Optogenetic Inhibition of Striatal GABAergic Neuronal Activity Improves Outcomes After Ischemic Brain Injury

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Background and Purpose—Striatal GABAergic neuron is known as a key regulator in adult neurogenesis. However, the specific role of striatal GABAergic neuronal activity in the promotion of neurological recovery after ischemic stroke remains unknown. Here, we used optogenetic approach to investigate these effects and mechanism.

Methods—Laser stimulation was delivered via an implanted optical fiber to inhibit or activate the striatal GABAergic neurons in *Gad2-Arch-GFP* or *Gad2-ChR2-tdTomato* mice (n=80) 1 week after 60-minute transient middle cerebral artery occlusion. Neurological severity score, brain atrophy volume, microvessel density, and cell morphological changes were examined using immunohistochemistry. Gene expression and protein levels of related growth factors were further examined using real-time polymerase chain reaction and Western blotting.

Results—Inhibiting striatal GABAergic neuronal activity improved functional recovery, reduced brain atrophy volume, and prohibited cell death compared with the control ($P<0.05$). Microvessel density and bFGF (basic fibroblast growth factor) expression in the inhibition group were also increased ($P<0.05$). In contrast, activation of striatal GABAergic neurons resulted in adverse effects compared with the control ($P<0.05$). Using cocultures of GABAergic neurons, astrocytes, and endothelial cells, we further demonstrated that the photoinhibition of GABAergic neuronal activity could upregulate bFGF expression in endothelial cells, depending on the presence of astrocytes. The conditioned medium from the aforementioned photoinhibited 3-cell coculture system protected cells from oxygen glucose deprivation injury.

Conclusions—After ischemic stroke, optogenetic inhibition of GABAergic neurons upregulated bFGF expression by endothelial cells and promoted neurobehavioral recovery, possibly orchestrated by astrocytes. Optogenetically inhibiting neuronal activity provides a novel approach to promote neurological recovery.

Visual Overview—An online visual overview is available for this article. **(*Stroke*. 2017;48:3375-3383. DOI: 10.1161/STROKEAHA.117.019017.)**

Key Words: basic fibroblast growth factor ■ brain injury ■ GABAergic neuron ■ optogenetics ■ stroke

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Ischemic stroke is a leading cause of death and disability worldwide.[1] Effective treatment of ischemic stroke remains a major challenge largely because the narrow treatment window of currently available treatment approaches is limited to the acute phase. Ischemia can cause cell death and brain tissue damage and result in behavioral functional deficit, leaving the stroke survivors with long-term disability. In recent years, in addition to acute treatments, other strategies aiming at promoting functional recovery in the subacute and the chronic phase are being explored. Neural stem cells (NSCs) transplantation, as well as means to augment endogenous neurogenesis, showed promise in animal models.[2,3] Gene therapies and endothelial progenitor cell transplantation that promotes angiogenesis had also been shown to enhance functional recovery after ischemic brain injury.[4,5] Recently, optogenetic technique has been applied in experimental ischemic stroke studies that established its ability in regulating blood flow,[6] promoting neurogenesis,[7,8] and enhancing the survival and migration of NSCs.[9] By using cell-type promotors and transgenic mouse lines, it is now possible to investigate the specific roles played by a given type of neurons in the structural and functional recovery after brain injury.

Striatal GABAergic neurons project axons to adjacent subventricular zone (SVZ) and regulate the adult neurogenesis.[10,11] The NSCs in the SVZ were also shown to migrate to the peri-infarct area during ischemic stroke.[12] Inhibition

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of striatal neuronal activity promoted the functional recovery after stroke.[7] However, this inhibition is not neuron type–specific and, therefore, not able to elucidate the specific roles played by GABAergic neurons even though they constitute the majority of striatal neurons.

To pinpoint the effects of GABAergic neuronal activities on poststroke recovery, we used optogenetic tools to specifically regulate the activities of GABAergic neurons using transgenic mice with cell type–specific promoter-driven opsin expression.[13] Using Arch (archaerhodopsin) or ChR2 (channelrhodopsin-2) expressed under the control of glutamate decarboxylase-2 (*GAD2*) promoter, the GABAergic neuronal activity can be silenced or activated when a 530 nm green laser or 473 nm blue pulse laser was applied. The effect of inhibiting or exciting GABAergic neurons on neurobehavioral outcomes after ischemic injury was assessed. Cellular and molecular changes were examined. The in vivo experimental data suggested that the inhibition of striatal GABAergic neuronal activity attenuated ischemic brain atrophy and promoted functional recovery, with increased microvessel density and level of endothelial bFGF (basic fibroblast growth factor) observed in the inhibition group. The intercellular communication between the components of neurovascular unit, namely neurons, astrocytes, and endothelial cells (ECs), was tested in cell coculture systems. Results revealed that augmented bFGF expression by ECs occurred only when the neuronal activities of cocultured GABAergic neurons were optically inhibited and with the presence of astrocytes. Furthermore, the conditioned medium (CM) from the aforementioned coculture protected neurons from oxygen glucose deprivation (OGD) damage. These in vitro experimental results showed that EC received information from neurons via astrocytes and responded by changing its secretion profile to change its microenvironment, which could in turn regulate neuronal survival.

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Materials and Methods

We will or make our data, analytic methods, and study materials available to other researchers on request through the corresponding authors.

Animals

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University, China, and conformed to the ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments). Three strains of transgenic mice, *Gad2-Cre* (010802#), *Arch-GFP* (012735#), and *ChR2-tdTomato* (012567#), from the Jackson Laboratory were used. Homozygous Gad2-Cre male mice were bred with Arch-GFP and ChR2-tdTomato female mice. The *Gad2-Arch-GFP* and *Gad2-ChR2-tdTomato* heterozygous were used in the experiments when they were 16 weeks old and weighting 25 to 28 g. Only male mice were used in this work because of housing constrains.

