

Decreased Astrocytic CCL5 by MiR-324-5p Ameliorates Ischemic Stroke Injury via CCR5/ERK/CREB Pathway

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Abstract

Following ischemic stroke, Ccl5 mRNA expression increased, while miR-324-5p expression decreased in the peri-infarct cortex of middle cerebral artery occlusion (MCAO) mice. However, the roles of CCL5 and miR-324-5p in stroke remain unclear. Here, we show that inhibiting CCL5 using antibodies or miR-324-5p not only reduced infarct area and preserved neurological function in MCAO mice but also attenuated astrocyte and microglia activation, protected dendritic structures, and maintained spine density. In an astrocyte-neuron co-culture system after oxygen-glucose deprivation (OGD), knockdown astrocytic CCL5 expression by antibody or miR-324-5p decreased neuronal apoptosis and preserved dendritic architecture. Importantly, the suppression of CCL5 enhanced the activation of the ERK/CREB pathway both in vivo and in vitro. Consistent with these findings, the application of Maraviroc, a CCR5 antagonist, reduced infarct size, decreased neuronal apoptosis, and upregulated the ERK/CREB pathway in neurons treated with OGD. In conclusion, targeting the CCL5 pathway via miR-324-5p represents a promising therapeutic strategy for alleviating ischemic stroke damage through modulation of neuronal CCR5/ERK/CREB pathway.

eLife assessment

This study presents a **useful** finding on the interplay of CCL5 and miR-324-5p during ischemic stroke injury. Despite its importance, the evidence supporting the claims of the authors is **incomplete**. In particular, the lack of methodological information, inappropriate statistical testing, a flawed culture system, and the temporal mismatch in the expression of CCL5 and miR-324-5p following stroke have hindered further evaluation of the claims. The work will be of interest to neuroscientists working on brain injury such as stroke.

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Introduction

Stroke is known to be one of the leading causes of disability and death ¹. The most common type of stroke is ischemic stroke; however, the pathogenesis of ischemic stroke is not fully clear, and treatment is limited ². It is well known that astrocytes, the most abundant glial cells in the brain, serve diverse functions in the central nervous system (CNS) including forming the blood-brain barrier, synthesizing neurotransmitters, providing structural and nutritional support, and modulating synaptic plasticity ³. Following a stroke, astrocytes are pivotal in mediating both the progression of neural injury and the protective responses in the post-stroke environment, highlighting their critical and versatile functions within the pathophysiology of stroke.

Previous works divide the recovery time of the stroke into acute, subacute, and chronic phases ^{4,5}. The acute phase and the early subacute phase after stroke are critical periods of neuroplasticity ⁶. In the acute phase of ischemic stroke, a large number of neurons undergo apoptosis, glial cells transform into activate state, the blood-brain barrier is damaged, inflammatory factors elevated, and inflammatory cells gather at the site of injury ^{7,8}. During subacute period, some neurons in the ischemic injury area undergo secondary death due to excitotoxicity or oxidative stress, while some neurons maintain survival, and their function gradually recovers. Glial cells remain continuously activate during this period, playing a “double-edged sword” role. They may provide beneficial effects such as recruiting inflammatory cells, engulfing debris of dead cells, and regulating ion metabolism in the brain. However, they may also lead to exacerbation of the inflammatory response and induce harmful effects like excessive oxidative stress ^{9–12}.

MicroRNAs (miRNAs) are small (~22nt) non-coding RNAs that bind to the 3'-UTR of target mRNA and result in mRNA degradation or translational repression ¹³. MiRNAs possess characteristics such as high conservation, simple structure, ease of synthesis, and ability to freely cross the blood-brain barrier, making them intriguing targets for clinical diagnosis and disease treatment ¹⁴. Under pathological condition, miRNAs alter in the ischemic brain ^{15,16} and modulating astrocytic miRNA therapy demonstrates neuroprotection against stroke-related inflammation, glutamate excitotoxicity and glial scarring ^{17–20}. Our previous research has demonstrated that miR-324-5p regulates C-C Motif Chemokine Ligand 5 (CCL5, also known as RANTES: Regulated on Activation, Normal T Cell Expressed and Secreted) expression, crucially affecting synaptic formation by inhibiting CCL5 secretion from astrocytes ²¹. However, in the context of stroke, the impact of astrocytes regulating CCL5 expression via miR-324-5p on neurological function and neuronal downstream pathways is largely unknown.

Chemokines, including CCL5, are pro-inflammatory cytokines with chemoattractant properties that have been described as important regulators of peripheral and central immune responses ^{22–24}. CCL5 is produced by various cells such as T lymphocytes, platelets, endothelial cells, smooth muscle cells, and glial cells ²⁵. Regarding whether circulating CCL5 levels climb or fall following ischemic stroke, different reports show contradictory data ²⁵. Some studies have reported elevated levels of this biomarker were detected in both blood and cerebrospinal fluid ^{26–29}, while others have found no significant differences in CCL5 levels compared to controls or between ischemic stroke patients over time ^{25,30,31}.

In the CNS, CCL5 and its receptors have multiple functions, including promoting neuroinflammation, modulation of synaptic activity, and neuroprotection against various neurotoxins ³². Studies using CCL5-knockout mice have shown a reduction in ischemic area volume and significantly limited blood-brain barrier permeability, along with reduced leukocyte and platelet adhesion ³³. Additionally, administration of CCL5 inhibitor in experimental stroke models has been demonstrated to decrease infarct size and improve neurological outcomes post-

stroke ³⁴. Conversely, elevated CCL5 levels may confer neuroprotection through mechanisms such as vasodilation, inhibition of platelet aggregation, and promotion of angiogenesis ²⁵. Moreover, previous research has indicated that CCL5 can increase neurotrophic factors such as brain-derived neurotrophic factor and epidermal growth factor ²⁷. Overexpression of CCL5 in wild-type mice has been associated with enhanced memory-cognition performance, as well as increased hippocampal neuronal activity and connectivity ³⁵. The contradictory conclusions underscore the significant interest in the regulation mechanism of CCL5 and the complexity of its functions, emphasizing the need for more detailed investigations to fully elucidate its roles in the brain following a stroke.

CCL5 interacts with multiple receptors, notably C-C Motif Chemokine Receptor 5 (CCR5), CCR1, CCR3, and GPR75 ³⁵. CCR5, a predominant receptor for CCL5, is a seven-transmembrane G-protein-coupled receptor crucial for regulating pro-inflammatory responses by modulating immune cell behavior, survival, and tissue retention ³⁶. CCR5 is also expressed in non-immune cells such as astrocytes, microglia, and neurons, where it influences neuronal survival and differentiation ³⁷. The CCR5 mRNA and protein levels were increased in brain, spinal cord, and blood plasma at 22 hours after middle cerebral artery occlusion (MCAO) in mice ³⁸. Stroke induces an upregulation of CCR5 expression in neurons and a simultaneous downregulation in microglia, indicating complex, cell-specific changes in CCR5 signaling during the recovery phase ³⁹.

In this study, building upon our earlier work on miR-324-5p-mediated regulation of astrocytic CCL5 expression, we investigated the neuroprotective effects of CCL5 knockdown via antibodies or miR-324-5p in vivo in MCAO mice. Then, we examined the role of reduced astrocytic CCL5 expression in inhibiting neuronal apoptosis and preserving dendritic structure using the oxygen-glucose deprivation (OGD) model in vitro. Lastly, we explored the interaction between astrocytic miR-324-5p or CCL5 and the neuronal ERK/CREB pathway via CCR5 following ischemic stroke.

Results

We divided the cortex of MCAO mice into four regions (**Figure 1B**): Ipsilateral Core (IC), Ipsilateral Penumbra (IP), Contralateral Core (CC) and Contralateral Penumbra (CP). We then separately analyzed gene expressions and cell phenotypes separately in each region. The IC region, directly affected by oxygen deprivation, experiences significant neuronal death. The IP region, also influenced by oxygen deprivation, may undergo secondary neuronal death following stroke or potentially survive and regain normal function.

As depicted in **Figure 1C**, the RT-qPCR results indicated that in MCAO mice, the expression levels of Ccl5 mRNA in the CC and CP regions significantly increased on D1 compared to the sham control. By D3 and D7, Ccl5 mRNA levels decreased in these regions, showing no significant differences compared to the sham control. In the IC and IP regions, Ccl5 mRNA levels continuously increased from D1 to D7. Specifically, the expression level of Ccl5 mRNA in the IC region was consistently higher than that in the IP region at each time point. Compared to the CP region, Ccl5 mRNA levels in the IP region was significantly lower on D1, but significantly higher on D3 and D7. These findings suggest that in the contralateral cortex, Ccl5 mRNA levels increase post-stroke and return to baseline by D3, whereas in the injured cortex, they continue to rise from D1 to D7, with markedly higher levels in the IC region compared to the IP region.

As shown in **Figure 1D**, the RT-qPCR results demonstrated that the expression levels of miR-324-5p in the cortex of MCAO mice were highest on D1, decreased significantly by D3, and then increased again by D7. On D1, miR-324-5p levels in the IC region were significantly lower than that in the IP region. By D3, the miR-324-5p expression levels in both regions decreased to similar levels. By D7, although the expression levels in both regions increased, the expression levels in the

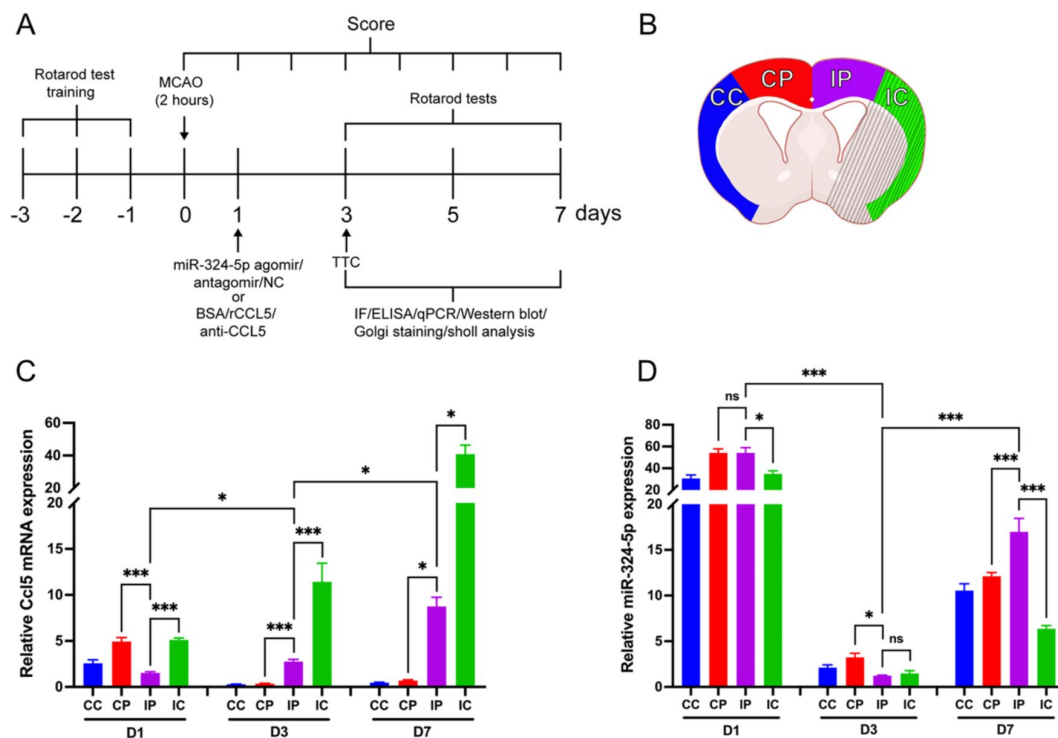


Figure 1.

