Low-intensity focused ultrasound stimulation promotes stroke recovery via astrocytic HMGB1 and CAMK2N1 in mice

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ABSTRACT
Background Low-intensity focused ultrasound stimulation (LIFUS) has been developed to enhance neurological repair and remodelling during the late acute stage of ischaemic stroke in rodents. However, the cellular and molecular mechanisms of neurological repair and remodelling after LIFUS in ischaemic stroke are unclear.

Methods Ultrasound stimulation was treated in adult male mice 7 days after transient middle cerebral artery occlusion. Angiogenesis was measured by laser speckle imaging and histological analyses. Electromyography and fibre photometry records were used for synaptogenesis. Brain atrophy volume and neurobehaviour were assessed 0–14 days after ischaemia. iTRAQ proteomic analysis was performed to explore the differentially expressed protein. scRNA-seq was used for subcluster analysis of astrocytes. Fluorescence in situ hybridisation and Western blot detected the expression of HMGB1 and CAMK2N1.

Results Optimal ultrasound stimulation increased cerebral blood flow, and improved neurobehavioural outcomes in ischaemic mice (p<0.05). iTRAQ proteomic analysis revealed that the expression of HMGB1 increased and CAMK2N1 decreased in the ipsilateral hemisphere of the brain at 14 days after focal cerebral ischaemia with ultrasound treatment (p<0.05). scRNA-seq revealed that this expression pattern belonged to a subcluster of astrocytes after LIFUS in the ischaemic brain. LIFUS upregulated HMGB1 expression, accompanied by VEGFA elevation compared with the control group (p<0.05). Inhibition of HMGB1 expression in astrocytes decreased microvessels counts and cerebral blood flow (p<0.05). LIFUS reduced CAMK2N1 expression level, accompanied by increased extracellular calcium ions and glutamatergic synapses (p<0.05). CAMK2N1 overexpression in astrocytes decreased dendritic spines, and aggravated neurobehavioural outcomes (p<0.05).

Conclusion Our results demonstrated that LIFUS promoted angiogenesis and synaptogenesis after focal cerebral ischaemia by upregulating HMGB1 and downregulating CAMK2N1 in a subcluster of astrocytes, suggesting that LIFUS activated specific astrocyte subcluster could be a key target for ischaemic brain therapy.

INTRODUCTION
Stroke is the second leading cause of death and the leading cause of disability globally. After a stroke, the neurovascular unit was disrupted, leading to an injury to capillaries and neurons, and loss of synapses, causing brain injury and impairing long-term motor and cognitive function. Even if patients are treated in the acute phase, they also faced long-term injuries. Therefore, in addition to the treatment in the acute stage, delayed therapies aimed to repair neurovascular units are also potentially significant and urgent needs after stroke.

Recently, neuromodulation techniques mainly including transcranial magnetic stimulation, transcranial electrical stimulation and transcranial focused ultrasound have been developed. Among them, TUS showed the great advantage of high spatial specificity, high penetration depth and non-invasive properties, which widely attracted the attention of neurobiologists. Ultrasound stimulation of the frontal–temporal cortex significantly improved the patient’s mental status and pain, and further reduced ischaemic injury. Ultrasound neuromodulation
significantly decrease parkinsonian-related activity in mice models and focused ultrasound thalamotomy treatment was approved by FDA for patients with essential tremor who did not respond to medication. These results suggested that cell-type-specific activation combined with Ca$^{2+}$-dependent molecular pathways could be expanded to pathological conditions. Motor function, neural activity and haemodynamic responses showed a linear coupling with ultrasound stimulation of the mouse cortex. Incomplete motor function recovery was one of the major pathological features of ischaemic stroke, and low-intensity focused ultrasound stimulation (LIFUS) has been shown to promote neurorehabilitation after ischaemic stroke.

The promotion of angiogenesis and neurogenesis is the main targets for the later stage of stroke therapy. Synaptogenesis is a cardinal component of neurogenesis. LIFUS was shown to modulate brain activity, with the most focused on the induction of motor responses and neuro-modulation function. Recently, the promotion of angiogenesis and neurogenesis by LIFUS was demonstrated in many experiments in vitro and in vivo with a variety of parameters. In vitro, LIFUS was proved to promote the secretion of neurotrophic factors from astrocytes such as vascular endothelial growth factor (VEGF) and brain-derived growth factor (BDNF). In vivo, LIFUS could induce angiogenesis to ameliorate infarction area in the acute stage of stroke, and inhibit brain oedema formation. These studies intrigued us to explore which optimal parameter of LIFUS would be suitable for late-stage therapy for ischaemic stroke and its underlying mechanism.

Here, we investigated the effects and molecular mechanisms of LIFUS in angiosynaptic regeneration after 7 days of ischaemic stroke in mice.