Experimental Design

The experimental design with the overall study timeline is shown in Figure I in the online-only Data Supplement. A 60-minute transient middle cerebral artery occlusion (tMCAO) surgery was performed to induce a focal cerebral ischemic injury. Optical fiber was implanted at 5 days after tMCAO, and laser stimulation was performed at 7 to 13 days after tMCAO, twice a day, with 15 minutes each session, followed by a daily 5-bromo-2′-deoxyuridine injection (50 mg/kg; Sigma, St Louis, MO). A modified neurological severity score test was performed to assess the neurological function before brain injury and at 1, 3, 7, and 14 days after tMCAO by a researcher blind to the animal treatment design.

Transient Middle Cerebral Artery Occlusion in Mice

Eighty adult male transgenic mice (inhibition group, n_1=21; noninhibition control group, n_2=17; activation group, n_3=23; and nonactivation control group, n_4=19) were used in this study. A formal sample size and power calculations were not performed because this was the first investigation using this intervention. The tMCAO surgery was performed as described previously,[14,15] which is described in more detail in the online-only Data Supplement.

Optrode Implantation and Electrophysiology

Optrode implantation and light stimulation were performed according to a previous study, with minor modification.[7] Five days after tMCAO model, a hole, AP=−0.02, ML=−2.50, DV=−2.00 mm relative to the bregma, was drilled for fiber implantation into peri-infarct area. The activity of neurons was recorded using eight 35 µm diameter nichrome electrodes around the fiber. The raw signals were sampled at 30 kHz and transformed into digital signals by an analog to digital converter (ADC; Plexon, Dallas, Texas). The local field potential was obtained from the raw signal with a fourth-order Butterworth band-pass filter from 250 to 3000 Hz. A detection threshold method was used to detect the neuronal Spikes.

Optical Fiber Implantation and Laser Stimulation

Optical fiber was implanted into peri-infarct area at 5 days after tMCAO, as previously described.[7] Animals were randomly assigned to laser stimulation groups or nonstimulation control groups. Continuous 530 nm green laser was used to inhibit the activity of GABAergic neurons in *Gad2-Arch-GFP* transgenic mice with 1 mW power. A 473 nm pulse blue laser with 0.5 mW power was used to activate the GABAergic neuronal activity in *Gad2-ChR2-tdTomato* transgenic mice. The laser power was measured by an optical power meter (Thorlabs). The laser power levels were sufficient to elicit action potentials or inhibit neuronal activity without causing adverse effects. It has been shown that optogenetic stimulation in this power range causes no physical damage to the tissue.[16,17]

Neurological Function Evaluation and Brain Atrophy Volume Assessment

Neurobehavioral tests were performed before tMCAO and at 1, 3, 7, and 14 days after tMCAO by an investigator blind to the experimental design using the modified neurological severity score (online-only Data Supplement).

Mouse brain was perfused with 0.9% saline and followed by fixation with 4% paraformaldehyde (Sinopharm Chemical Reagent, Shanghai, China). After perfusion, 30-µm-thick brain sections were cut using a microtome and kept in antifreeze solution (20% glycerol, 30% ethylene glycol, 50% PBS, vol/vol). Slides were stained with 0.1% Cresyl Violet for brain atrophy measurement. The brain volume was calculated by the cubature formula of circular truncated cone as

$$V = \sum h/3\left[\Delta S_n + \left(\Delta S_n \times \Delta S_{n+1}\right)^{1/2} + \Delta S_{n+1}\right].$$

Immunostaining and Quantification

Immunostaining was performed as described previously[18] and in more detail in the online-only Data Supplement. Antibodies for cluster of differentiation 31 (CD31), Nestin, DCX (doublecortin), 5-bromo-2′-deoxyuridine, neuronal nuclei, bFGF, GFAP (glial fibrillary acidic protein), Iba-1 (ionized calcium binding adapter molecule 1), and GAD were used to assess neurogenesis, angiogenesis, and growth factor production. For apoptosis analysis, TUNEL staining (terminal dexynucleotidyl transferase mediated dUTP nick end labeling) was performed by using an in situ Cell Death Detection Kit (Roche,

Diagnostics, Basel, Switzerland). Zo-1 (zonula occludens-1) antibody (1:200; Invitrogen) was used for the integrity of blood brain barrier measurement, and the corresponding brain samples were not treated with paraformaldehyde, instead of frozen with methyl alcohol. Gap formation in microvessels was quantified as the ratio of gap length to the whole length of microvessels as previously described.[15] Three fields in the ischemic boundary zone in each slice were imaged with confocal microscope (Leica, Solms, Germany). Photomicrographs were taken using the same parameters. The number of Nestin$^+$ cells, TUNEL$^+$ cells, and microvessel were quantified by an investigator who was blind to the identity of each image. The amount of DCX$^+$ cells were quantified based on the area of fluoresce signal, using Image *J* software (NIH, Bethesda, MD). All photographs were taken under the same condition.

Western Blot and Real-Time Polymerase Chain Reaction Analysis

The ipsilateral striatum was collected to extract protein with protein lysate (RIPA with cocktail, phosphatase inhibitor) as described previously[19] and used for Western blot analysis.

The cortex and striatum of each hemisphere are separated, and the total RNA from the ipsilateral striatum was extracted using a TRIzol reagent (Invitrogen). The cDNA was synthesized by reverse transcriptase polymerase chain reaction (PCR) using a SYBR Premix Ex Taq Kit (Takara, Dalian, China). All procedures were performed following the manufacturer's protocol. The primers sequences are given in Table I in the online-only Data Supplement.