Increased Ccl5 RNA expression and decreased miR-324-5p expression in the peri-infarct cortex in MCAO mice. (A) Illustration of animal experimental protocol. (B) Schematic of the brain showing CC, CP, IP, IC region of the cerebral cortex. The shadow part indicated the ischemic region. (C and D) QRT-PCR analysis of Ccl5 mRNA expression (C) and miR-324-5p expression (D) in the cortex on D1, D3 and D7 after MCAO (n=5). The data were normalized by the sham mice; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by unpaired two-tailed Student's *t* test.

IC region remained significantly lower than that in the IP region. When comparing the CP and IP regions, the expression levels of miR-324-5p were similar on D1. By D3, the expression levels in the CP regions were significantly higher than that in the IP regions. However, by D7, although both regions showed increased levels, those in the CP regions were significantly lower than that in the IP regions.

Combining **Figures 1C** and **1D**, within 24 hours post-MCAO, abundant expressions of miR-324-5p were observed in the ipsilateral cortex, potentially participating in the post-transcriptional regulation of Ccl5 gene expression and contributing to the reduced levels of Ccl5 mRNA at this time point. During the acute phase from D1 to D3 following stroke, the significant decrease in miR-324-5p levels may diminish their inhibitory effect on Ccl5 expression, leading to an increase in Ccl5 mRNA levels in the ipsilateral cortex of ischemic injury. In the subacute phase from D3 to D7, the overall increase in miR-324-5p expression in the cortex may exert a stronger inhibitory effect on Ccl5 mRNA expression levels. However, during this period, Ccl5 expression in the ipsilateral cortex continued to increase despite the elevated miR-324-5p expression.

To investigate the potential effects of the miR-324-5p/CCL5 axis on MCAO mice, recombinant mouse CCL5 protein (rCCL5), CCL5 antibody (anti-CCL5), or BSA was intracortically injected into the ipsilateral cortex within 24 hours post-surgery (**Figure 1A**). Subsequently, structural and functional analysis were conducted from D3 to D7. Additionally, 5nmol of the CCR5 antagonist MVC was injected intraperitoneally at 24, 48, and 72 hours post-surgery to evaluate its impact on recovery after ischemic stroke.

CCL5 protein levels in the cortical proteins of MCAO mice were detected using ELISA on D3 and D7 (**Figure 2A**). On D3, in the IC cortex regions, CCL5 concentrations were notably highest in the rCCL5 group compared to the other groups. Conversely, significant reductions in CCL5 concentration were observed in the IP, CC, and CP areas of the anti-CCL5 group mice. Furthermore, CCL5 concentrations significantly decreased in the IP area of the MVC group. By D7, significant increased CCL5 concentrations were observed in the IC, IP and CC area of the rCCL5 injection group. Besides, CCL5 concentrations in all groups within the IP area were significantly higher than the concentrations on D3. Furthermore, significant reductions in CCL5 concentration were observed in the IP and CP areas of the anti-CCL5 group compared to the BSA control group by D7. These results suggest that the injection of CCL5 antibody in the peri-infarct cortex can significantly decrease CCL5 concentrations from D3 to D7, whereas rCCL5 injection significantly increases CCL5 protein concentration in the IC area from D3 to D7, and in the IP area at D7. Blocking CCR5 continuously from D1 to D3 with MVC led to decreased CCL5 concentrations in the IP region of MCAO mice at D3 but not at D7.

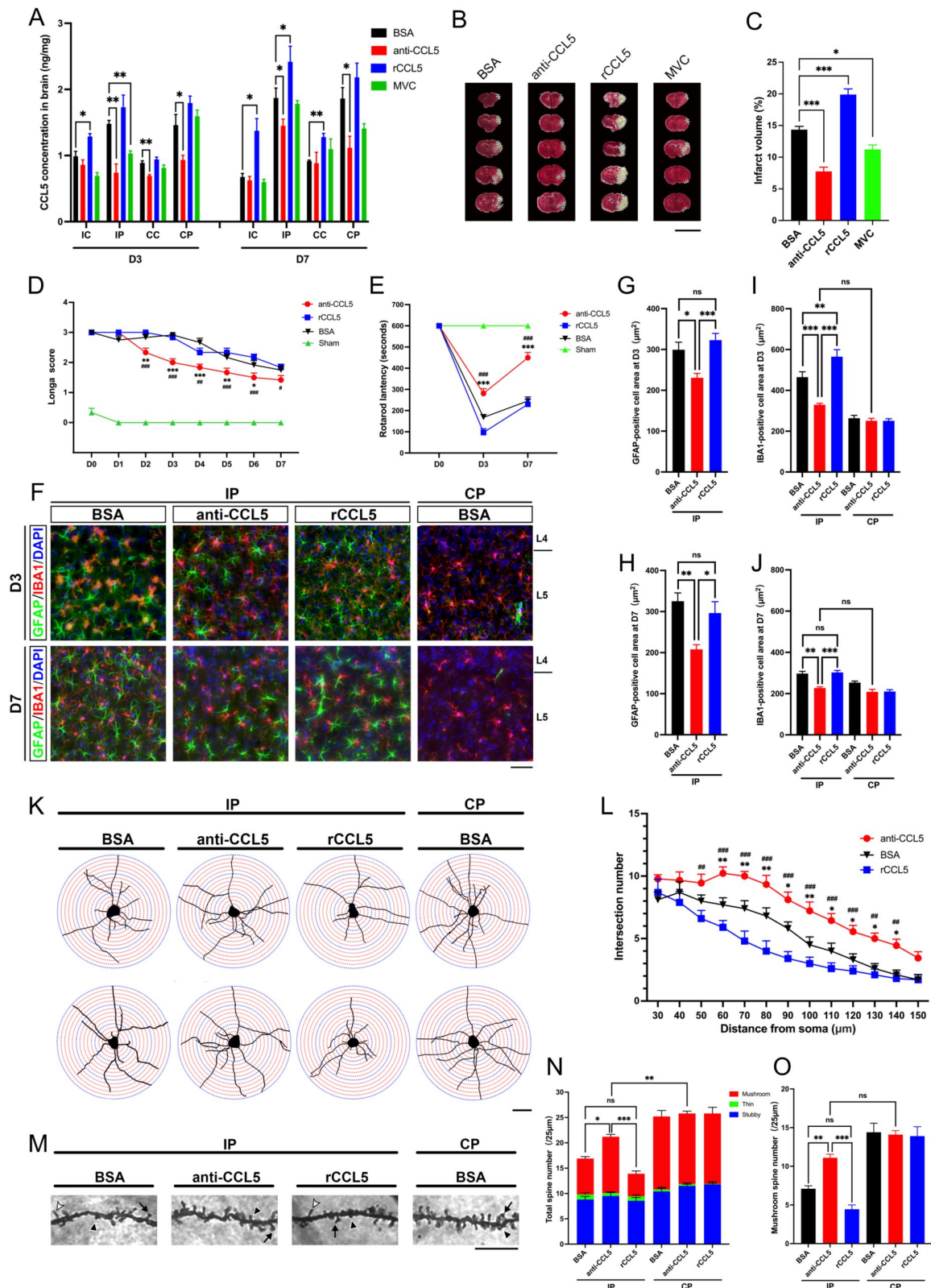


Figure 2.

Intracerebral CCL5 antibody delivery ameliorated ischemic stroke injury. (A) ELISA analysis of CCL5 concentration in the cerebral cortex of BSA, CCL5 antibody, rCCL5, and MVC treated mice on D3 and D7 after MCAO (n=3). (B and C) Representative TTC stained serial sections (B) and infarct volume (C) in brains from BSA, CCL5 antibody, rCCL5, and MVC treated mice on D3 after surgery (n=6). Scale bar: 1cm. (D) Longa scoring of BSA, CCL5 antibody, rCCL5 treated mice, and sham control from D0 to D7 after surgery (n=12). (E) Rotarod test of BSA, CCL5 antibody and rCCL5 treated mice, and sham control mice on D0, D3 and D7 from surgery (n=7). (F) Representative images of immunofluorescent double labeling of GFAP and IBA1 in the IP cortical region of BSA, CCL5 antibody and rCCL5 treated mice, and in the CP cortical region of BSA treated mice on D3 and D7 after MCAO. Scale bar: 50 μ m. (G and H) The surface area of GFAP⁺ activated astrocytes in the IP region of BSA, CCL5 antibody and rCCL5 treated mice on D3 (G) and D7 (H) after MCAO (n=10). (I and J) The surface area of IBA1⁺ microglia cells in the IP region of BSA, CCL5 antibody and rCCL5 treated mice on D3 (I) and D7 (J) after MCAO (n=10). (K) Representative schematic showing dendritic branch structure in the IP cortical region of BSA, CCL5 antibody and rCCL5 treated mice, and in the CP cortical region of BSA treated mice on D7 after MCAO. Scale bar: 20 μ m. (L) Sholl analysis of the dendritic branch structure in the IP region of BSA, CCL5 antibody and rCCL5 treated mice on D7 after MCAO (n=10). (M) Representative images of basal dendritic spines in the IP region of BSA, CCL5 antibody and rCCL5 treated mice, and in the CP cortical region of BSA treated mice on D7 after MCAO. Mushroom spines marked by arrows, stubby spines marked by black arrowheads and filopodial-like thin spines marked by white arrowheads. Scale bar: 10 μ m. (N and O) Quantification of the total spine density (N) and mushroom spine density (O) in the IP and CP region of BSA, CCL5 antibody and rCCL5 treated mice on D7 after MCAO (n=10). The composition of mushroom spines, stubby spines and thin spines within the total dendritic spine is indicated; * p <0.05, ** p <0.01 and *** p <0.001 by one-way ANOVA with Tukey's post-hoc test. In A, * p <0.05, ** p <0.01 and *** p <0.001 by unpaired two-tailed Student's t test. In D, E and L, * p <0.05, ** p <0.01 and *** p <0.001 vs. BSA group, ## p <0.01 and ### p <0.001 vs. rCCL5 group by two-way ANOVA with Tukey's post-hoc test.

The TTC results indicated that at D3, the infarct area in the anti-CCL5 group significantly decreased compared to the BSA control (**Figure 2B-C**). In contrast, following the rCCL5 injection, the infarct area significantly increased, reaching approximately 1.4 ± 0.1 times that of the BSA group. Furthermore, after MVC injection, the infarct area in MCAO mice significantly decreased compared to the BSA controls. These results demonstrate that reducing CCL5 protein expressions in the cortex or blocking the CCR5 receptor post-MCAO significantly reduced neuronal death after ischemic injury, thereby exerting a protective effect against ischemic brain damage. Conversely, increasing CCL5 protein levels significantly increased the infarct area post-ischemic injury, indicating its function of exacerbating ischemic cortical damage.

Following the injection of the CCL5 antibody, MCAO mice exhibited significantly lower Longa scores compared to both the BSA group and the rCCL5 group from D2 to D6 (**Figure 2D**). No statistic difference in the score was observed between the BSA group and the rCCL5 group. Besides, sham mice recovered to score of 0 within 24 hours post-operation and maintained this score until D7, suggesting that aside from occlusion, the surgery did not significantly impact the motor behavior of the mice. These results indicate that reducing CCL5 expression in the ipsilateral cortex of MCAO mice significantly protects motor function after ischemic brain injury.