**MATERIALS AND METHODS**

Parameters of LIFUS including stimulation frequency, intensity, duration and relative protocols were generated and listed as shown in the online supplemental materials. For in vivo experiments, mice (n=120) were randomly allocated into experimental treatment or control groups, and n=12 mice per group were analysed for neurobehavioural tests and laser speckle imaging; other experiments were around n=4 mice per group, the details we put in the online supplemental materials. Animal experiments were planned using G*Power software (Heinrich-Heine-Universität Düsseldorf, V.3.1.9.6, setting α at 0.05 and power at 0.8) combined with the pre-experiment to determine sample size. Adult male mice (8–10 weeks, C57BL/6J) were used for MCAO and other experiments, and aged male mice were 22 months. Animals were randomly distributed into required groups receiving either scramble or the pAAV viral inhibitor or overexpression, in each intervention test, and a control group was performed to fulfil reconfirmation. Endpoints for the animal experiments were determined in accordance with institutionally approved criteria.

For the specific analysis of the underlying molecular mechanisms in MCAO mice following LIFUS, multiomics approaches including iTRAQ proteomic analysis were performed to detect the key different proteins and pathways, and single-cell RNA sequencing analysis was performed to identify the potential clusters of different cell types and groups. For functional outcomes, laser speckle imaging, electromyography (EMG) and fibre photometry records were applied at different time points in MCAO mice.

Last, proof-of-concept studies were performed in vivo. Outcomes were determined by assessing molecular factors, histological phenotype, electrophysiology and neurobehavioural function performed in a blinded manner 14 days after the ischaemic brain injury. The molecular mechanisms in the ipsilateral hemisphere in response to LIFUS were determined by Fluorescence in situ hybridisation (FISH), immunofluorescence staining, qPCR, Western blot and corresponding quantification. All analyses were performed in an unbiased fashion. The details of methods are mainly presented in the online supplemental materials.

**RESULTS**

**Optimised LIFUS parameters for ischaemic stroke therapy in mice**

Experiments were designed as shown in online supplemental figure 1. A LIFUS with 500 kHz centre frequency was suitable for rodent research (Ultrasound Neurostimulation System, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China). The x-y diameter of ultrasound was 4.5 mm and 5 mm, respectively, the depth of ultrasound irradiation was 4 mm, and the x-y diameter was 2 mm when normalised sound pressure was more than 50% of total irradiation energy (figure 1A–C). The sequence diagram of the ultrasound stimulation was shown. 500 kHz pulse repetition frequency (PRF), 300 ms sonication duration (SD) and 50% duty cycle (DC) were used across all the ultrasound experiments (figure 1D).

Additional combinations of pressure (MPa) were used to make $I_{\text{rms}}$ different (online supplemental table S1). We combined different ultrasonic intensity (UI; 22, 101, 201 mW/cm$^2$) and stimulation duration (1, 3, 5 min) to treat stroke mice in the late acute stage (online supplemental table S2). We measured the cerebral blood flow (CBF) after ultrasound stimulation using laser speckle imaging. The result showed that CBF increased after immediate ultrasound stimulated ipsilateral hemisphere for 3 or 5 min with 101 and 201 mW/cm$^2$ day 7 after stroke (online supplemental figure 2A). The motor function was evaluated by modified neurological severity score (mNSS) before and up to 14 days after MCAO using different stimulation parameters, results showed that only 101 mW/cm$^2$ improved neurobehavioural outcomes. One day of...
Figure 1  Establishing optimal LIFUS parameters for neuronal repair and remodelling in MCAO mice. (A) 2D distribution of the ultrasound field in oblique and longitudinal cross-sections. The x-axis and y-axis of the ultrasound spot were ~4.5 mm and ~5 mm, respectively. (B) Depth of ultrasound irradiation was 4 mm. (C) X-Y diameter was 2 mm when normalised sound pressure was more than 50% of the total irradiation energy. (D) Schematic diagram of ultrasound, PRF was 1 kHz and 1/PRF was 1 s, SD was 300 ms, and DC was 50%. (E) Cresyl violet-stained brain sections and (F) quantification of atrophy volume at 14 days following MCAO. Dashed lines indicated brain atrophy area, (n=3–7 mice/group). (G) Representative CD31 (red) and Ki67 (green) immunostaining images in the perifocal region. (H) Quantitative analysis of capillary number, (I) surface area, (J) Ki67, and (K) Ki67+/CD31 signals in the perifocal region of ipsilateral hemisphere after 14 days following MCAO in mice, (n=4 mice/group. Scale bar=150 µm). (L) mNSS, (M) tail suspension, (N) grid walking and (O) rotarod test for neurobehavioural outcomes in each group (n=3 mice/group in sham groups, n=10–12 mice/group in the IS groups). US1, −2 to –3=mice treated with different dose of ultrasound. IS, ischaemic stroke mice; IS US, ischaemic stroke mice treated with US. Data are mean ± SD. LIFUS, low-intensity focused ultrasound stimulation; mNSS, modified neurological severity score; PRF, pulse repetition frequency; SD, sonication duration.
ultrasound stimulation interval and 3 or 5 min of stimulation duration achieved a better recovery in neurobehavioural outcomes compared with the MCAO mice (IS, online supplemental figure 2B–D).