Cells Culture and Laser Stimulation In Vitro

Primary neuron and astrocytes were prepared as previously described[20] with minor modification. Briefly, the striatum was isolated from *Gad2-Arch-GFP* transgenic mice at postnatal day 0, digested with 0.25% trypsin for 10 minutes at 37°C, and terminated with 10% fetal bovine serum (FBS; GIBCO, Carlsbad, CA). After a quick rinse with PBS, the tissue was dissociated and suspended in DMEM (with 4.5 g/L glucose), followed by a filtration step using a 40 µm strainer. The cells (5×10^5 cells/mL) were plated in a 6-well plate precoated with poly-D-Lysine (Sigma). Mouse brain endothelial cells (1×10^5/mL) were plated in a 6-well plate at 9 days after the plating of neurons/astrocytes to prepare the 3-cell cocultured system. Cocultured cells were stimulated for 5 minutes with a 530 nm continuous laser. The laser stimulation was performed every 12 hours for a total of 3 sessions. The laser power was 0.6 to 0.8 mW, measured by an optical power meter (Thorlabs). Protein and RNA were extracted at 6 hours after the last stimulation for real-time PCR analysis. To investigate the role of astrocytes in the intercellular communication of this system, in a separate experiment, 2 µmol/L cytosine arabinoside (Sigma) was added to the neuron culture medium at 3 days after neuron/astrocytes plating to inhibit the proliferation of astrocytes to obtain predominant neuron culture. Mouse brain endothelial cells were then plated in the neuron culture to create a neuron/EC coculture setup. Photoinhibition of the neuronal activity was performed in the neuron/astrocyte and neuron/EC systems using the same stimulation parameters. RNA was extracted at 6 hours after the last stimulation for reverse transcriptase PCR analysis. The CM from inhibited or noninhibited neuron/astrocytes coculture was collected every 12 hours and was used to treat EC cells to test its ability to induce gene expression in ECs. Six hours after CM treatment, RNA was extracted from the ECs for PCR analysis.

Oxygen Glucose Deprivation and Cytotoxicity Assessment

To test whether inhibiting the neurons in the 3-cell cocultured system would promote trophic factor secretion and prevent injury, the CM was collected from the 3-cell cocultured system at 24 hours after laser inhibition. Another neuron/astrocyte/EC coculture was subjected to OGD for 30 minutes. The CM was used to treat the neuron/astrocyte/EC coculture at 12 hours prior, 0, and 12 hours after OGD. Six hours after the last CM treatment, the supernatant of the OGD cells was collected for cytotoxicity assessment using a lactate dehydrogenase kit (LDH; Beyotime, Shanghai, China). All procedures were performed according to the manufacturer's protocol.

Statistical Analysis

All data were presented as mean±SEM. Two-tailed unpaired Student's *t* test was performed, and a value of $P<0.05$ was considered significantly different. All statistical analysis was performed with SPSS v21.0 (SPSS Inc, Chicago, IL)

Results

Striatal GABAergic Neurons Can Be Effectively Inhibited or Activated by Laser Stimulation

Because >95% of striatal neurons are GABAergic neurons,[21] most part of the striatum expressed Arch-GFP in *Gad2-Arch-GFP* transgenic mice (Figure 1A). To ensure that GABAergic neurons expressed Arch-GFP, we used GAD, a specific marker of gamma amino butyric acid (GABA), to stain GABAergic neurons in brain sections. GAD is the key enzyme for GABA synthesis. Its cellular localization is mainly in the areas that near the nucleus (Figures IIA and IIB in the online-only Data Supplement). The GAD staining of laser-stimulated brains is also performed (Figure III in the online-only Data Supplement). The staining result showed that the cells expressing Arch-GFP were GABAergic neurons. To demonstrate that

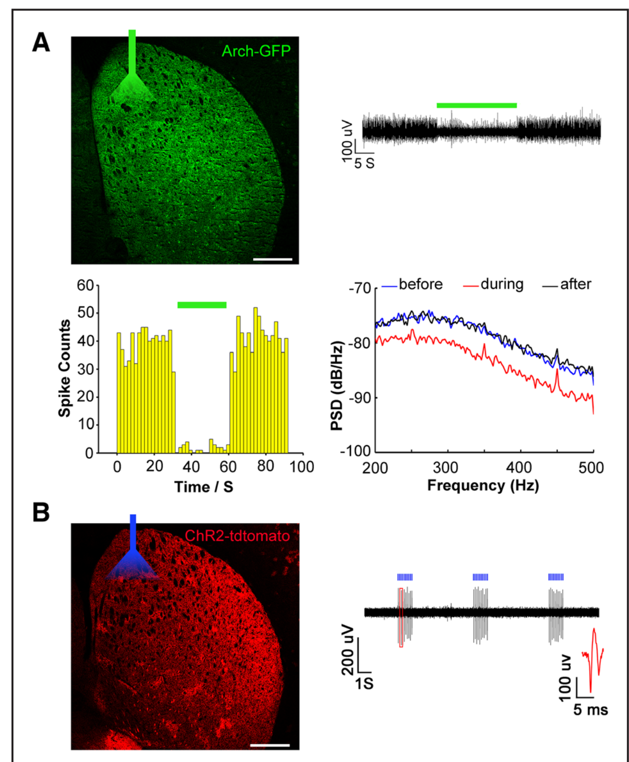

Figure 1. Successful regulation of striatal GABAergic neuronal activity by optogenetic tools. **A**, Fluorescent image of the left striatum of Gad2-Arch-GFP transgenic mice and local field potential (LFP) signal before, during, and after laser-induced neuronal inhibition (**upper**). **Lower**, The spike counts corresponding to power spectral density (PSD) analysis of LFP. **B**, Fluorescent image of the left striatum of *Gad2-ChR2-tdTomato* transgenic mice and LFP signal during pulsed laser stimulation. The rectangles (green) in (**A**) and the funnel in (**A**) and (**B**) represent laser projection, bar=250 µm.

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laser stimulation can inhibit or activate the neuronal activity successfully, a homemade optrode was used to stimulate the GABAergic neurons and simultaneously record their activity. The local field potential signals showed that the neuronal activity was inhibited during the 530 nm laser stimulation. The corresponding power spectral density during laser stimulation was also decreased compared with before or after laser stimulation. In addition to the continuous local field potential signal, the discrete SPIKE also showed that the firing rate was reduced during stimulation (Figure 1A). Laser stimulation of 473 nm in *Gad2-ChR2-tdTomato* transgenic mice activated striatal neuronal activity as expected (Figure 1B).

Photo Inhibition of GABAergic Neuronal Activity Improved Neurological Function and Reduced Brain Atrophy Volume After Ischemic Stroke

The details of the modified neurological severity scoring criteria were described previously.[22] In our experiments, the 4 experimental groups included inhibition (Arch/light$^+$) group, noninhibition (Arch/light$^-$) group, activation group (ChR2/light$^+$), and nonactivation (ChR2/light$^-$) group. We found that after a 7-day stimulation, the modified neurological severity score was lower in the Arch/light$^+$ group compared with the Arch/light$^-$ group (Figure 2A; P<0.01), indicating that the inhibition of striatal GABAergic neuronal activity improved neurobehavioral recovery. In contrast, the activation of striatal GABAergic neuronal activity hindered functional recovery (Figure 2C; P<0.01).