All mice successfully completed the Rotarod test before MCAO surgery and remained on the rod without falling. At both D3 and D7, mice in the anti-CCL5 group showed significantly longer latency-to-fall on the Rotarod compared to the rCCL5 and BSA control groups (**Figure 2E**). In contrast, significantly shorter latency-to-fall on the Rotarod were detected in the rCCL5 group compared to the BSA control group at D3 (not shown on the figure). By D7, no statistically significant differences were detected between the two groups. Besides, sham mice were able to remain on the Rotarod without falling at both D3 and D7, indicating that aside from occlusion the

surgical procedure did not significantly affect the Rotarod performance of the mice. These findings indicate that reducing CCL5 expression in the ipsilateral cortex improved neurobehavioral recovery in MCAO mice.

The GFAP/IBA1 immunofluorescence staining was employed to label the activated astrocytes and microglia in the IP cortex (**Figure 2F** [↗](#)). Statistical analysis indicated that at both D3 and D7, the surface areas of GFAP-positive astrocytes and IBA1-positive microglia were significantly reduced in the anti-CCL5 group (**Figure 2G-J** [↗](#)). Moreover, within the anti-CCL5 group, no statistical differences were observed in the surface area of microglia between the IP and CP cortical regions (**Figure 2I-J** [↗](#)). Conversely, the surface area of astrocytes in the rCCL5 group showed no significant difference compared to the BSA group (**Figure 2G-H** [↗](#)). Notably, at D3, the surface area of microglia in the rCCL5 group was significantly larger than in the BSA control group (**Figure 2I** [↗](#)). Whereas by D7, the surface areas of microglia in both groups decreased to comparable levels (**Figure 2J** [↗](#)). Besides, no significant differences were detected in the density of activated astrocytes or microglia in the IP cortex among the BSA, anti-CCL5, and rCCL5 group at both D3 and D7 (Supplementary Figure S1A-D). These experimental results suggest that reducing CCL5 concentration in the injured cortex of MCAO mice suppresses the activation of astrocytes and microglia, potentially reducing brain inflammation after ischemic injury.

The Sholl analysis performed post-Golgi staining revealed that at D7, the dendritic branch complexity of cortical neurons in the IP region of anti-CCL5 group was significantly higher within the 70μm to 150μm range from cell body compared to the rCCL5 and BSA control groups (**Figure 2K-L** [↗](#)). No significant differences were found in the dendritic branch complexity of cortical neurons in the CP cortex among the three groups (Supplementary Figure S1F). In the IP region of the rCCL5 group, dendritic branch complexity was significantly lower compared to that in the BSA group within the 50μm to 80μm range from cell body (not shown in the figure). These results indicate that inhibiting CCL5 expression within the ischemic cortex significantly protects the dendritic structure of neurons, while excess CCL5 expression further compromised the dendritic structure of cortical neurons.

Subsequently, we analyzed differences in dendritic spine density and morphology in the cortical neurons among the groups. In the IP region of anti-CCL5 group at D3 and D7, both the total dendritic spine density and the density of mature mushroom-type dendritic spines were significantly higher compared to the rCCL5 and BSA control groups, while the densities of thin-type and stubby-type immature dendritic spines showed no significant differences across the groups (**Figure 2M-O** [↗](#), Supplementary Figure S1G-H). Furthermore, no statistical differences were observed in total dendritic spine density or in the proportions of different morphological types of dendritic spines between the rCCL5 group and the BSA group in the IP area. Besides, there were no statistical differences in the total dendritic spine density or in the proportions of the three different morphological types of dendritic spines in the CP cortical neurons among the groups. These results suggest that inhibiting CCL5 expression in the injury cortex after ischemic stroke has a significant protective effect on the quantity of neuronal dendritic spines, particularly the mature mushroom-type dendritic spines.

In the preceding sections, we have investigated the effects of overexpressing or knocking down CCL5 protein on neuronal structure, glial cell activation, and neurological function following ischemic stroke. Subsequently, we administered in situ injections to the IP cortex of MCAO mice to modulate miR-324-5p levels. ELISA assay results revealed a significant reduction in CCL5 protein concentration in the ipsilateral cortex of the miR-324-5p agomir group mice on D3 and D7 (**Figure 3A** [↗](#)). Furthermore, decreased CCL5 expression was also observed in the CP area of the same group on D7. Conversely, the miR-324-5p antagomir group exhibited a significant increase in CCL5 concentration in the IP, CC, and CP areas at D3, and in the IC, IP, and CC areas at D7. These findings indicate that miR-324-5p agomir injection significantly reduces CCL5 expression in the ischemic cortex, whereas miR-324-5p antagomir injection markedly increases CCL5 expression.

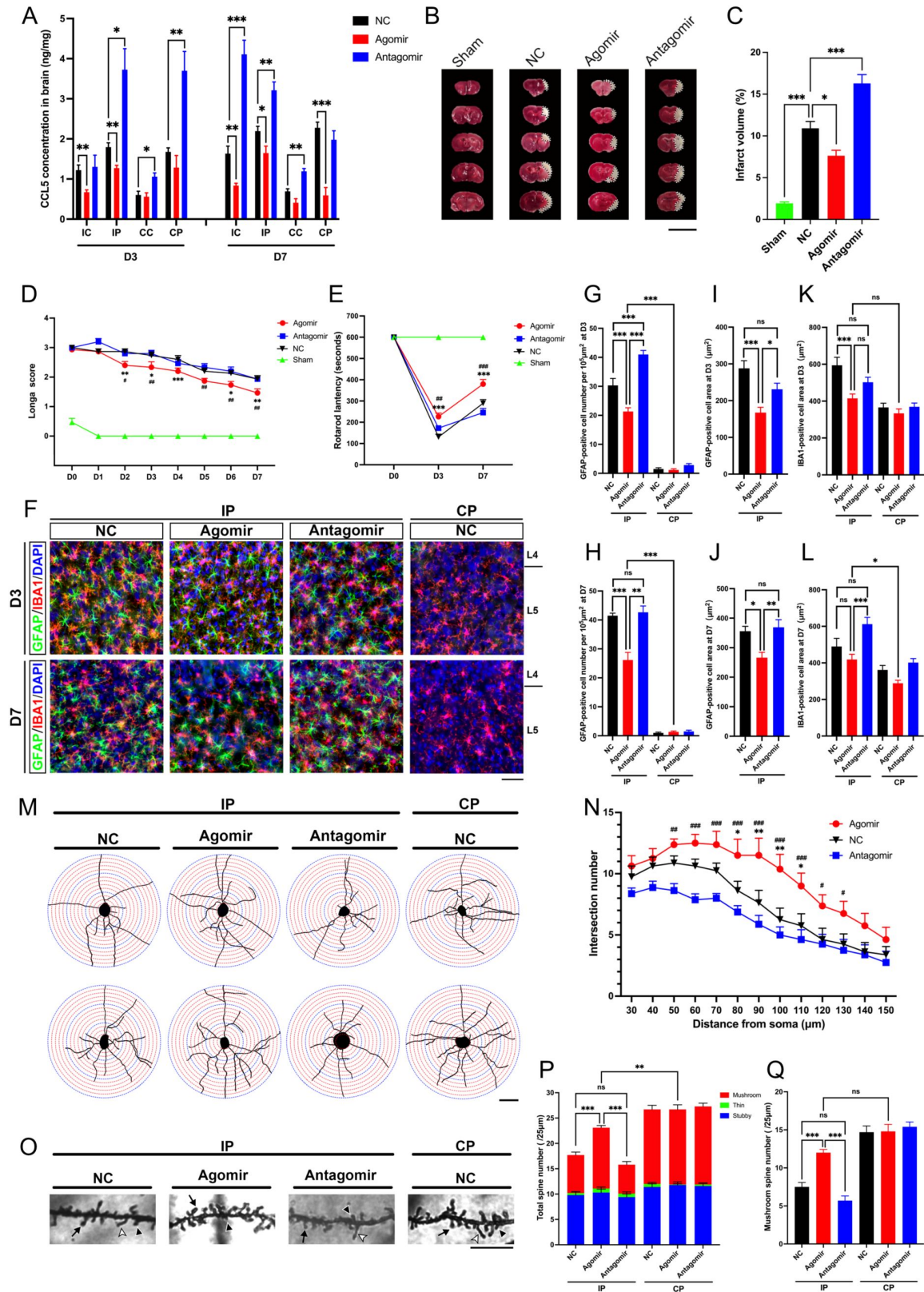


Figure 3.

Intracerebral miR-324-5p agomir delivery ameliorated ischemic stroke injury. (A) ELISA analysis of CCL5 concentration in the cerebral cortex of NC agomir, miR-324-5p agomir and miR-324-5p antagonist treated mice on D3 and D7 after MCAO (n=4). (B and C) Representative TTC stained serial sections (B) and infarct volume (C) in brains from sham control mice, NC agomir, miR-324-5p agomir and miR-324-5p antagonist treated mice on D3 after surgery (n=6). Scale bar: 1 cm. (D) Longa scoring of NC agomir, miR-324-5p agomir and miR-324-5p antagonist treated mice, and sham control from D0 to D7 after surgery (n=15). (E) Rotarod test of NC agomir, miR-324-5p agomir and miR-324-5p antagonist treated mice, and sham control on D0, D3 and D7 from surgery (n=7). (F) Representative images of immunofluorescent double labeling of GFAP and IBA1 in the IP cortical region of NC agomir, miR-324-5p agomir and miR-324-5p antagonist treated mice, and in the CP cortical region of NC agomir treated mice on D3 and D7 after MCAO. Scale bar: 20 μ m. (G and H) The number of GFAP⁺ activated astrocytes per 10⁵ μ m² in the IP and CP region of NC agomir, miR-324-5p agomir and miR-324-5p antagonist treated mice on D3 (G) and D7 (H) after MCAO (n=6). (I and J) The surface area of GFAP⁺ activated astrocytes in the IP region of NC agomir, miR-324-5p agomir and miR-324-5p antagonist treated mice on D3 (I) and D7 (J) after MCAO (n=10). (K and L) The surface area of IBA1⁺ microglia cells in the IP and CP region of NC agomir, miR-324-5p agomir and miR-324-5p antagonist treated mice on D3 (K) and D7 (L) after MCAO (n=10). (M) Representative schematic showing dendritic branch structure in the IP region of NC agomir, miR-324-5p agomir and miR-324-5p antagonist treated mice, and in the CP cortical region of NC agomir treated mice on D7 after MCAO. Scale bar: 50 μ m. (N) Sholl analysis of the dendritic branch structure in the IP region of NC agomir, miR-324-5p agomir and miR-324-5p antagonist treated mice on D7 after MCAO (n=8). (O) Representative images of basal dendritic spines in the IP region of NC agomir, miR-324-5p agomir and miR-324-5p antagonist treated mice, and in the CP cortical region of NC agomir treated mice on D7 after MCAO. Mushroom spines marked by arrows, stubby spines marked by black arrowheads and filopodial-like thin spines marked by white arrowheads. Scale bar: 10 μ m. (P and Q) Quantification of the total spine density (P) and mushroom spine density (Q) in the IP and CP region of NC agomir, miR-324-5p agomir and miR-324-5p antagonist treated mice on D7 after MCAO (n=10). The composition of mushroom spines, stubby spines and thin spines within the total dendritic spine is indicated; **p*<0.05, ***p*<0.01 and ****p*<0.001 by one-way ANOVA with Tukey's post-hoc test. In A, **p*<0.05, ***p*<0.01 and ****p*<0.001 by unpaired two-tailed Student's *t* test. In D, E and N, **p*<0.05, ***p*<0.01 and ****p*<0.001 vs. NC agomir group, # *p*<0.05, ##*p*<0.01 and ###*p*<0.001 vs. miR-324-5p antagonist group by two-way ANOVA with Tukey's post-hoc test.