To explore whether longer time of ultrasound stimulation would have better or side effect in MCAO mice, we added a group of 10-min stimulation with 101 mW/cm² every other day. Then, we labelled ultrasound stimulation 3, 5 and 10 min with 101 mW/cm² every other day. Cresyl violet staining results showed that US3 damaged the ipsilateral hemisphere in both young and aged healthy mice compared with the control, indicated that 10 min was an osterization of stimulation. For MCAO (IS) groups, US1 showed better potential than US2 to reduce the brain atrophy volume (figure 1F). The instant change of CBF and mNSS results showed that US1 and US2 were better stimulation to improve stroke recovery. To explore the effect of LIFUS on CBF of MCAO mice, we measured the spatiotemporal changes of CBF using laser speckle imaging, and chose the perifocal regions as ROI (online supplemental figure 2E). For the sham group, there was no difference in CBF changes in immediate, 7 and 13 days, and 13 days endpoint after US1 and US2. For the IS groups, both US1 and US2 groups of mice showed better CBF recovery in the ipsilateral hemisphere of the IS mice than that of IS group, while US1 exhibited increased CBF at day 7 to the endpoint of 13 days after MCAO (online supplemental figure 2F).

The increase of cerebral blood volume is attributed to a surge of angiogenesis. We investigated angiogenesis in the perifocal region of the ipsilateral hemisphere after MCAO (figure 1G). The number of microvessels and surface CBF did not increase in the sham groups after US1 or US2 treatment (figure 1H,I). The number of Ki67+ cells and Ki67+/CD31+ microvessels increased in perifocal region of the ipsilateral hemisphere at 14 days of MCAO after US1 or US2 treatment, but they were more prominent after US1 treatment (figure 1J,K).

To further determine which was the best stimulation parameter of LIFUS for the neurobehavioural recovery in the MCAO mice, we applied a number of neurobehavioural tests including mNSS, tail suspension test, foot fault and rotarod to comprehensively evaluate the sensorimotor functions up to 14 days after MCAO (figure 2A–D). Both US1 and US2 stimulation did not induce significant change compared with that in the sham groups. For IS groups, both US1 and US2 showed better outcomes than that in IS groups, while US1 exhibited fewer motor deficits and better neurological recovery in all the four neurobehavioural tests in the MCAO mice (figure 2L–O). qPCR results also showed that mRNA level of VEGF, BDNF and endothelial nitric oxide synthase (eNOS) increased in the MCAO mice after US1 stimulation compared with the IS group (online supplemental figure 2G). Taken together, these data suggested that US1 stimulation attenuated neurological deficits and promoted functional recovery at both the histological and neurobehavioural levels. Therefore, US1 was chosen as the optimal ultrasound stimulation method in the following experiments.

**LIFUS upregulated HMGB1 and downregulated CAMK2N1 in a new cluster of astrocytes**

To explore the mechanism of ultrasound effect on CBF recovery and newly formed microvessels, we performed iTRAQ-based proteomic analysis to detect the mechanism in the protein level. Genes with a p value<0.05 and |fold change|≥1.5 were regarded as DEGs. Overall, 261 genes were upregulated and 848 genes were downregulated after LIFUS compared with the IS mice (online supplemental figure 3A). Volcano plot displayed 184 genes involved in angiogenesis and synapse pathway (figure 2A). We further conducted gene ontology (GO) pathway analyses to display the enrichment of 15 terms clustering from all different expression genes that we were interested in the IS US group compared with the IS group, indicating that ultrasound truly change the physiological process after ischaemic stroke significantly, and correlated to VEGF and calcium related pathways (figure 2B). Heatmap displayed changed genes, which related with angiogenesis and synapse signal pathways in the IS US group compared with the IS group (figure 2C).

To further explore the mechanism at single-cell level, cell lineage analysis by comprehensive single-cell RNA-sequencing was performed to gain information of the transcriptional profile in different treated groups. Cluster analysis using a uniform manifold approximation and projection for dimension reduction (UMAP) revealed the difference in global gene expression profiles of cell types in four different groups, and identified clusters of cells with unique genetic signature (figure 2D, online supplemental figure 3B). Gene expression profiles of healthy and injured region with or without ultrasound stimulation were shown by UMAP, suggesting that the expression profiles after LIFUS both in physiological and pathological conditions were different (online supplemental figure 3C).