The brain atrophy volume was measured after 14 days of tMCAO. The brain atrophy volume in the Arch/light$^+$ group was much smaller than that of the Arch/light$^-$ group (Figure 2B), while the brain atrophy volume was larger in the ChR2/light$^+$ group than that of the ChR2/light$^-$ group (Figure 2D). The ratios of ipsilateral to contralateral brain volume were also calculated and compared across the 4 groups. The results are consistent with those from the absolute brain atrophy volume measurements.

Photo Inhibition of GABAergic Neuronal Activity Increased Vascular Density in the Peri-Infarct Region and Reduced Apoptosis

We examined the number of microvessels in the peri-infarct area in each group of mice. The results showed that Arch/

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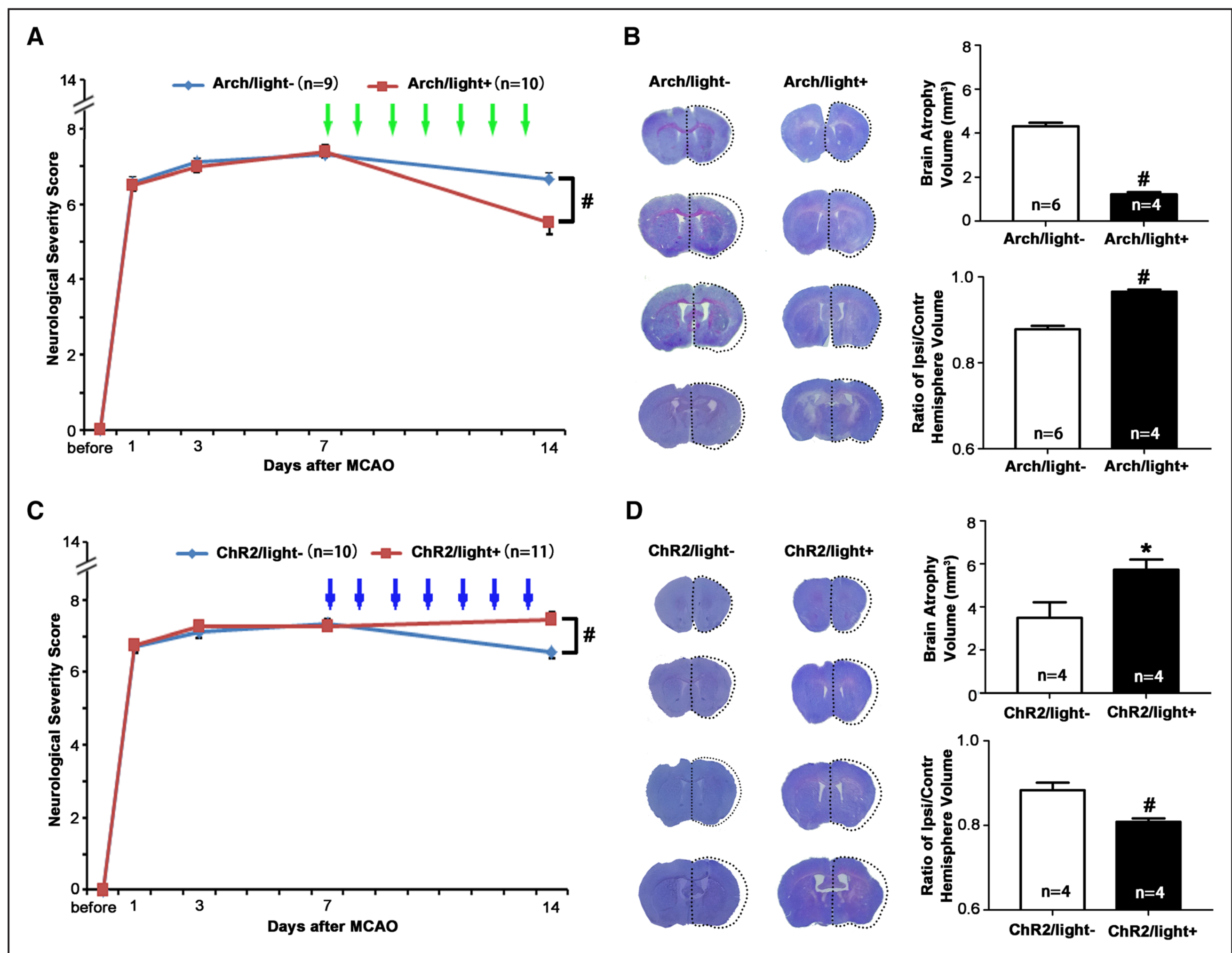

Figure 2. Inhibition of striatal GABAergic neuronal activity promoted the neurobehavioral recovery and reduced atrophy volume. **A** and **B**, The modified neurological severity score (mNSS) test and atrophy volume of Arch/light$^-$ and Arch/light$^+$ group, respectively (n=4–10 per group). **C** and **D**, The mNSS test and atrophy volume of ChR2/light$^-$ and ChR2/light$^+$ group, respectively (n=4–11 per group), *P<0.05, #P<0.01. Arch indicates archaerhodopsin; ChR2, channelrhodopsin-2; and MCAO, middle cerebral artery occlusion.

light⁺ group had a significantly higher vascular density than the Arch/light⁻ group. In contrast, the ChR2/light⁺ group had a significantly lower vascular density than the ChR2/light⁻ group (Figure 3A). We quantified the apoptotic cells in the peri-infarct area at 14 days after ischemic stroke by TUNEL staining. DAPI⁺ and TUNEL⁺ double-positive cells were verified as apoptotic cells (Figure IV in the online-only Data Supplement). The results showed that much less apoptotic cells were detected in the Arch/light⁺ group than in the Arch/light⁻ group. In contrast, the activation of striatal GABAergic neuronal activity led to increased apoptosis (Figure 3B). We also checked the integrity of blood brain barrier by double staining Zo-1 protein and CD31. The results showed that there are no statistically significant differences between the groups but a trend of reduced gap formation in Arch/light⁺ group comparing to Arch/light⁻ group and a trend of increased gap formation in ChR2/light⁺ versus ChR2/light groups (Figure V in the online-only Data Supplement).

