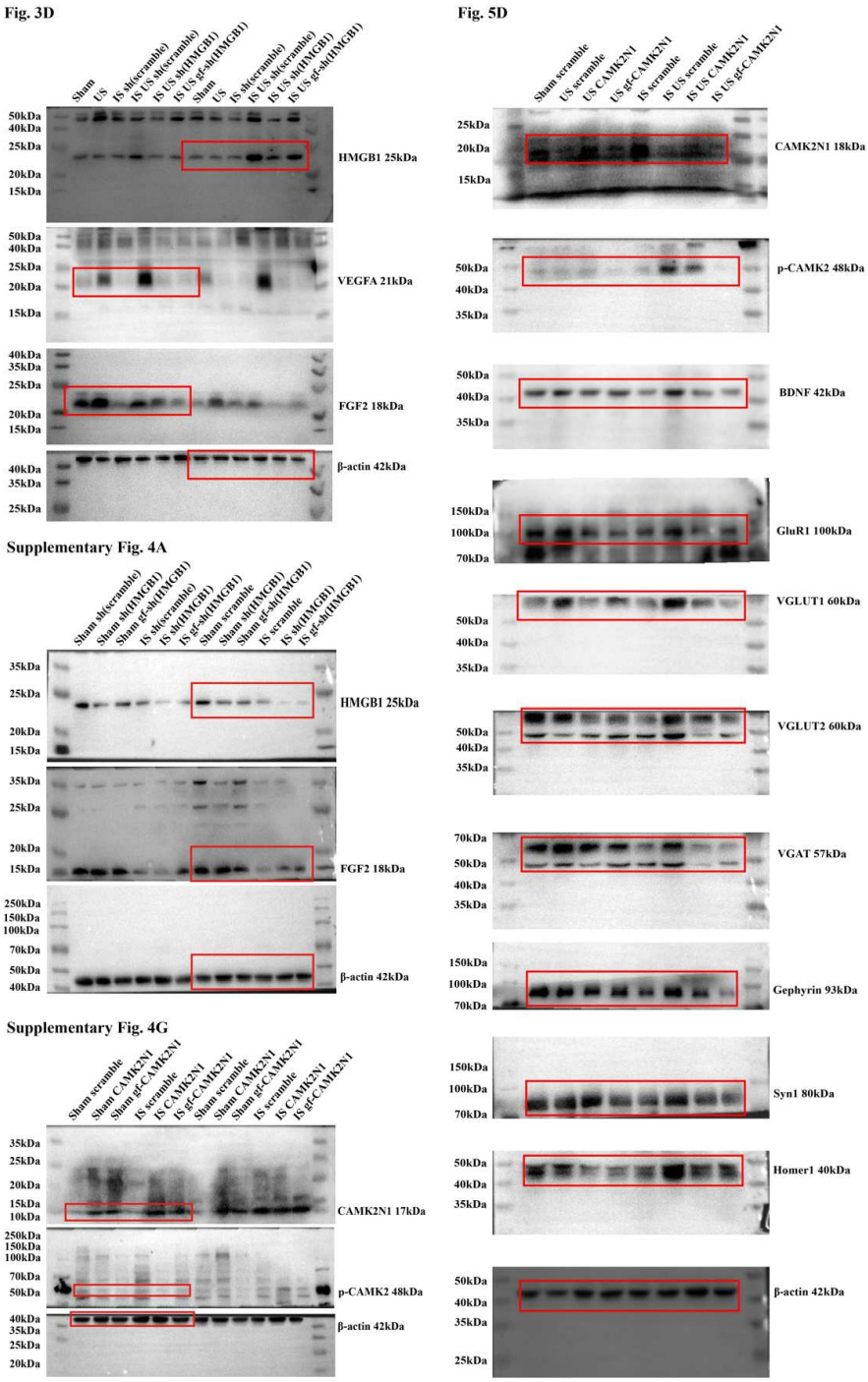

Supplementary Figure 6. Raw data of western blot.

374 **Supplementary Tables**

375 **Table S1 Ultrasonic parameters design.**

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I_{SPTA} (mW/cm^2)	f (kHz)	PRF (kHz)	SD (ms)	DC (%)	Pr (Mpa)
22	500	1	300	50	0.117
101	500	1	300	50	0.250
201	500	1	300	50	0.352

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379 **Table S2 Ultrasonic parameters combinations.**

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I_{SPTA} (mW/cm^2)	Stimulation time (min)	Interval (d)	Total time (d)
22/101/201	1	0	7
	1	1	5
	1	2	3
	3	0	7
	3	1	5
	3	2	3
	5	0	7
	5	1	5
	5	2	3

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383 **Table S3 Ultrasonic parameters setting for further examination.**

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I_{SPTA} (mW/cm^2)	Group	Stimulation time (min)	Interval (d)	Total time (d)
101	US1	3	1	4
101	US2	5	1	4
101	US3	10	1	4

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387 **Table S4 Antibodies used in western blots.**

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Antibody	Host	Catalogue Number	Vendor	Concentration
Hmgb1	Rabbit	10829-1-AP	Proteintech	1:1000
VEGFA	Rabbit	19003-1-AP	Proteintech	1:1000
FGF2	Mouse	05-118	Millipore	1:1000
CAMK2N1	Rabbit	PA5-23740	Thermo Fisher	1:1000
p-CaMKII	Mouse	sc-32289	Santa Cruz	1:500
BDNF	Mouse	sc-65514	Santa Cruz	1:1000
Synapsin I	Rabbit	AB1543	Millipore	1:1000
Homer1	Rabbit	ab184955	Abcam	Abcam
Glutamate receptor 1	Rabbit	ab31232	Abcam	1:1000
VGLUT1	Mouse	MAB5502	Millipore	1:1000
VGLUT2	Mouse	MAB5504	Millipore	1:1000
VGAT	Rabbit	AB5062P	Millipore	1:1000
Gephyrin	Rabbit	ab177154	Abcam	1:1000
b-actin	Mouse	66009	Proteintech	1:2000

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