Since previous studies showed that astrocyte mediated the effects of ultrasonic neuromodulation, we then concentrated on the subclusters of astrocytes in four groups of astrocytes. Heatmap showed 17 differentially expressed genes overlapped with genes of iTRAQ proteomic analysis related to angiogenesis and synapse pathways in astrocytes of the IS US mice compared with that in the IS mice (figure 2E). We found that most of the other significantly differential genes were phenotypic genes such as Pecam1 or pathway genes such as Pik3c2a/Atp5f1d. We aimed to find the initial genes that directly response to ultrasound as the target genes therefore, then focused on HMGB1 and CAMK2N1. HMGB1 was the top differential expressed gene related to angiogenesis, and CAMK2N1 was the top differential gene related to synaptogenesis, suggesting that these two genes were...
Figure 2  LIFUS upregulated HMGB1 and downregulated CAMK2N1 in a new cluster of astrocytes. (A) Volcano plot demonstrated fold change of protein level of HMGB1 and CAMK2N1 in the IS US group compared with the IS group. (B) Bar chart of GO terms showed angiogenesis and synapse related pathways including BP (biological process, green), CC (cell component, blue), and MF (molecular function, red). (C) Heatmap showed different protein expression in the IS US group compared with IS group. (D) UMAP plot showed the expression profiles in the left ipsilateral by clustering cell types in the ipsilateral hemisphere of mouse brain. (E) Heatmap showed differentially expressed genes (DEGs) in the IS US group compared with IS group of astrocytes. (F) Bar chart of GO terms showed enriched HMGB1 and (G) CAMK2N1-related pathways of astrocytes in the IS US group. (H) Expression profiles of HMGB1 and CAMK2N1 in astrocytes organised into groups, and coloured based on gene expression patterns. (I) Bar chart showed the proportions of three subgroups in four different groups. (J) Violin plots represented the expression distributions of HMGB1 and CAMK2N1 in astrocytes organised into groups. GO, gene ontology; IS, ischaemic stroke mice; IS US, ischaemic stroke mice treated with US; US, mice treated with ultrasound; LIFUS, low-intensity focused ultrasound stimulation; UMAP, uniform manifold approximation and projection; VEGF, vascular endothelial growth factor.
involved in the promotion of angiogenesis and synaptogenesis in MCAO mice with LIFUS. Bar chart of GO terms showed enriched HMGBl and CAMK2N1-related pathways in astrocytes from IS US group, including angiogenesis-related, calcium-related and synapse-related pathways which we focused on (figure 2E,G). The secondary profiling of astrocytic subclusters yielded three subtypes with distinct functional cell identities (online supplemental figure 3D). Feature plot showed HMGBl and CAMK2N1 expression in secondary profiles of four different groups (figure 2H). Among the three subclusters, the proportion of subcluster 2 (C2) astrocytes in the IS US mice was ~70%, more than IS group (figure 2I), suggesting that IS US promote the C2 positive new subcluster. Heatmap showed expressed genes that HMGBl increased and CAMK2N1 decreased in subcluster 2 compared with other subclusters (1 and 3) in astrocytes, which was consistent with iTRAQ proteomics analysis (online supplemental figure 3E). The distributions of HMGBl and CAMK2N1 expression in astrocytes, microglia and endothelial cells were displayed by violin plots (figure 2J), online supplemental figure 3F). Heatmap of top differentially expressed genes in astrocytes related to angiogenesis and synapse pathway were shown in the US group compared with sham group, while there has no change of HMGBl and CAMK2N1 expression (online supplemental figure 3G). GO terms displayed enriched pathways in all cell types in US group compared with the sham group, and showed that enriched C2 promote angiogenesis, neuroregeneration, immune and inflammatory related pathways after stroke, and other cell types could be involved in angiogenesis and synaptogenesis via different signal molecules in physiological condition (online supplemental figure 3H).

**Astrocytic HMGBl inhibition decreased angiogenesis-related factor expression and reversed neurobehaviour recovery after LIFUS in MCAO mice**

To investigate whether LIFUS increased angiogenesis by upregulating HMGBl in astrocytes of MCAO mice, we knocked down HMGBl in whole-brain cells by pAAV-U6-shRNA (HMGBl)-CMV-WPRE virus, and in astrocytes by pAAV-GfaABC1D-3xFLAG-miR30shRNA (HMGBl)-WPRE virus. pAAV-U6-shRNA-CMV-WPRE virus was used as a control.

FISH was used to colocalise targeted gene with astrocytes in RNA level. FISH results showed that HMGBl significantly expressed in astrocytes compared with the corresponding controls at 14 days after MCAO (figure 3A). Expression of HMGBl increased after US treatment compared with the IS group of mice and decreased in the IS US sh (HMGBl inhibition in the whole brain) and IS US gf-sh groups (HMGBl inhibition in astrocytes) in mRNA level (figure 3B), which demonstrated that viral inhibition was effective. By quantifying the number of HMGBl+/GFAP+ astrocytes in the perifocal region at 14 days after MCAO by FISH, we found that 64% of HMGBl+ cells were astrocytes and 36% HMGBl+ cells were other type of cells (figure 3C).

We then analysed the expression of HMGBl, VEGFA and FGF2 in perifocal regions in MCAO mice. VEGFA and FGF2 expression showed an increasing trend in the sham group after LIFUS. The expression of HMGBl, VEGFA and FGF2 significantly increased in LIFUS treated groups compared with the IS US group at 14 days after MCAO, which was reversed by HMGBl inhibition in IS US sh and IS US gf-sh groups of mice (figure 3D,E). The expression of other proteins and neurobehavioural outcomes was not affected by HMGBl inhibition in the sham group (online supplemental figure 4A-F). For IS groups, ultrasound-treated groups showed better neurobehavioural outcomes including mNSS, tail suspension test, foot fault and rotarod than that in the IS group at 14 days after MCAO (figure 3F-I), and reversed by the inhibition of HMGBl in the whole brain and astrocytes.

**LIFUS-upregulated HMGBl in astrocytes promoted CBF and lectin+ microvessels after MCAO**

To determine whether LIFUS-induced angiogenesis was correlated with upregulated HMGBl in astrocytes, we further measured the spatiotemporal changes of CBF and brain microvasculature in ipsilateral perifocal region (figure 4). Surface CBF increased in the IS US group compared with the IS US group of mice (figure 4A,B). We also qualified the ratio of the ipsilateral to the contralateral of ROI; the results also showed that CBF increased at day 7 and day 13 after MCAO (figure 4D,E). Increased CBF induced by LIFUS were reversed by inhibiting HMGBl in the whole brain cells as well as astrocytes (figure 4A,B).