The TTC assay results revealed that the infarct area was significantly smaller in the miR-324-5p agomir group compared to the NC agomir group at D3 (**Figure 3B-C**). Conversely, after miR-324-5p antagonist injection, the infarct area significantly increased. Besides, the infarct area in the sham group mice was less than 2.5% of the whole brain area, suggesting that aside from occlusion, the surgery operation did not cause profound infarct area in mice. These results demonstrate that miR-324-5p overexpression following ischemic stroke can reduce cortical neuronal death, thus exerting a protective effect, while its absence exacerbates neuronal death, increasing the infarct area in the brain following stroke.

The Longa score results indicated that miR-324-5p agomir mice consistently exhibited significantly lower scores than the NC agomir control group on D2, D3, D4, D6, and D7 (**Figure 3D**). Additionally, these scores were significantly lower when compared to the antagonist group on D2, D3, D5, D6, and D7. In contrast, no significant differences were observed between the antagonist and NC control group. These data suggest that miR-324-5p overexpression significantly enhances neurological function following ischemic stroke.

The Rotarod tests indicated on D3 and D7, mice in the agomir group exhibited significantly longer latency-to-fall compared to both the antagonist and NC groups (**Figure 3E**). However, no statistically significant differences were found in the Rotarod performance between the antagonist and NC groups. These results suggest the role of miR-324-5p in improving somatosensory-motor functional outcomes following ischemic brain injury.

Statistical analysis revealed that following the injection of miR-324-5p agomir, the density of activated astrocytes with positive GFAP staining in the IP area significantly decreased on D3 and D7, compared to both the NC control and the antagomir groups (**Figure 3G-H**). Besides, the density of activated astrocytes in the IP area in the antagomir group was significantly higher than that in the NC control group on D3. However, due to an increase in activated astrocytes in the NC group by D7, no statistical differences were observed in the density of activated astrocytes between the antagomir group and the NC group. Furthermore, the surface area of activated astrocytes within the IP region was smaller in the agomir group compared to the NC group on both D3 and D7, while there was no statistical differences between the antagomir group and the NC group (**Figure 3I-J**).

On D3, the surface area of microglia in the agomir group was significantly smaller than that in the NC group (**Figure 3K**). By D7, due to decreased surface area of microglia in the NC group and increased surface area of microglia in the antagomir group, no significant difference between the agomir group and the NC group was detected, but the surface area of microglia in the agomir group significantly smaller than in the antagomir group (**Figure 3L**). On both D3 and D7, there were no statistically significant differences in the surface area of activated microglia in the IP area between the antagomir group and the NC group. Moreover, no significant differences were detected in the density of activated microglia between the agomir and NC group in IP regions on both D3 and D7 (Supplementary Figure S2A-B). These results indicate that overexpression of miR-324-5p following ischemic stroke significantly inhibited the activation and proliferation of astrocytes in the ipsilateral cortex, and also reduced microglia activation, as evidenced by the reduction in microglial surface area on D3.

Sholl analysis of Golgi-stained cortical neurons in the IP region revealed that on D3 and D7, the dendritic field complexity in the agomir group was significantly higher than that in the antagomir group and the NC group (**Figure 3M-N**, Supplementary Figure S2C). On D7, the dendrite intersections of cortical neurons in the IP area of the agomir group were significantly more than that in the NC control group within the range of 80µm-110µm from the cell body, and significantly more than that in the antagomir group within the range of 50µm-130µm from the cell body. Conversely, in the antagomir group, the dendrite intersections of cortical neurons in the IP area were significantly less than that in the NC group within the range of 90 µm-100µm and 140µm-150µm from the cell body (not shown in the figure). These findings suggest that overexpression of miR-324-5p after ischemic brain injury contributes to the preservation of dendritic structures of neurons, potentially facilitating the recovery of neuronal function following ischemic stroke.

Analysis of dendritic spine density and morphology in the cortical neurons of the IP area demonstrated that on D3 and D7, both the total dendritic spine density and the density of mushroom- type mature dendritic spines in the agomir group were significantly higher than that in both the NC control and the antagomir groups (**Figure 3O-Q**, Supplementary Figure S2E-F). No significant differences were observed in the total dendritic spine density and mushroom-type mature dendritic spine density between the antagomir group and the NC group at D3 and D7. These results underscore the significant protective effects of increasing miR-324-5p expression on neuronal dendritic spine densities post-ischemic stroke, particularly on mature mushroom-type dendritic spine densities.

We established an in vitro co-culture model of primary cortical astrocytes and neurons (**Figure 4A**). Following 1hr OGD treatment, the co-culture media were supplemented with BSA, CCL5 antibody, or rCCL5 to examine the effects of modulating CCL5 expression on neuronal apoptosis and dendritic structure. ELISA was conducted to measure CCL5 concentration in the co-cultured medium at D3 post-OGD (**Figure 4B**). Results showed that the CCL5 concentration in the un-OGD- treated control group was approximately 45.0 ± 5.4 pg/ml. Following OGD treatment, CCL5 concentration significantly increased in the BSA control group, reaching 145.0 ± 7.6 pg/ml. The

addition of CCL5 antibody significantly decreased CCL5 concentrations in the OGD-treated co-cultured medium compared to the BSA control, reducing them to 48.3 ± 6.8 pg/ml. Conversely, the supplementation with rCCL5 led to a significant increase in CCL5 concentration, reaching $829.6 \pm$

37.1 pg/ml. These findings indicate that astrocytic CCL5 expression significantly increases after OGD treatment. The introduction of CCL5 antibody can substantially reduce CCL5 concentrations in the extracellular milieu of the astrocyte-neuron co-culture system, whereas the exogenous addition of rCCL5 substantially elevates the CCL5 concentrations. Moreover, when MVC was added to the co-culture media post-OGD, CCL5 concentration was significantly reduced. This result suggests that blocking astrocytic CCR5 reduces CCL5 production, thereby downregulating its functions following OGD or ischemic injury.

TUNEL staining indicated significantly less apoptosis neurons in the CCL5 antibody group compared to both the BSA control group and the rCCL5 group (**Figure 4C-D**). The apoptosis neuron ratio in the rCCL5 group was significantly higher than that in the BSA control group. Simultaneous addition of rCCL5 and MVC (MVC+rCCL5) in the co-culture system resulted in significantly lower apoptosis density compared to the rCCL5 and BSA control groups. These results demonstrate that reducing CCL5 levels in the extracellular milieu of the astrocyte-neuron co-culture system after OGD treatment, or inhibiting the CCR5 receptor, both exert a protective effect on neuronal survival following OGD injury. Conversely, excessive CCL5 leads to increased neuronal death post-OGD.

Sholl analysis revealed that on D3 and D6 post-OGD, dendrite field complexity in co-cultured neurons treated with CCL5 antibody was significantly higher compared to both the BSA control and rCCL5 groups (**Figure 4E-F**, Supplementary Figure S3A). At D6, the dendritic intersections of neurons in the anti-CCL5 group were significantly greater than that in the BSA control and rCCL5 groups at distances of $60\mu\text{m}$ - $200\mu\text{m}$ from the cell body. Compared to the rCCL5 group and BSA group, neurons in the MVC+rCCL5 group showed significantly increased dendrite intersections at distances of $80\mu\text{m}$ to $200\mu\text{m}$ from the cell body (not shown in the figure). These results indicate that reducing CCL5 levels in the extracellular milieu of the co-culture system or inhibiting the CCR5 receptor significantly protects the dendritic structure of neurons after OGD injury.

MAP2/SYN1 immunofluorescence staining revealed that at D6 post-OGD treatment, neurons in the CCL5 antibody group exhibited a significantly higher synaptic density than those in both the BSA control and rCCL5 groups (**Figure 4G-H**). No significant differences were observed in synaptic densities between the rCCL5 and BSA control groups. Notably, neuronal density in the MVC+rCCL5 group was markedly higher compared to the rCCL5 and BSA groups. These findings suggest that reducing CCL5 expression or inhibiting the CCR5 receptor can significantly preserve neuronal synaptic density following OGD injury.

Following OGD treatment, the co-culture media of astrocytes and neurons were supplemented with NC agomir, miR-324-5p agomir, or miR-324-5p antagomir. On D3 and D6 post-OGD, the transfection efficiency of agomirs in the co-culture system reached approximately 60% (Supplementary Figure S3B). On D3 post-OGD, ELISA was employed to detect CCL5 concentrations in the co-culture media (**Figure 5A**). Results indicated that CCL5 levels in the agomir-transfected group was significantly lower than that in both the NC group and antagomir groups. Conversely, CCL5 concentrations were significantly elevated in the antagomir-transfected group compared to the NC group. These findings demonstrate that agomir transfection significantly reduces CCL5 expression in the astrocyte-neuron co-culture media, whereas antagomir transfection significantly increases CCL5 expression.

TUNEL assay indicated that at D6 post-OGD, apoptotic neurons were significantly fewer in the agomir group compared to both the NC and antagomir groups (**Figure 5B-C**). In contrast, the antagomir group displayed more apoptotic neurons than the NC group. These results demonstrate

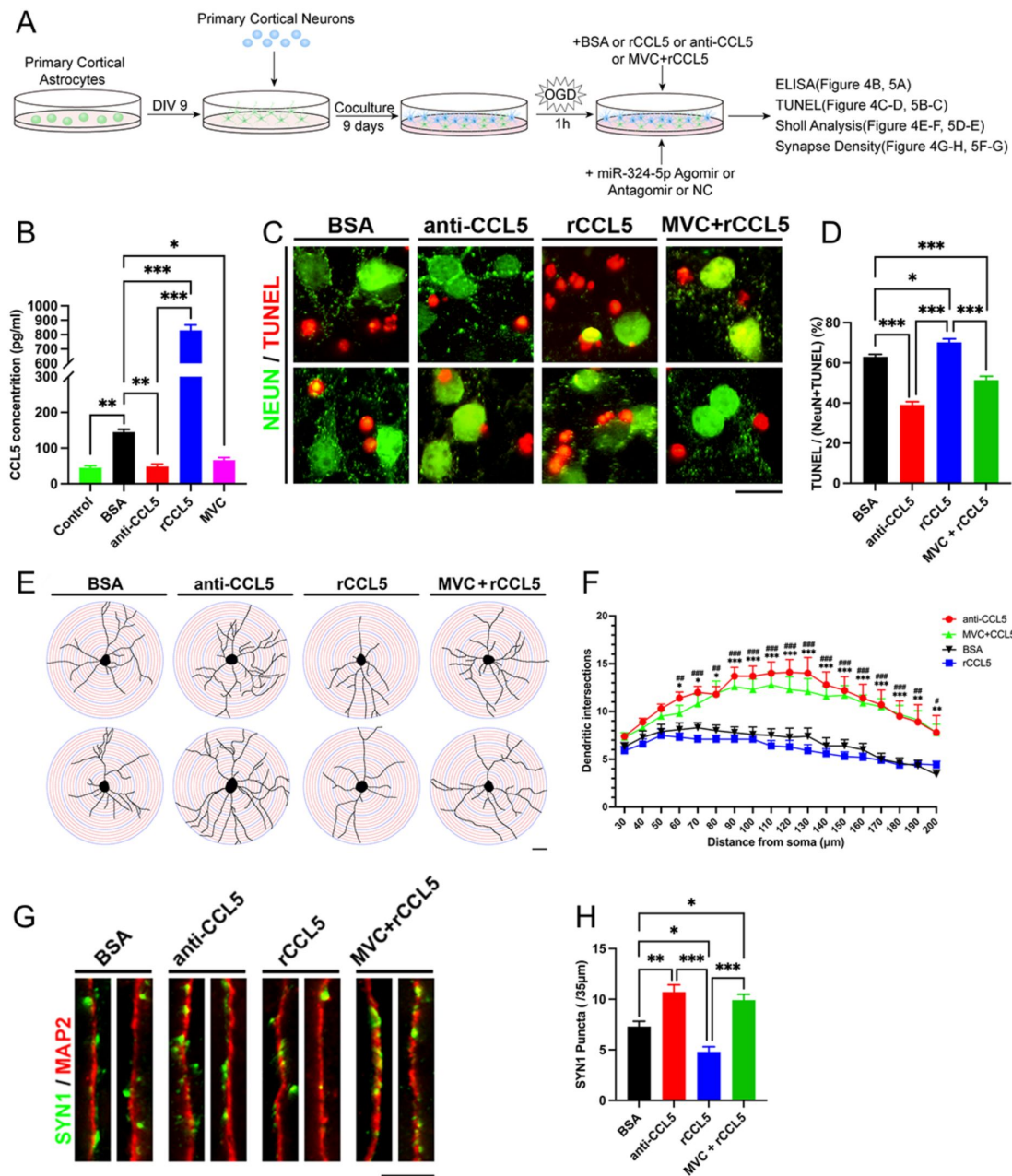


Figure 4.