Photo Inhibition of GABAergic Neuronal Activity Did Not Improve Neurogenesis in the Subacute Phase

We examined the number of NSCs and neural progenitor cells in the SVZ and peri-infarct area. The results showed that the Arch/light⁺ group had more Nestin⁺ NSCs in the SVZ, and the ChR2/light⁺ group has fewer NSCs than that of corresponding unstimulated control group (Figure 3C). However, the numbers of DCX⁺ cells in the SVZ and the peri-infarct region in the Arch/light⁺ group were both smaller than those of the Arch/light⁻ group (Figure 3D). Activation of striatal GABAergic neuronal activity resulted in opposite consequences. We performed 5-bromo-2′-deoxyuridine/neuronal nuclei double staining to examine the newborn mature neurons, but few

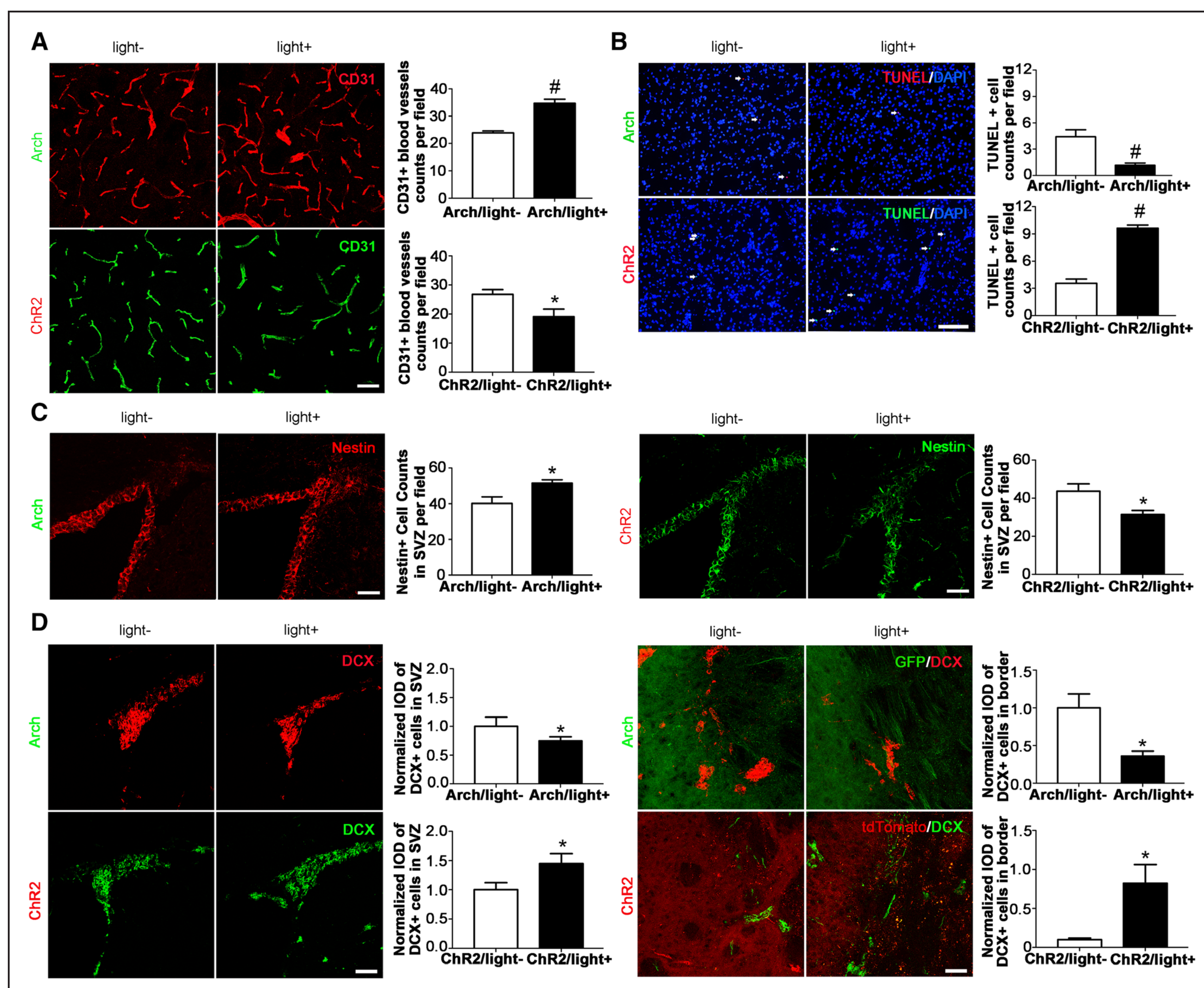

Figure 3. Striatal GABAergic neuronal activity regulated the microvessel density and cell death in the peri-infarct area and influenced the number of Nestin⁺ or DCX⁺ cells in subventricular zone (SVZ) and the peri-infarct area. **A**, Cluster of differentiation 31 (CD31) fluorescent staining of microvessels (red) in the peri-infarct area of the 4 groups and the corresponding quantitative analysis bar graph, bar=50 μm. **B**, TUNEL staining (indicated by white arrow) in the peri-infarct area of the 4 groups and the quantitative analysis bar graph, bar=100 μm. **C**, Nestin⁺ fluorescent staining (red) in the SVZ from Arch (archaerhodopsin) groups and ChR2 (channelrhodopsin-2) groups, respectively. **D**, DCX⁺ fluorescent staining in the SVZ (**left**) and peri-infarct area (**right**) of the 4 groups and the corresponding quantitative analysis, bar=50 μm, *P<0.05, #P<0.01. DCX indicates doublecortin; GFP, green fluorescent protein; and TUNEL, terminal dexynucleotidyl transferase mediated dUTP nick end labeling.