We further investigated functional angiogenesis by injecting tomato lectin. Lectin+ microvessels increased in the IS US group compared with IS group of mice which was reversed after HMGBl inhibition (figure 4C). The quantification of lectin+ capillary number and surface area increased in the US groups compared with the IS, IS US sh and IS US gf-sh groups (figure 4D,E). Ki67+ cells colabelled with lectin+ microvessels emerged in the perifocal regions, indicating newly formed microvessels. Ki67+ cells and Ki67+/lectin+ microvessels increased in the perifocal region of the IS US group of mice compared with the other 3 groups of mice at day 14 following MCAO (figure 4F,G). scRNA results showed that HMGBl mRNA has no difference in sham groups with or without US treatment (online supplemental figure 3G), suggesting that ischaemic mice easily respond to LIFUS, and finally led to improve outcomes.

**Astrocytic CAMK2N1 overexpression reversed the synapses increase after LIFUS in MCAO mice**

To determine whether LIFUS promoted synaptogenesis via decreasing CAMK2N1 in astrocytes after MCAO, we overexpressed CAMK2N1 in the brain cells by pAAV-CAG-P2A-CAMK2N1-3xFLAG-WPRE and in astrocyte by pAAV-GfaABC1D-P2A-CAMK2N1-3xFLAG-WPRE. pAAV-P2A-3xFLAG-WPRE was used as a control.

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IS US gf-sh groups of mice (figure 3D,E). Ki67+ cells colabelled with lectin+ microvessels emerged in the perifocal regions, indicating newly formed microvessels. Ki67+ cells and Ki67+/lectin+ microvessels increased in the perifocal region of the IS US group of mice compared with the other 3 groups of mice at day 14 following MCAO (figure 4F,G). scRNA results showed that HMGBl mRNA has no difference in sham groups with or without US treatment (online supplemental figure 3G), suggesting that ischaemic mice easily respond to LIFUS, and finally led to improve outcomes.

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We investigated the CAMK2N1 localisation and expression by FISH and Western blot (figure 5). FISH results showed that CAMK2N1 expressed in astrocytes at 14 days after MCAO (figure 5A). CAMK2N1 expression was downregulated in the IS US group compared with the IS group at 14 days after MCAO, and successfully increased in the IS US sh and the IS US gf-sh groups compared with the IS US control (figure 5B). Quantification of the number of CAMK2N1+/GFAP+ astrocytes showed that 80% of CAMK2N1+ cells were astrocytes and 20% of CAMK2N1+ cells were other cells in the perifocal regions at 14 days after MCAO (figure 5C).

Western blot analysis showed that CAMK2N1 was downregulated in the IS US groups compared with the IS group, which was reversed in the IS US CAMK2N1 (CAMK2N1 overexpression in the whole brain) and the IS US gf-CAMK2N1 (CAMK2N1 overexpression in astrocytes) groups (figure 5D,E). Meanwhile, IS US groups exhibited higher expression of Phospho-Ca2+/calmodulin-dependent protein kinase II (p-CAMK2),