Administration of CCL5 antibody alleviated OGD injury in cortical neurons co-cultured with astrocytes. (A) Illustration of astrocyte-neuron co-culture experimental protocol. Primary cortical astrocytes and neurons were isolated and co-cultured. After OGD treatment, BSA, CCL5 antibody, rCCL5, or MVC+rCCL5 was added to the culture medium. Alternatively, NC agomir, miR-324-5p agomir, or miR-324-5p antagomir was added to the culture medium post-OGD treatment. Samples were collected at D3 and/or D6 after OGD. (B) ELISA analysis of CCL5 concentration in the culture medium of BSA, CCL5 antibody, rCCL5, and MVC+rCCL5 group co-cultured cells on D3 after OGD, and in the culture medium of un-OGD-treated control group (n=5). (C and D) Representative images of NeuN immunofluorescence and TUNEL staining (C), and the ratio of apoptotic neurons (D) in BSA, CCL5 antibody, rCCL5, and MVC+rCCL5 group co-cultured cells on D3 after OGD (n=10). (E and F) Representative schematic (E) and Sholl analysis (F) of dendritic branch structure in BSA, CCL5 antibody, rCCL5, and MVC+rCCL5 group co-cultured cells on D6 after OGD (n=10). (G and H) Representative images of immunofluorescent double labeling of MAP2 and SYN1 (G) and quantification of the synapse puncta density (H) in BSA, CCL5 antibody, rCCL5, and MVC+rCCL5 group co-cultured neurons on D6 after OGD (n=10); * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by one-way ANOVA with Tukey's post-hoc test. In F, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. BSA group, # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. rCCL5 group by two-way ANOVA with Tukey's post-hoc test.

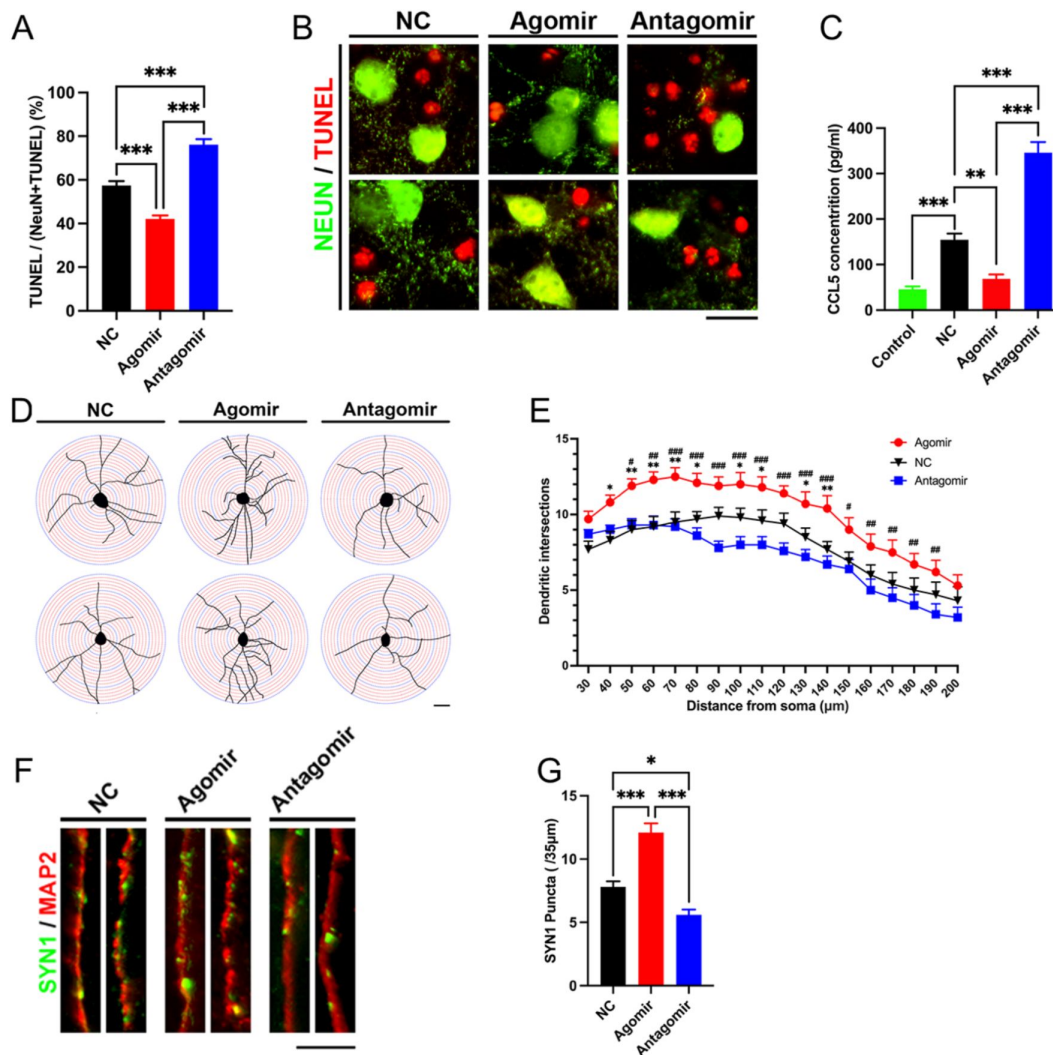


Figure 5.

Administration of miR-324-5p agomir alleviated OGD injury in cortical neurons co-cultured with astrocytes. (A) ELISA analysis of CCL5 concentration in the culture medium of NC agomir, miR-324-5p agomir and miR-324-5p antagomir group co-cultured cells on D3 after OGD, and in the culture medium of un-OGD-treated control group (n=5). (B and C) Representative images of NeuN immunofluorescence and TUNEL staining (B), and the ratio of apoptotic neurons (C) in NC agomir, miR-324-5p agomir and miR-324-5p antagomir group co-cultured cells on D3 after OGD (n=10). (D and E) Representative schematic (D) and Sholl analysis (E) of dendritic branch structure in NC agomir, miR-324-5p agomir and miR-324-5p antagomir group on D6 after OGD (n=10). (F and G) Representative images of immunofluorescent double labeling of MAP2 and SYN1 (F) and quantification of the synapse puncta density (G) in NC agomir, miR-324-5p agomir and miR-324-5p antagomir group neurons on D6 after OGD (n=10); * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by one-way ANOVA with Tukey's post-hoc test. In E, * $p < 0.05$ and ** $p < 0.01$ vs. NC agomir group, # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. miR-324-5p antagomir group by two-way ANOVA with Tukey's post-hoc test.

that upregulating miR-324-5p expression in the co-culture system reduce neuronal apoptosis after OGD, thereby exerting a protective effect on neuronal survival. Conversely, suppressing miR-324-5p expression exacerbates neuronal apoptosis following OGD injury.

Sholl analysis demonstrated that on D3 and D6 post-OGD, dendritic arbor complexity in the agomir-treated group was significantly greater than in both the NC group and antagomir groups (**Figure 5D-E**, Supplementary Figure S3C). Specifically, on D6, neurons in the agomir group exhibited significantly more dendritic branches within distances of 40µm-80µm, 100µm-110µm, and 130µm-140µm from the soma compared to those in the NC group. Moreover, within the 50µm-190µm range from the soma, the agomir group neurons displayed significantly more dendritic branches than those in the antagomir group. No significant differences were detected between the antagomir and NC groups. These findings indicate that miR-324-5p upregulation significantly preserves neuronal dendritic structures after OGD injury, whereas its downregulation simplifies dendritic structure, potentially impeding functional recovery of neurons after OGD treatment.

MAP2/SYN1 staining results revealed that at D6 post-OGD, synaptic densities in the agomir group were significantly higher than that in both the NC group and antagomir groups (**Figure 5F-G**). Conversely, neurons in the antagomir group exhibited significantly reduced synaptic densities compared to the NC group. These findings indicate that enhancing miR-324-5p expression in the co-culture system significantly preserves synaptic density post-OGD, while its inhibition leads to greater synaptic loss.

To assess the impact of regulating the miR-324-5p/CCL5 axis on intracellular signaling pathways in the cortical region after ischemic injury, we collected total proteins from the IC, IP, CC, and CP regions of MCAO mice injected with BSA, CCL5 antibody, rCCL5, or MVC (**Figure 6A**). SDS-PAGE electrophoresis and Western blot analysis revealed that on D3 post-MCAO, the levels of activated ERK, as indicated by the phosphorylated ERK (p-ERK) to total ERK (t-ERK) ratio, were significantly lower in the ipsilateral cortex (IC and IP regions) compared to the contralateral cortex (CC and CP regions; not shown in the figure) in the BSA control group. In the anti-CCL5 group, p-ERK ratios were significantly higher than that in the BSA control group in the ipsilateral cortex, and approached levels similar to those in the contralateral cortex (**Figure 6B**). Following MVC injection, an increase in the p-ERK ratio in the ipsilateral cortex was observed, with no significant differences in the contralateral region. In the rCCL5 group, p-ERK ratio showed a significant increase in the IP region, with no statistical differences observed in the remaining cortical regions compared with the BSA control.

In the BSA group, no significant differences in phosphorylated CREB (p-CREB) to total CREB (t-CREB) ratios were observed among different cortical regions at D3 post-injury (not shown in the figure). In the anti-CCL5 group, levels of activated CREB in the ipsilateral cortex were significantly higher compared to the BSA control group, while no significant differences in activated CREB levels were observed between the two groups in the contralateral cortex (**Figure 6C**). Following MVC injection, significant upregulation of activated CREB levels were observed in the IC region, whereas no significant differences were observed in the remaining parts of the cortex. In the rCCL5 group, p-CREB ratios were significantly lower in the IP, CC and CP regions compared to the BSA control group. These results indicate the downregulated ERK pathway in the ipsilateral cortex following ischemic stroke. Inhibiting CCL5 expression or blocking CCR5 receptor could restore the ERK/CREB regulatory pathway within the ipsilateral cortex of MCAO mice, potentially exerting a protective effect on the structure and function of neurons following ischemic injury.