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double-positive newborn mature neurons were observed, neither Arch/light⁻ nor Arch/light⁺ group, in areas including the SVZ, the peri-infarct, and the core region (Figure VI in the online-only Data Supplement).

Photoinhibition of GABAergic Neuronal Activity Increased bFGF Expression in the Peri-Infarct Region

The fact that the Arch/light⁺ group had higher microvascular density in the peri-infarct region point to the tissue protection and proangiogenic effects of the intervention. To further explore the mechanisms of such effect, we extracted RNAs from the ipsilateral striatum to evaluate the expression of growth and trophic factors, including VEGF (vascular endothelial growth factor), bFGF, PDGF (platelet-derived growth factor), and BDNF (brain-derived neurotrophic factor). Reverse transcriptase PCR results showed that bFGF was significantly overexpressed in the Arch/light⁺ group comparing to the Arch/light⁻ group, while no difference was observed for other factors (Figure 4A). Western results confirmed that the Arch/light⁺ group had a higher bFGF expression than the Arch/light⁻ group, and the activation group had an opposite consequence (Figure 4B). The immunostaining results also presented a similar consequence (Figure 4C). To determine the cellular source of bFGF, we performed bFGF/CD31 (Figure 5A), bFGF/GFAP (Figure 5B), and bFGF/Iba-1 (Figure 5C) double staining. The results showed that the increased bFGF was mainly secreted by ECs in microvessels. It was noted that the activation of striatal GABAergic neuronal activity led to a decrease of bFGF in microvessels.

Photoinhibition of GABAergic Neuronal Activity Promoted the Expression of bFGF by EC Cells in the Presence of Astrocytes In Vitro

To better understand the regulation of bFGF expression by GABAergic neuronal activity, we cocultured neurons/astrocytes/ECs in vitro. The primary neurons were extracted from *Gad2-Arch-GFP* newborn P0 transgenic mice, expressing Arch-GFP on the membrane of GABAergic neurons. After laser stimulation, RNAs were extracted and analyzed using real-time PCR. The results showed that the inhibition of GABAergic neuronal activity promoted the expression of bFGF (Figure 6A; $P<0.05$). When we omitted the ECs and kept all other conditions the same, no significant change of bFGF mRNA level was detected in the neuron/astrocyte Arch/light⁺ group (Figure 6B; $P=0.66$). When we deleted the astrocytes and kept all other conditions the same, no significant change of bFGF mRNA level was detected in the neuron/EC Arch/light⁺ group either (Figure 6C; $P=0.83$). The omitting efficiency of astrocytes is shown in Figure VII in the online-only Data Supplement. We then treated ECs with the CM collected from the light⁺ or light⁻ neuron/astrocytes coculture. Results showed that CM from light⁺ neuron/astrocyte group promoted the bFGF expression in ECs (Figure 6D). These results demonstrated

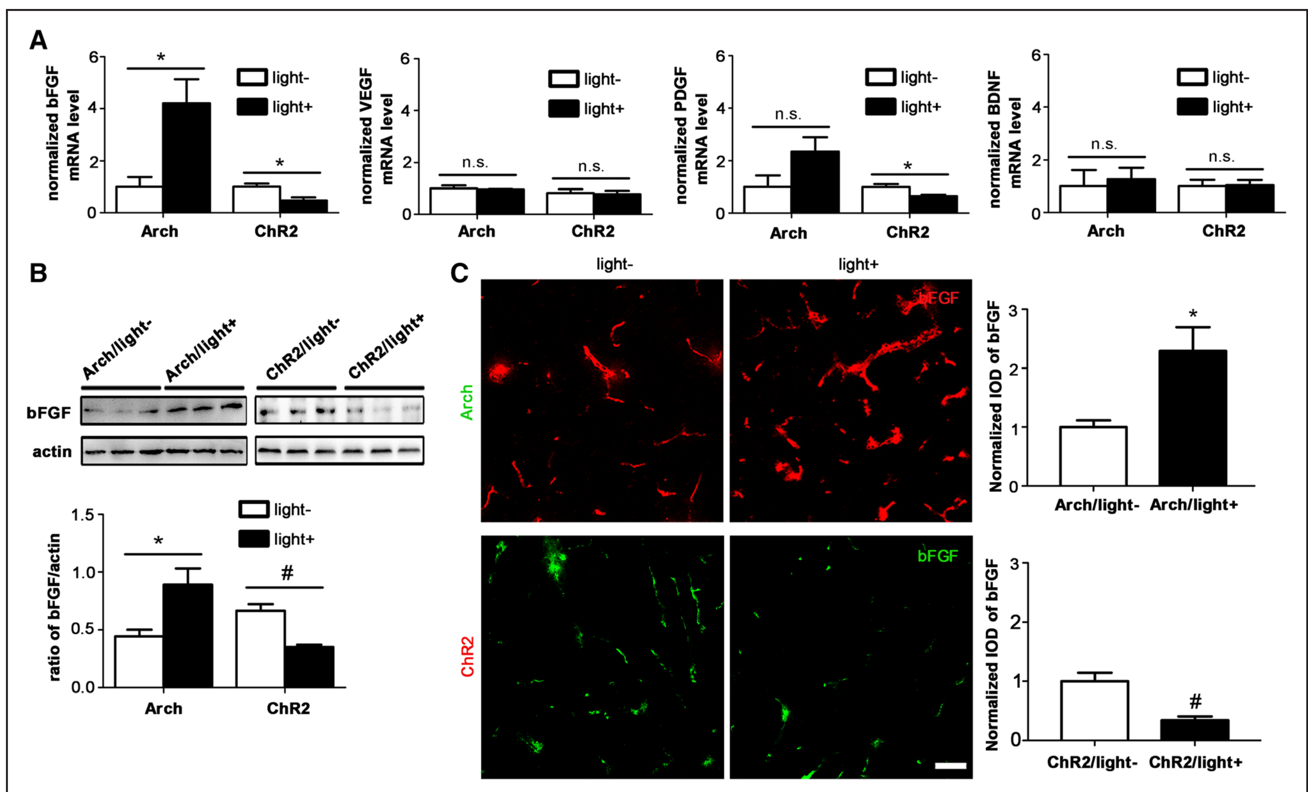

Figure 4. Striatal GABAergic neuronal activity influenced the bFGF (basic fibroblast growth factor) secretion in vivo. **A**, The mRNA level of bFGF, VEGF (vascular endothelial growth factor), PDGF (platelet derived growth factor), and BDNF (brain-derived neurotrophic factor) from the 4 groups. **B**, Western blot result of bFGF in the 4 groups and the quantitative gray value ratio analysis of bFGF bands to corresponding actin bands. **C**, bFGF fluorescent staining in the peri-infarct area from the 4 groups and the quantitative analysis, bar=50 μm, *$P<0.05$, #$P<0.01$. Arch indicates archaerhodopsin; and ChR2, channelrhodopsin-2.