Figure 3  Inhibiting astrocytic HMGB1 attenuated angiogenesis and reversed neurobehavioural outcomes after LIFUS in MCAO mice. (A) Representative in situ hybridisation images of HMGB1 (green) signals and GFAP+ astrocytes (red) in the IS scramble, IS US scramble, IS US sh (HMGB1), and IS US gf-sh (HMGB1) mice. Scale bar=75 µm. (B) Corresponding semiquantification of HMGB1 mRNA expression level in different groups and (C) percentile of astrocytes (n = 3 mice/group). (D) Western blotting (E) and quantification of HMGB1, VEGFA and FGF2, protein levels (from left to right, normalised to corresponding sham) in ipsilateral mice brain, n=4 mice/group for all groups. (F) mNSS, (G) tail suspension, (H) grid walking and (I) rotarod test of neurobehavioural outcomes in each group (n=3 mice/group in sham groups, n=12 mice/group in the IS groups). IS, ischaemic stroke mice; IS US, ischaemic stroke mice treated with US; US, mice treated with ultrasound. Data are mean ± SD. LIFUS, low-intensity focused ultrasound stimulation.
Figure 4  LIFUS upregulated astrocytic HMGB1 via increased CBF and lectin+ microvessels in MCAO mice. (A) Representative images showed immediate CBF changes and endpoint CBF of ROI at 7 days and 13 days by laser speckle imaging in the sham, sham US, IS scramble, IS US scramble, IS US sh (HMGB1), IS US gf-sh (HMGB1) groups. (B) Quantification of CBF normalised to sham. Start row was the quantification of CBF at 7 days and 13 days, respectively. End row was the quantification of immediate CBF changes followed ultrasound at 7 days and 13 days respectively, (n=6 mice/group in the sham groups, n=10 mice/group in the IS groups). (C) Representative lectin (red) and Ki67 (green) immunostaining images, scale bar=150 µm. (D) Quantitative analysis of capillary number, (E) surface area showing angiogenesis in perifocal region. (F) Quantification of the Ki67+ cells and (G) Ki67+/lectin+ signals to exhibit newly formed endothelial cells and microvessels, (n=3 mice/group in sham groups, n=4 mice/group in the IS groups). Sham groups indicated sham group and US group. IS groups indicated IS scramble group, IS US scramble group, IS US sh(HMGB1) group and IS US gf-sh (HMGB1) group. Data are mean ± SD. IS, ischaemic stroke mice; IS US, ischaemic stroke mice treated with US; US, mice treated with ultrasound; LIFUS, low-intensity focused ultrasound stimulation.
Figure 5  Astrocytic CAMK2N1 overexpression reversed the synapses increase after LIFUS in MCAO mice. (A) Representative images of CAMK2N1 (green) signals and GFAP+ astrocytes (red) in IS scramble, IS US scramble, IS US CAMK2N1, and IS US gf-CAMK2N1 mice. Scale bar = 75 µm. (B) Corresponding quantification of CAMK2N1 mRNA expression level in different groups and (C) percentile of astrocytes, (n= 3 mice/group). (D) Western blotting and (E) quantification of CAMK2N1, p-CAMK2, BDNF, GluR1, VGLUT1, VGLUT2, VGAT, Gephyrin, Syn1, Homer1, respectively, from left to right and up to down, relative to β-actin and normalised to corresponding sham in ipsilateral hemisphere of mouse brain, (n=4 mice/group). Sham groups indicated sham scramble group, US group, US CAMK2N1 group and US gf-CAMK2N1 group. IS groups indicated IS scramble group, IS US scramble group, IS US sh (HMGB1) group and IS US gf-sh (HMGB1) group. Data are mean ± SD. IS, ischaemic stroke mice; IS US, ischaemic stroke mice treated with US; US, mice treated with ultrasound; LIFUS, low-intensity focused ultrasound stimulation.
which was also reversed after CAMK2N1 overexpression (figure 5E). In addition, LIFUS increased BDNF, excitatory synapse-related protein glutamate receptor 1 (GluR1), VGLUT1 and VGLUT2 expression. Inhibitory synapse protein (VGAT and Gephyrin), and total synapse protein (Synapsin I and Homer1) had similar expression pattern (figure 5E). At the same time, CAMK2N1 overexpression did not affect other protein expression or neurobehaviour in the sham mice (online supplemental figure 4G–L).

**LIFUS-driven astrocytic CAMK2N1 downregulation promoted electrical signals and increased dendritic spine density after MCAO**

To investigate morphological changes in neuron after increased synaptic protein levels, we performed Golgi-Cox staining to visualise neuronal dendritic spines in the peri-focal region (figure 6A). The number of total spines on secondary dendrites was shown and calculated (figure 6B). Results showed that the number of total spines was significantly increase in the IS US group compared with the IS group, which was reversed in the US and the IS US groups (figure 6C), suggesting that inhibition of downregulated CAMK2N1 in whole brain or astrocyte was beneficial for dendritic spines increase after ischaemic stroke.

Since GluR1 is a calcium-permeable neurotransmitter receptor and plays a key role in synaptic plasticity, we also applied fibre photometry records to detect Ca\(^{2+}\) changes after LIFUS. The results showed that frequency change of the calcium signals slightly increased in the US group (figure 6D), while it was significantly higher in the IS US groups than that in the IS, IS US CAMK2N1 and IS US gf-CAMK2N1 groups during 3-min LIFUS (figure 6E). Heatmap displayed variance of three mice in one group, suggesting that there was a relatively strong increase of frequency after LIFUS, and this enhancement could be reversed by CAMK2N1 overexpression in whole brain cells and astrocytes (figure 6F,G). Furthermore, we also evaluated nerve-to-muscle signal transmission of motor neurons by electromyography (EMG) amplitude. Results showed that the amplitude increased during LIFUS both in the US and the IS US groups at day 7 and day 13 after MCAO, which was consistent with Ca\(^{2+}\) level changes in the brain. LIFUS-induced enhancements were inhibited in US CAMK2N1 and US gf-CAMK2N1 groups (figure 6H–K). Calcium signals frequency and EMG amplitude of original waves were stable before LIFUS in the different groups (online supplemental figure 5).

These results suggested that LIFUS induced calcium changes and increased neuronal activities. Neurobehavioural outcomes including the mNSS, tail suspension test, grid walking and rotarod test were better in the IS US group than that in the IS US CAMK2N1 and IS US gf-CAMK2N1 groups of mice (figure 6L–O). The recovery was reversed by CAMK2N1 overexpression in the whole brain and astrocytes.

**DISCUSSION**

In this study, we demonstrated that LIFUS enhanced HMGB1 expression in a subcluster of astrocytes of ischaemic mouse brain, which promoted microvascular repair and remodelling. At the same time, downregulated CAMK2N1 increased neural dendritic spines and synaptic generation, which eventually leads to neurological functional recovery. We highlighted a molecularly targeted mechanism for the LIFUS therapy at the single-cell level and implies a therapeutic rationale for neurogenesis, angiogenesis and synaptogenesis for the preclinical trials, and provided a theoretical basis for LIFUS to be applied to other diseases.