Brain tissue protein is comprised of proteins from various cell types, including astrocytes, neurons, microglia, and endothelial cells. To investigate the impact of regulating astrocytic miR-324-5p/CCL5 axis on the intracellular signaling pathways of neurons following ischemic injury, we isolated primary cortical neurons and co-cultured them with ACM. After OGD treatment, neurons were co-cultured with OGD-ACM for three days, followed by supplementation with BSA, rCCL5,

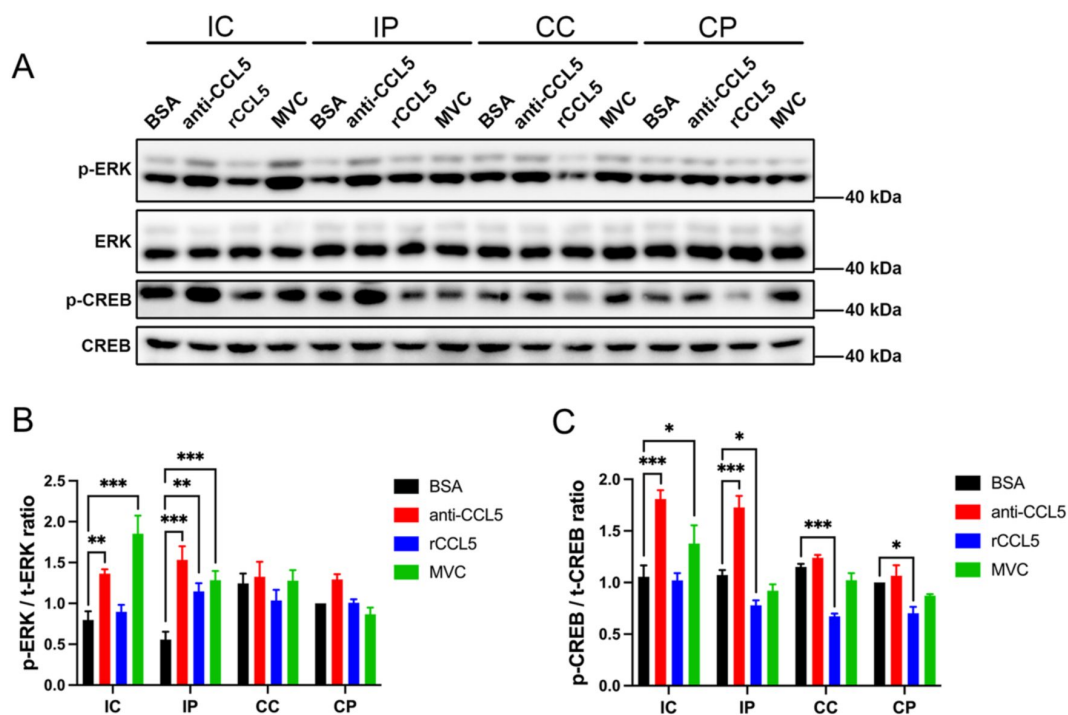


Figure 6.

Intracerebral CCL5 antibody delivery enhanced the ERK/CREB pathway in the ipsilateral cortex of MCAO mice. (A) Representative Western blot of p-ERK, ERK, p-CREB, and CREB expression in the IC, IP, CC, and CP cortical proteins from BSA, CCL5 antibody, rCCL5, and MVC treated mice on D7 after MCAO (n=5). (B and C) The expression ratio of p-ERK to ERK (B), and p-CREB to CREB (C) in the IC, IP, CC, and CP cortical proteins from BSA, CCL5 antibody, rCCL5, and MVC treated mice on D7 after MCAO (n=5). All protein expression levels were normalized to the expression in the CP region; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by two-way ANOVA with Tukey's post-hoc test.

CCL5 antibody, or a combination of MVC and rCCL5 in the neuronal cultural media (**Figure 7A** [↗](#)). Neuronal proteins were collected, and changes in the expression of the ERK/CREB pathway were examined. As shown in **Figure 7B-D** [↗](#), primary cortical neurons co-cultured with OGD-ACM exhibited significantly decreased levels of activated ERK and CREB after treated with rCCL5 for 10-60 minutes on D3 post-OGD. Conversely, the addition of CCL5 antibody or the simultaneous addition of 5nM MVC and rCCL5 led to increased levels of activated ERK and CREB in neurons. These results suggest that post-OGD, CCL5 inhibits neuronal ERK/CREB pathway via the CCR5 receptor. Decreasing CCL5 expression or blocking neuronal CCR5 receptor both restored the ERK/CREB pathway in neurons post-OGD injury.

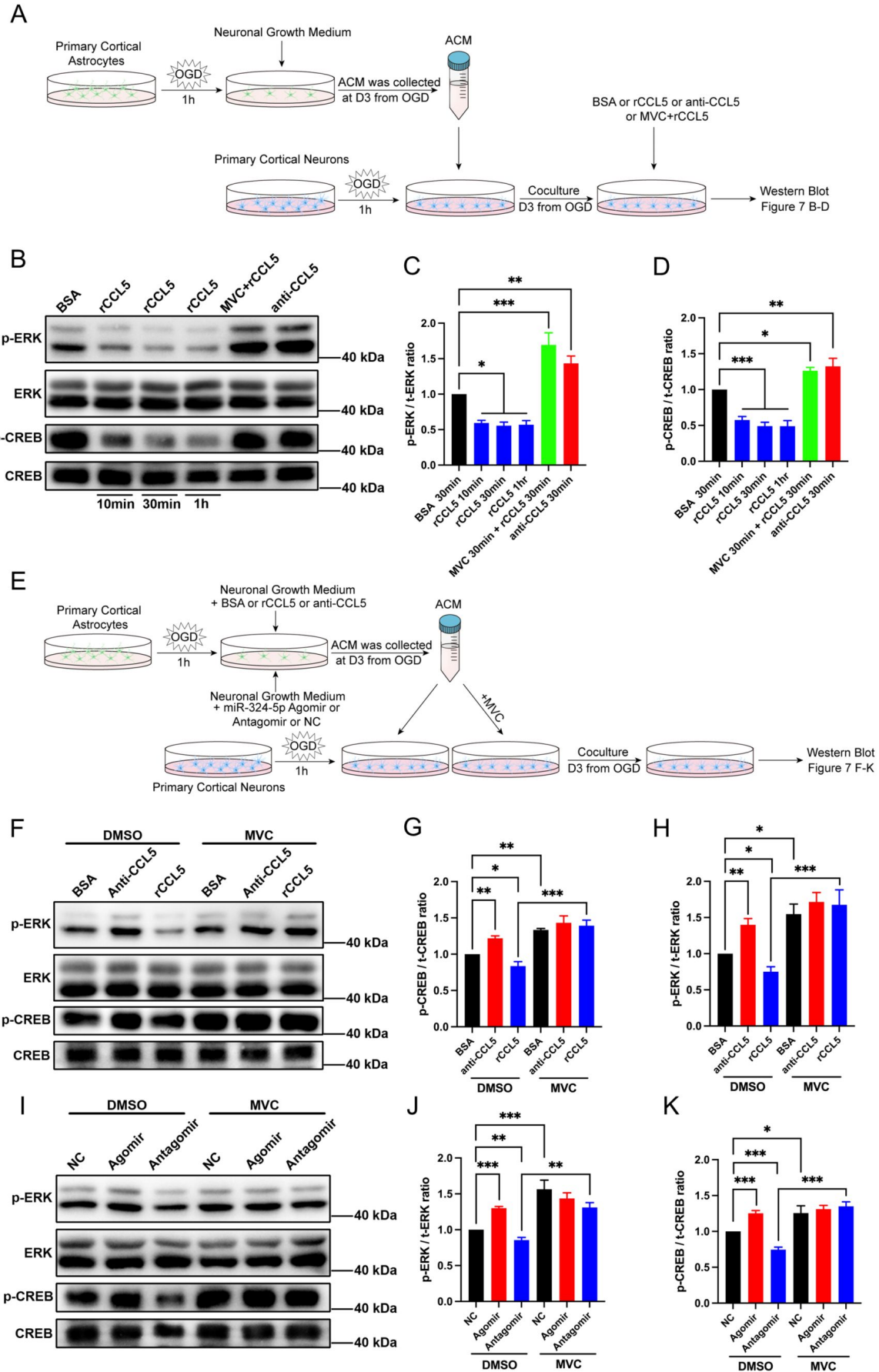


Figure 7.

Administration of miR-324-5p, CCL5 antibody and MVC enhanced neuronal ERK/CREB pathway after OGD injury. (A) Illustration of ACM-neuron co-culture experimental protocol for [Figure 7B-D](#). After OGD treatment, primary cortical neurons were co-cultured with OGD-ACM for 3 days, then BSA, CCL5 antibody, rCCL5, or MVC+rCCL5 was supplemented in the culture medium before neuronal protein was collected. (B) Representative Western blot of p-ERK, ERK, p-CREB, and CREB expression in the BSA, CCL5 antibody, rCCL5, and MVC+rCCL5 treated neuronal proteins. (C and D) The expression ratio of p-ERK to ERK (C), and p-CREB to CREB (D) in the BSA, CCL5 antibody, rCCL5, and MVC+rCCL5 treated neuronal proteins (n=6). (E) Illustration of ACM-neuron co-culture experimental protocol for [Figure 7F-K](#). After OGD treatment, primary cortical neurons were co-cultured with BSA OGD-ACM, anti-CCL5 OGD-ACM, or rCCL5 OGD-ACM for 3 days, with MVC or DMSO added to the medium. (F) Representative Western blot of p-ERK, ERK, p-CREB, and CREB expression in neurons co-cultured with BSA OGD-ACM, anti-CCL5 OGD-ACM, and rCCL5 OGD-ACM. (G and H) The protein expression ratio of p-ERK to ERK (G), and p-CREB to CREB (H) in neurons co-cultured with BSA OGD-ACM, anti-CCL5 OGD-ACM, and rCCL5 OGD-ACM (n=5). (I) Representative Western blot of p-ERK, ERK, p-CREB, and CREB expression in neurons co-cultured with NC agomir OGD-ACM, miR-324-5p agomir OGD-ACM, and miR-324-5p antagomir OGD-ACM. (J and K) The protein expression ratio of p-ERK to ERK (J), and p-CREB to CREB (K) in neurons co-cultured with NC agomir OGD-ACM, miR-324-5p agomir OGD-ACM, and miR-324-5p antagomir OGD-ACM (n=5). Protein expression levels were normalized to the BSA group in C and D, to the BSA group added with DMSO in G and H, to the NC agomir group added with DMSO in J and K. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ by one-way ANOVA with Tukey's post-hoc test. In C and D, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ by two-way ANOVA with Tukey's post-hoc test.

After OGD treatment of primary cortical astrocytes, the culture medium was supplemented with BSA, CCL5 antibody, or rCCL5. The respective OGD-ACMs (BSA OGD-ACM, anti-CCL5 OGD-ACM, and rCCL5 OGD-ACM) were collected, and OGD-treated neurons were co-cultured with these OGD-ACMs for three days ([Figure 7E](#)). Subsequent Western blot results showed significantly increased levels of activated ERK and CREB in neurons co-cultured with anti-CCL5 OGD-ACM compared to the BSA OGD-ACM control group ([Figure 7F-H](#)). Conversely, decreased levels of activated ERK and CREB were observed in neurons co-cultured with rCCL5 OGD-ACM.