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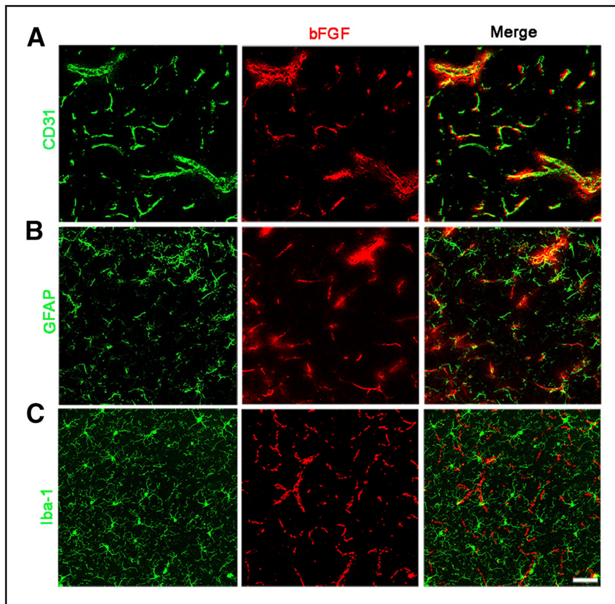

Figure 5. bFGF (basic fibroblast growth factor) was mainly produced by blood vessels. **A**, Double staining of CD31 (**upper left**, green) and bFGF (**upper middle**, red). **B**, Double staining of GFAP (glial fibrillary acidic protein; **middle left**, green) and bFGF (**middle**, red). **C**, Double staining of Iba-1 (**bottom left**, green) and bFGF (**bottom middle**, red). The 3 images in the right column were the corresponding merging images (CD31/bFGF, GFAP/bFGF, and Iba-1/bFGF), bar=50 μm. CD31 indicates cluster of differentiation 31; and Iba-1, ionized calcium binding adapter molecule 1.

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that inhibition of GABAergic neuronal activity promoted the bFGF expression from ECs, and this regulation was dependent on the presence of astrocytes. Our working hypothesis is that astrocytes relay the signal from striatal GABAergic neurons to regulate the secretion of bFGF by ECs, as illustrated in Figure VIII in the online-only Data Supplement.

CM From Arch/light+ Neuron/Astrocyte/EC Cocultures Protected Cells From OGD Injury

We cocultured neuron, astrocytes, and ECs and subjected the system to OGD for 1 hour to induce cell injury. Then we treated the injured cells with CM collected from another neuron/astrocyte/EC coculture with or without light inhibition. The results showed that the CM from light+ group improved the viability of cells (Figure 6D). This protective effect is consistent with the results observed in vivo.

Discussion

In this work, we demonstrated that inhibiting striatal GABAergic neuronal activity promoted neurobehavioral recovery and reduced brain atrophy volume at 14 days after tMCAO in mice, while activating striatal GABAergic neurons exacerbated brain injury. Such regulation of the striatal GABAergic neuronal activities is likely to initiate biochemical changes in other components of the neurovascular unit, namely astrocytes, ECs, and pericytes. Using an in vitro 3-cell coculturing system that includes GABAergic neurons, astrocytes, and ECs, we were able to confirm that the presence of astrocytes is necessary for this signal relay between the neurons and the ECs, supporting that the neuronal activity serves as a trigger of the repair process that involves multiple components.

Multiple factors contribute to improved neurobehavioral outcomes and reduced brain atrophy volume at different phases after ischemic brain injury. In the acute phase, anti-inflammatory strategies and blood brain barrier protection have been shown to be beneficial for reducing brain injury in animal experiments.[23–26] In the subacute phase and recovery phase, treatments aimed at promoting blood flow, tissue regeneration, and neuroplasticity had shown promising results.[3,6–8,27] Optogenetic technique has been used as a powerful tool to delineate the contribution of specific neuronal types in the recovery after ischemic stroke. Cheng et al[6] have shown that targeted optogenetic stimulation of the ipsilesional primary motor cortex produces functional recovery in rodent stroke models, with increased level of the plasticity markers BDNF, NGF (nerve growth factor), and NTF3 (neurotrophin-3) at 14 days after stroke. We showed that inhibiting striatal neuronal activity promoted functional recovery and neurogenesis at 5 weeks after tMCAO in mice.[7] Recent report demonstrated that stimulating endogenous glutamatergic activity in the striatum triggered glutamate release into the SVZ region and increased proliferation of SVZ neuroblasts.[8] Here, we revealed that GABAergic neuronal activity regulated the secretion profile of brain ECs via astrocytes and protects the neurovascular units against ischemic injury.