Previous studies showed that LIFUS with 400 mW/cm\(^2\) preconditioning mitigated focal cerebral ischaemia in rats.\(^{26}\) LIFUS pretreatment could also significantly decrease the neuronal cell apoptosis, downregulation of apoptosis-related signalling molecules and upregulation of BDNF in the ischaemic brain tissue with I\(_{sppa}\) = 528 mW/cm\(^2\).\(^{27}\) It is noted that I\(_{sppa}\) = 39 mW/cm\(^2\) for mice\(^{26}\) or I\(_{sppa}\) = 2.6 W/cm\(^2\)\(^{28}\) for rats in the post ischaemic stage were proved protective when LIFUS with 12-hour or 24-hour interval. LIFUS with 193 mW/cm\(^2\) used to the whole brain 3 times in the first week after MCAO upregulated neurotrophies including VEGF and eNOS.\(^{20}\) Our study supported that 3 min with 101 mW/cm\(^2\) every other day was optimal ultrasound parameter for neurorehabilitation in the late acute stage (after 7 days) of MCAO, which provided a safe and effective treatment for ischaemic stroke.

Based on proteomic protein expression and enrichment pathways, we set the indicators as angiogenesis and synaptogenesis, the key factor for stroke recovery, to observe the effects of ultrasound stimulation. Therefore, we first considered the components of neurovascular units, including astrocyte-endothelium, pericytes, neurons and microglia.\(^{26–31}\) Microglia-enriched pathways mainly promote inflammation and immune-related functions, neurons and endothelial cell are the target cell for angiogenesis and synaptogenesis, astrocytes are cardinal mediator between neurons and endothelial cells, and played a vital role in the neurovascular unit remodelling after ischaemic stroke.\(^{32,33}\) Astrocyte-mediated neurovascular coupling signals could stimulate neurons, leading to a calcium efflux and release of vasoactive substances onto vessels.\(^{34–36}\) Increasing evidence showed that astrocytes are major cell type affected by ultrasound.\(^{18,38}\) At the same time, we observed significant changes of HMGB1 and CAMK2N1 in ischaemic mice after ultrasound only in astrocytes, but not in other cells, which intrigue us to conduct cellular level experiments and related validation in astrocytes.

We also found that angiogenesis and neurogenesis (synaptogenesis) are the most significantly enriched pathways in proteomic analysis by ultrasound stimulation after stroke, then HMGB1 and CAMK2N1 were identified as the most significantly differential genes in both pathways. Furthermore, we performed scRNA sequencing to further
Figure 6  Astrocytic CAMK2N1 downregulated by LIFUS promoted electrical signals and increased dendritic spine density after LIFUS in MCAO mice. (A) Presentative Golgi staining images and quantitative analysis. Low magnification of Golgi staining images of neurons in the perifocal region of ipsilateral hemisphere. Scale bar=75 µm. (B) Representative images of dendritic spines and (C) a bar graph showed the number of total spines in the sham, US, US CAMK2N1, US gf-CAMK2N1, IS, IS US, IS US CAMK2N1, IS US gf-CAMK2N1 mice at 14 days after MCAO, (n=4 mice/group). (D) Average Ca2+ transients (delta F/F) and (E) variance of GCaMP6s signals were displayed at day 7 and day 13 after MCAO. (F) and (G) Heatmap displayed variance of the calcium activity of neurons during a 5-min records after ultrasound stimulation and CAMK2N1 overexpression respectively (n=3 mice/group). (H, I) Electromyography (EMG) records showed average EMG amplitude (delta A/A) and (J, K) variance heatmap during a 5-min records, (n=3 mice/group). (L) mNSS, (M) tail suspension, (N) grid walking (O) and rotarod test showed that neurobehavioural outcomes in different groups, (n=3 mice/group in the sham groups, n=12 mice/group in the IS groups). Ctrl (black line), Ctrl US (blue line), Ctrl US CAMK2N1 (yellow line), and Ctrl US gf-CAMK2N1 (red line) mice. Data are mean ± SD. IS, ischaemic stroke mice; IS US, ischaemic stroke mice treated with US; US, mice treated with ultrasound; LIFUS, low-intensity focused ultrasound stimulation.
identify ultrasound-induced post-stroke changes at the cellular level, as well as pathways affected by ultrasound stimulation. Interestingly, we demonstrated that HMGB1 and CAMK2N1 are also the significantly changed genes in astrocytes. Previous studies showed that ultrasound stimulation mediated by astrocytes to promote angiogenesis and synaptogenesis, and HMGB1 and CAMK2N1 involved in this process. Therefore, we identified these two genes as targets for further experiments. Overall, HMGB1 and CAMK2N1 are the overlapping targets of proteomics and scRNA sequencing in ischaemic stroke mice, which promoted angiogenesis and synaptogenesis after ultrasound stimulation. Other studies showed that HMGB1 could release from astrocytes for neurovascular remodelling and angiogenesis through TLR4 and RAGE signalling pathways after ischaemic injury. CAMK2 (calcium/calmodulin-dependent protein kinase 2) was critical for transducing Ca\(^{2+}\) signals and contributing to cellular calcium homeostasis after injury. In our study, LIFUS promoted angiogenesis-related genes’ expression including PECAM1 and VEGFR1/Fil1 in both iTRAQ proteomics and scRNA-seq. Furthermore, LIFUS also promoted synaptogenesis-related genes like Atp5f1, Gabarap, Nyap1, Ryr3 and Trpv2, which could increase Ca\(^{2+}\) influx compared with IS groups. Increased brain calcium could activate or lead to a high concentration of CAMK2 translocates from the dendritic shaft to postsynaptic densities of dendritic spines, which played a critical role in calcium signalling regulation in learning and synaptic plasticity, and cell proliferation. CAMK2N1 translocated from the nuclei to the cytoplasm could directly interact with CAMK2 to inhibit its phosphorylation, and CAMK2N1-CAMK2 signalling restricts synaptic progression.