Furthermore, when MVC was added to the ACM-neuron co-culture systems at a final concentration of 5nM in each group immediately post-OGD, Western blot results revealed significantly upregulated ERK/CREB pathway in neurons co-cultured with both BSA OGD-ACM and rCCL5 OGD-ACM compared to the corresponding groups without MVC treatment. These results further indicate that both inhibiting CCL5 expression and blocking the CCR5 receptor could significantly enhance the ERK/CREB pathway in neurons post-OGD.

After OGD treatment, primary cortical astrocytes were transfected with NC agomir, miR-324-5p agomir, or miR-324-5p antagomir, respectively. The respective OGD-ACMs (NC OGD-ACM, agomir OGD-ACM, and antagomir OGD-ACM) were collected, and OGD-treated neurons were co-cultured with these OGD-ACMs for three days. Western blot results showed significantly increased levels of activated ERK and CREB in neurons co-cultured with agomir OGD-ACM compared to the NC OGD-ACM group ([Figure 7I-K](#)). Additionally, decreased levels of activated ERK and CREB were detected in antagomir OGD-ACM co-cultured neurons.

Following MVC treatment, significant upregulation of the ERK/CREB pathway was observed in neurons co-cultured with NC OGD-ACM and antagomir OGD-ACM, compared to the corresponding groups without MVC treatment. Moreover, no statistical differences were detected in the activated ERK and CREB levels among neurons co-cultured with agomir OGD-ACM, either with or without MVC addition, and neurons co-cultured with NC OGD-ACM with MVC addition (not shown in the figure). This indicated that astrocytic miR-324-5p primarily affects co-cultured neuronal ERK/CREB pathway through influencing the CCR5 receptor. Taken together, these results indicate that

overexpressing miR-324-5p in astrocytes can significantly upregulate the neuronal ERK/CREB pathway via CCR5 receptor post-OGD, whereas inhibiting astrocytic miR-324-5p decreased the neuronal ERK/CREB pathway (**Figure 8** [↗](#)).

Discussion

The main findings of this study are: 1. Following an ischemic stroke, Ccl5 mRNA expression increased from D1 to D7, while miR-324-5p greatly reduced after D1 in the ipsilateral cortex of MCAO mice; 2. Inhibiting CCL5 expression through intracerebral CCL5 antibody or miR-324-5p delivery reduced the infarct area, protected neurological functions, suppressed activation of astrocytes and microglia, and preserved both dendritic field complexity and dendritic spine density in MCAO mice; 3. In the astrocyte-neuron co-culture system, inhibiting astrocytic CCL5 expression with CCL5 antibody or miR-324-5p administration after OGD treatment decreased neuronal apoptosis and preserved dendritic field structure and synapse density; 4. Inhibiting CCL5 expression by intracerebral CCL5 antibody delivery led to upregulated the ERK/CREB pathway in the ipsilateral cortex of MCAO mice; 5. Inhibiting astrocytic CCL5 expression by CCL5 antibody or miR-324-5p administration upregulated the ERK/CREB pathway in ACM-cocultured neurons after OGD treatment; 6. MVC application reduced infarct area in MCAO mice, decreased neuronal apoptosis, preserved dendritic field structure and synapse density in OGD-treated neurons in vitro; 7. MVC application upregulated the ERK/CREB pathway in ACM-cocultured neurons after OGD treatment.

Our study reinforces the view that CCL5 knockdown in experimental stroke mice reduced infarct size, improved neurological functions, suppressed glial activation, and preserved both dendritic field complexity and dendritic spine density after stroke. Ccl5 mRNA expression in the ipsilateral cortex after ischemic stroke was elevated from D1 to D7 (**Figure 1C** [↗](#)). Besides, CCL5 protein concentrations in the IP regions were increased from D3 to D7 (**Figure 2A** [↗](#)). CCL5 knockdown revealed reduced infarct size, improved neurological scores and elevated motor functions in MCAO mice (**Figure 2B-E** [↗](#)). While CCL5 overexpressed MCAO mice showed exacerbated infarct size (**Figure 2B-C** [↗](#)). Decreased astrocyte cell area and microglia cell area were detected in CCL5 knockdown MCAO mice, while CCL5 overexpression increased microglia cell area at D3 post stroke (**Figure 2G-J** [↗](#)). Furthermore, increased dendritic field complexity and dendritic spine density were found after CCL5 knockdown in MCAO mice, while CCL5 overexpression showed decreased dendritic field complexity.

Besides, rCCL5 injection within 24hr after MCAO surgery caused elevated death rate from D1 to D3. Severe infarct size and excessive glia activation was detected in these mice (data not shown), emphasizing that CCL5 concentration should be seriously restricted after ischemic stroke.

In our previous work, we verified astrocytic miR-324-5p downregulated Ccl5 mRNA level ²¹[↗](#). Therefore, we detected whether overexpressing miR-324-5p in ipsilateral cortex could mimic the effect of CCL5 knockdown in MCAO mice. Indeed, miR-324-5p agomir reduced CCL5 protein concentration, while miR-324-5p antagomir elevated CCL5 concentration in ipsilateral cortex after ischemic stroke (**Figure 3A** [↗](#)). MiR-324-5p agomir application reduced infarct size, improved Longa scores and motor functions, while the same dose of miR-324-5p antagomir increased infarct size (**Figure 3B-E** [↗](#)). Both the CCL5 antibody and the miR-324-5p agomir reduced CCL5 expression in the ipsilateral cortex and improved motor functions in MCAO mice. When comparing these two groups, administration of the CCL5 antibody significantly decreased mortality rates after MCAO (data not shown). Furthermore, the CCL5 antibody group exhibited notably better neurological scores before D3, although by D7, both groups showed comparable scores (**Figures 2D** [↗](#), **3D** [↗](#)). Besides, the Rotarod test revealed better motor abilities in the CCL5 antibody group compared to the miR-324-5p agomir group at D3 and D7 post-MCAO (**Figure 2E** [↗](#), **3E** [↗](#)). These differences may be attributed to the immediate reduction of resident CCL5 by the antibody, whereas the miRNA

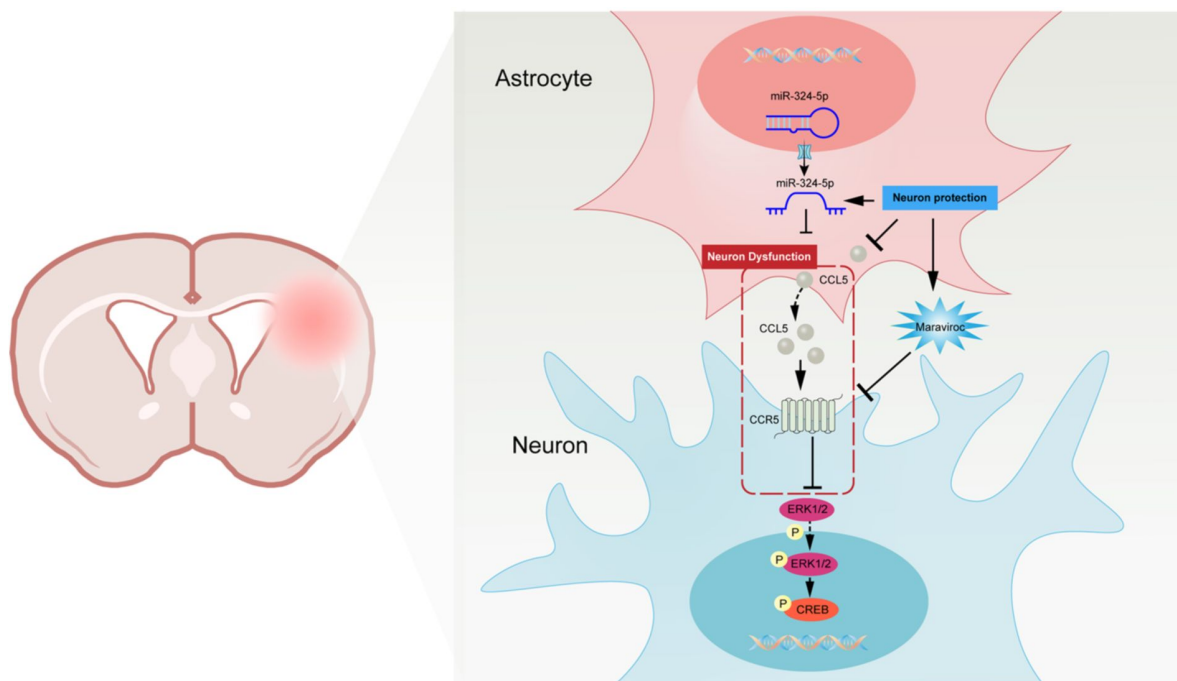


Figure 8.

Proposed model of the neuroprotective role of astrocytic miR-324-5p by inhibiting CCL5 expression and upregulating neuronal ERK/CREB pathway after ischemic stroke.

agomir inhibits subsequent CCL5 expression post-transcriptionally after transfection. Therefore, reducing CCL5 levels early after a stroke could be more beneficial in minimizing infarct size and enhancing motor functional recovery.

Decreased activated astrocyte density and cell area were found in miR-324-5p overexpressed mice from D3 to D7 (**Figure 3G-J**). In contrast, miR-324-5p antagomir induced excessive astrocyte activation in the early stage post stroke (**Figure 3G**). Moreover, miR-324-5p agomir kept cortical microglia at less-activated state in the early stage following stroke, as indicated by the reduced microglial surface area in **Figure 3K**.

The acute inflammatory response to stroke constitutes complex reactions to sterile tissue injury, which is essential for initiating processes that facilitate the clearance of damaged tissue and create an optimal environment for subsequent tissue repair⁴⁴. However, excessive inflammatory activation can lead to the overproduction of detrimental agents, including cytokines, chemokines, and reactive oxygen species (ROS). This inflammatory cascade following stroke may compromise vascular integrity, promote cellular apoptosis, and result in secondary brain damage, thereby exacerbating cerebral lesions⁴⁵.

Microglia, the resident immune cells of the brain, become rapidly activated following stroke, exhibiting both pro-inflammatory and anti-inflammatory roles at different stages of ischemic stroke⁴⁶. Initially, during the acute phase, activated microglia release a range of inflammatory mediators such as TNF, IFN- β , and IL-6, which contribute to a robust inflammatory response that impedes neural recovery⁷. Subsequently, in the subacute phase, microglia shift towards a protective role by secreting cytokines like neurotrophic factor IGF1, which aids in neural repair and remodeling following ischemic injury⁴⁷.

Moreover, activated microglia stimulate the formation of A1 astrocytes through the secretion of IL-1 α , TNF- α , and C1q⁴⁸. These A1 astrocytes then release pro-inflammatory factors such as IL-1 β , TNF- α , and NO, which exacerbate the neuroinflammatory response⁴⁹. Additionally, astrocytes are involved in reactive gliosis and the formation of glial scars post-stroke, which can hinder axon regeneration and neurological recovery⁴⁸. In contrast, A2 astrocytes exhibit anti-inflammatory properties and contribute to neuroprotection in MCAO mice⁵⁰. Therefore, astrocytes demonstrate a complex duality in stroke response, exhibiting both detrimental and beneficial effects through their distinct A1 and A2 phenotypes.