The inhibition of striatal GABAergic neuronal activity seems to trigger a cascade of downstream events, resulting in the increase of microvessel density and bFGF secretion in the peri-infarct region compared with the control. bFGF played an important role in brain repair and cell growth and could be secreted by many cell types, including ECs.[28–31] To identify the cellular source of bFGF in our model, we double-stained the brain sections with bFGF/GFAP, bFGF/Iba-1, and bFGF/CD31 antibodies and found that the majority of double-positive signal was detected in the bFGF/CD31 group but not in the bFGF/GFAP or bFGF/Iba-1 groups. To further investigate the effects of inhibiting GABAergic neuronal activity on bFGF secretion, we designed 3 coculture systems, neurons/astrocytes/ECs, neurons/ECs, and neurons/astrocytes, and treated the cocultures with a 530 nm green laser to inhibit the neuronal activity in vitro. We found that only in the neurons/astrocytes/ECs coculture system, bFGF was upregulated in Arch/light+ group when compared with Arch/light− control group. Neither the neurons/astrocytes nor the neurons/ECs coculture yielded significant increase of bFGF expression after laser stimulation. We treated ECs with the CM collected from neuron/astrocytes system and observed a similar level of bFGF upregulation compared with 3-cell coculture system. This result, together with the absence of bFGF upregulation in the neurons/ECs coculture, supported that astrocytes were essential for the signal relay between neurons and ECs. Given the important roles played by bFGF in brain repair,[32] vessel formation, and cell division,[33,34] the increased bFGF could account for the improved outcomes of the inhibition group. Previous reports showed that GABAergic neurons regulated blood pressure through various molecules, including GABA, nitric oxide synthase, neuropeptide Y, vasoactive intestinal peptide,

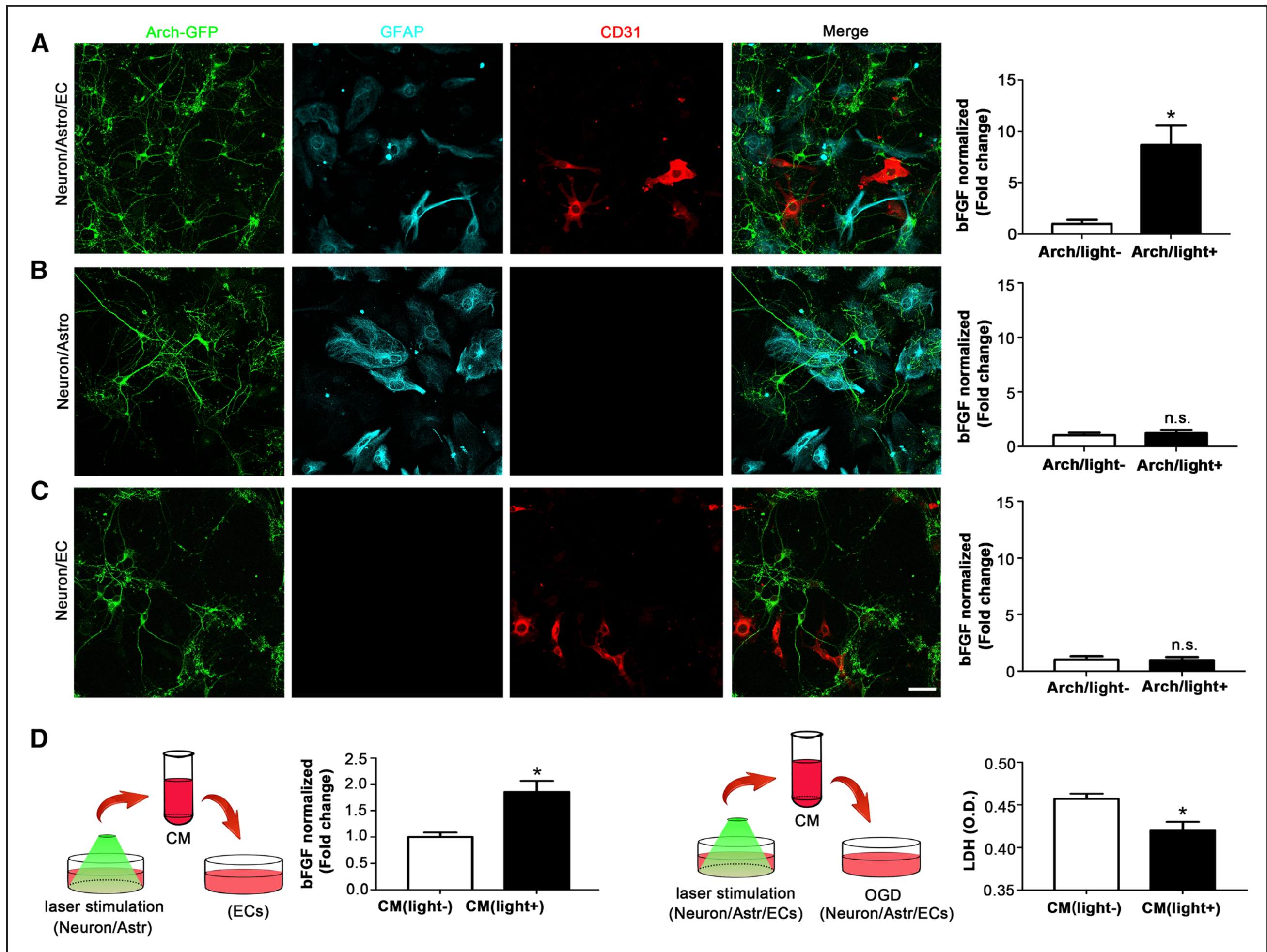

Figure 6. Inhibition of GABAergic neuronal activity promoted bFGF (basic fibroblast growth factor) expression in endothelial cells (ECs) dependent of astrocytes in vitro. **A**, The neurons/astrocytes/ECs 3-cell coculture system and quantitative analysis of bFGF. **B**, The neurons/astrocytes coculture system and the corresponding quantitative analysis of bFGF. **C**, The neurons/ECs coculture system and the corresponding quantitative analysis of bFGF, bar=50 µm. Conditioned medium (CM) from the stimulated neurons and astrocytes coculture system increased the bFGF expression (**D**, **left**) and promoted the viability of ECs after oxygen glucose deprivation (OGD; **D**, **right**), *$P<0.05$. Arch indicates archaerhodopsin; GFAP, glial fibrillary acidic protein; and LDH, lactate dehydrogenase.

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somatostatin, and cholecystokinin, acting through their corresponding receptors.[35–38] GABA was also believed to trigger the intracellular calcium ion influx and the translocation of endothelial nitric oxide synthase in ECs.[39] In this work, we demonstrated that inhibiting striatal GABAergic neuronal activity resulted in an increased microvessel density and heightened secretion of bFGF from ECs after tMCAO. Such regulation was shown to depend on the presence of astrocytes in vitro. The detailed mechanisms by which striatal GABAergic neuronal activity regulated bFGF, via astrocytes, warrant further study.

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Disclosures

None.

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