Clinical transformation and application are very important for the LIFUS. In fact, research on LIFUS for CNS disease therapy is still in the laboratory and preclinical stage (fusfoundation.org), especially in ischaemic stroke. Animal studies help to understand the efficacy, safety and the underlying mechanism. In addition, this research proved ultrasound stimulation could promote neurological function and physical rehabilitation, which provides an opportunity to treat patients who remain disabled after thrombolysis. We also have a series of further studies to explore the mechanism of ultrasound stimulation, which will further provide significant translational value for clinical trials eventually. Our results provided preliminary in vivo data for the potential clinical application of small and convenient ultrasound instrument to the human body. The efficacy of ultrasound treatment in the subacute stage of stroke in animals could be expanded to the clinical use. In future, the movement rehabilitation in patients who had a stroke can not only from limb rehabilitation to brain recovery, but also can from central angiogenesis and synaptogenesis induced by ultrasound to the limb rehabilitation. The mechanism by which ultrasound acts on astrocytes to promote angiogenesis and synaptogenesis can also be used as an experimental technique basis for determining the specific brain region that ultrasound stimulate in subsequent studies. In addition, our study provides an important reference for ultrasound treatment of neurodegenerative diseases such as Alzheimer’s Disease (AD), Parkinson’s disease (PD) and other brain diseases such as glioma.
CONCLUSIONS

Our findings suggest that LIFUS therapy promoted angiogenesis and synaptogenesis via astrocytic HMGB1 and CAMK2α (Figure 7). The results highlight a molecularly targeted mechanism for LIFUS therapy at the single-cell level, which provided a deeper understanding about the key molecular and cellular events of LIFUS-induced recovery after ischaemic stroke, and theoretical basis for extending LIFUS to other disease models and clinical settings.

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Contributors ZZ is responsible for the overall content. G-YT, ZZ, and JW conceived the project, designed the experiments, and edited the paper finally. LG designed and performed the experiments, analysed the data, and drafted the manuscript and figures. CW contributed to ultrasonic parameter design, electromyogram and fiber photometry records. LC contributed to ultrasonic parameter design, electromyogram and fiber photometry records. LC contributed to western blot. YT, and YW helped to design the experiment and interpret the data.

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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 \((\text{CBF}_{\text{End}} - \text{CBF}_{\text{Start}})/\text{CBF}_{\text{Start}} \times 100\%\). 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: \(V = \sum h/3*[\Delta S_n + (\Delta S_n * \Delta S_{n+1})^{1/2} + \Delta S_{n+1}]\), in which \(V\) represents volume, \(h\) represents the distance between the two adjacent brain sections, \(\Delta S_n\) and \(\Delta S_{n+1}\) 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™ 1200 system (Thermo Fisher). All analyses
were performed by a Q-Exact 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-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 batchelor. 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 MouseRNAsseqData 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 \[ \log 2 \text{-fold change} \] > 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 ImageJ software (NIH,
Bethesda, MD).

**Fluorescence in situ hybridization**

*In situ* hybridization was performed using one biotin-labeled riboprobe for HMGB1 (5′-
GCTTTCTTCTCAGGGCTTGTGTCATC -3’) and 2 mix biotin-labeled riboprobes
CAMK2N1 (5′- CCGTAGGGCCAGCACCTCCGACAT-3’ and 5′-
GGCTCAGTTCTGTCGGCGTA-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).
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 PreAmpifier 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 Ca²⁺ 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 ImageJ 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 ≥ 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**

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.
325 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.
Supplementary Figure 5. Fiber photometry records showed calcium waveforms of one representative mouse in the eight groups. (A) and (B) Original waveform was excited by 470 nm (green) and 410nm (blue) were detected in striatum perifocal region in 8 different groups at day 7 and 13 after MCAO. (C) and (D) Original waveform was detected in left hind leg of 8 different groups at day 7 and 13 after MCAO.
Supplementary Figure 6. Raw data of western blot.
### Supplementary Tables

**Table S1 Ultrasonic parameters design.**

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<th>$I_{SP_{TA}}$ (mW/cm²)</th>
<th>f (kHz)</th>
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Table S2 Ultrasonic parameters combinations.

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### Table S3 Ultrasonic parameters setting for further examination.

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Table S4 Antibodies used in western blots.

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