Our data revealed that both overexpression miR-324-5p and knockdown CCL5 could reduce activated astrocyte and microglia surface area after stroke. It is well-known that morphological features and function are tightly coupled in astrocyte and microglia^{51,52}. Astrocytic reactivity is characterized by the profound expression of GFAP and extending processed to the infarct region to form glia scar. Microglial reactivity encompasses their transformation from ramified to amoeboid morphology⁵³. The decreased glial cell size reflected the “low” activation status of these cells, and the alleviated inflammation state in the cortex. In comparison of miR-324-5p agomir mice and anti-CCL5 mice, miR-324-5p agomir application not only reduced activated astrocyte cell area, but also reduced activated astrocyte density. This difference perhaps resulted from miR-324-5p agomir could inhibit CCL5 expression persistently during the experimental period, and/or inhibit other genes coordinated with CCL5 to minimize activated astrocytes in the ischemic cortex.

It has been reported that the administration of exogenous RNA can induce activation of resident microglia in the cortex⁵⁴. Microglia density did not show a significant change in the anti-CCL5 group and in the agomir group compared with their respective controls (Supplementary Figures S1C-D, S2A-B). However, when comparing these two groups together, application of the CCL5 antibody resulted in reduced microglia cell area and density at D7 compared to the miR-324-5p agomir group (**Figures 2J**, 3L; Supplementary Figures S1D, S2B). This reduction was likely

caused by the excessive stimulation of microglia by the exogenous RNAs, and/or as a result of other genes targeted by miR-324-5p in the ischemic cortex. In summary, both CCL5 antibody and miR-324-5p agomir reduce glial activation following ischemic stroke. Specifically, miR-324-5p agomir administration exhibited a more pronounced inhibitory effect on astrocyte activation, whereas CCL5 antibody administration more effectively alleviated microglia activation.

Numerous studies have highlighted the correlation between neural network reorganization in the peri-infarct cortex and functional improvement in both stroke patients and in experimental stroke model ^{55,57}. In contrast, the increased neurodegeneration together with the reduced dendritic density and synaptic density may exacerbate brain damage and compromise neurological function in the subacute phase of brain injury ⁵⁸. In our study, injections of rCCL5 or miR-324-5p antagomir simplified the dendritic branch structure, although neither the total spine density nor the mushroom spine density showed statistical significance compared with the respective control mice (**Figures 2K-O**, **3M-Q**). Conversely, both miR-324-5p agomir and CCL5 antibody application significantly preserved dendritic field complexity and dendritic spine density after ischemic stroke on D3 and D7. This preservation of neuronal structure likely contributed to the reduced neurological deficits and infarct size observed in these group of mice.

Activation of CCR5 inhibits hippocampal neuron long-term potentiation and modulates MAPKs and CREB signaling ^{59,60}. In the HIV induced dementia mice model, neuronal CCR5 downregulation enhanced cortical neuron plasticity and facilitated recovery of learning and memory by elevating CREB protein levels ⁶⁰. Post-stroke knockdown of CCR5 in the premotor cortex facilitates motor control recovery, associated with dendritic spine preservation, new patterns of cortical projections to the contralateral premotor cortex, and enhanced CREB and DLK signaling ³⁹. Additionally, a loss-of-function CCR5 mutation in human patients improves stroke recovery on distinct measures of cognitive, motor, and sensory function that includes memory, verbal functioning, and attention ³⁹. Moreover, CCR5 blockade following stroke improved axon mapping and synaptic density, while attenuating reactive gliosis and peripheral immune cell infiltration ⁵⁸. Besides, activated CCR5 could induce neutrophil migration through the Akt/GSK-3 β pathway, aggravating neuroinflammation in the acute stroke stage ⁶¹. Blockade of CCR5 notably reduced the infiltration of peripheral immune cells and inhibited the reactive proliferation of glial cells after stroke ⁵⁸.

Maraviroc (MVC, APEXBIO, UK-427857), a CCR5 receptor antagonist widely used clinically, demonstrates safety and the ability to penetrate the blood-brain barrier ⁶². MVC inhibits CCR5 activation, thereby impeding inflammatory chemokine recruitment of immune cells and local inflammation exacerbation ^{63,64}. In ischemic stroke, MVC treatment attenuates the expression of pro-inflammatory factors IL-1 β , IL-6, and TNF- α , suggesting its function of inhibiting local nerve injury post-ischemia ⁵⁸. Our research further indicates that MVC reduces CCL5 expression in astrocytes following oxygen-glucose deprivation (OGD) treatment (**Figure 4B**). As CCL5 is pro-inflammatory factor and knockdown CCL5 downregulated astrocyte and microglia reactivity in MCAO mice, MVC could exert anti-inflammatory activity by inhibiting CCL5 production.

As reported, MVC has been observed to promote sustained motor recovery and reduce infarct volumes following MCAO ^{65,66}. MVC exerts these neuroprotective effects by inhibiting neuronal apoptosis, evidenced by increased Bcl-2 expression and decreased BAX levels after ischemic injury ⁶⁵. Consistent with previous studies, TUNEL staining confirmed that MVC treatment inhibited neuronal apoptosis after OGD (**Figure 4B-C**). Furthermore, MVC treatment in MCAO mice reduced infarct volume (**Figures 2B-C**), suggesting that blocking the neuronal CCR5 receptor benefits neuronal survival during ischemic stroke.

Both reducing CCL5 concentration by CCL5 antibody, and blocking CCR5 receptor by MVC preserved neuronal dendritic structure and synaptic density following OGD injury (**Figure 4E-H**). Besides, although CCL5 can active CCR1, CCR3 and CCR5, which are all expressed on

neurons, Sholl analysis and synapsin puncta showed no statistic differences between the two groups. This suggests that astrocyte-secreted CCL5 probably predominantly activates the CCR5 receptor to influence dendrite morphology and synapse density in OGD neurons. The addition of up to 20 ng/ml rCCL5 did not result in simplified dendrite structure or decreased synapse density after OGD treatment, indicating that astrocyte-secreted CCL5 after OGD may have already reached its maximum effect on inhibiting dendritic structure and synaptic density in neurons.

Transfection with miR-324-5p agomir reduced CCL5 concentration in the astrocyte-neuron coculture system, whereas miR-324-5p antagomir increased CCL5 concentration (**Figure 5A**). Besides, the transfected cells were predominantly astrocytes (Supplementary S3B). MiR-324-5p agomir could mimic the effects of CCL5 antibody in inhibiting neuronal apoptosis and in preserving dendritic arbor organization and synapse density (**Figure 5B-G**). In contrast, miR-324-5p antagomir exacerbated neuronal apoptosis and synapse loss after OGD injury. Together with previously mentioned finding that in vivo miR-324-5p agomir administration reduced infarct volume and improved neurological function in MCAO mice, our research suggests that inhibiting astrocytic CCL5 expression by miR-324-5p plays a neuroprotective role in ischemic stroke injury.

In neurodevelopment, the ERK/MAPK pathway plays a critical role in various processes including neuronal proliferation, synaptic growth, gliogenesis, and cerebral cortex development⁶⁷. Following ischemic stroke, activation of the ERK signaling pathway confers neuroprotection, enhances neuronal plasticity and migration, thereby significantly contributing to neural remodeling and repair⁶⁸. Besides, the ERK/MAPK pathway is crucial in modulating angiogenesis in brain microvascular endothelial cells, which potentially facilitates neuroprotection and neurological repair post-stroke⁶⁹.

Activation of the Gai pathway of CCR5 leads to the downregulation of intracellular cAMP and phosphorylated CREB in neurons^{60,70,71}. CREB plays a key role in enhancing neuronal excitability and long-term synaptic plasticity⁷²⁻⁷⁵. Furthermore, CREB serves as a central molecular node in the post-stroke response circuits that contribute to motor deficit recovery⁷⁶. Peak expression of p-CREB and CREB-induced gene expression occurs within the first two days following stroke⁷⁷, although CREB activation persists in glial cells for weeks post-stroke, playing roles in both neurogenesis and gliogenesis^{78,79}. Additionally, CREB transfection enhances the remapping of injured somatosensory and motor circuits and facilitates the formation of new connections within these circuits post-stroke. Consequently, increasing CREB levels in motor neurons enhances motor recovery, while blocking CREB signaling prevents stroke recovery⁷⁶.

In our study, levels of p-ERK and p-CREB increased in the ischemic cortex after CCL5 antibody or MVC treatment in MCAO mice (**Figure 6A-C**). To specifically examine the ERK/CREB pathway in neurons, primary cortical neurons were isolated and cultured with OGD-ACM after OGD treatment. Application of rCCL5 downregulated p-ERK and p-CREB in post-OGD neurons, whereas pre-treatment with MVC blocked the effects of rCCL5, resulting in elevated levels of neuronal p-ERK and p-CREB (**Figure 7B-D**, **7F-H**). Addition of CCL5 antibody also upregulated the ERK/CREB pathway in neurons cocultured with OGD-ACM. Collectively, these findings suggest that astrocytes upregulate and release CCL5 following OGD treatment, which inhibits the neuronal ERK/CREB pathway through the CCR5 receptor. This inhibition can be blocked by the CCR5 antagonist MVC. Knockdown of astrocytic CCL5 consequently upregulated the neuronal ERK/CREB pathway, potentially elucidating the molecular mechanism underlying the neuroprotective effects of CCL5 antibody in ischemic stroke mice.

Furthermore, application of MVC increased the ERK/CREB pathway in neurons co-cultured with miR-324-5p antagomir-treated OGD-ACM, but did not further elevate the pathway in neurons co-cultured with miR-324-5p agomir-treated OGD-ACM (**Figure 7I-K**). This indicated that astrocytic miR-324-5p modulated the neuronal ERK/CREB signaling pathway by suppressing CCL5 expression and inactivating the neuronal CCR5 receptor. In summary, our findings demonstrate the

neuroprotective effects of astrocytic miR-324-5p in preserving neuronal structure and enhancing motor recovery post-stroke by inhibiting CCL5 expression and subsequently upregulating neuronal ERK/CREB pathway through the CCR5 receptor (**Figure 8** [↗](#)).

Methods

All animal experiments were performed according to protocols approved by the Ethical Committee of the Experimental Animal Center of Shandong Second Medical University. The approval No. is 2020SDL077. All experiments were done according to institutional ethical guidelines on animal care.

Middle cerebral artery occlusion mouse model

To eliminate the influence of gender differences on MCAO surgery, male C57BL/6J mice (20-25 g) were purchased from the Experimental Animal Center of Shandong Second Medical University and housed in a controlled SPF level environment on a 12 hour light/dark cycle with free access to water and standard chow diet. Anesthesia was induced by isoflurane. Right common and external carotid artery were exposed and ligated. Embolus (Jialing, 1800AAA) was inserted through the common carotid artery into the internal carotid artery to a point approximately 18mm distal to the carotid bifurcation, thereby occluding the origin of middle cerebral artery. The occlusion was kept for 2 hours. Re-perfusion was achieved by carefully taking out the embolus to avoid bleeding. Then, the stitching and necessary sterilization were done. MCAO mice were evaluated for their neurological function following the Longa score system ⁴⁰ [↗](#) immediately post-surgery, with those exhibiting a score of 3 being randomly grouped for follow-up analysis. In sham group, mice received the same surgical procedure without the embolus insertion.

For intracerebral injections, mice were anesthetized with isoflurane and positioned in a stereotaxic apparatus. A volume of 2 μ l of either miRNA or protein was injected into the MCAO mouse brain using microsyringe (Hamilton, 65460-05) at a rate of 0.2 μ l/min. The injected miRNA was as follows: 200 μ M of negative control (NC) agomir (GenePharma), miR-324-5p agomir (GenePharma), or miR-324-5p antagomir (GenePharma). The concentration of injected protein was as follows: 20 ng/ μ l bovine serum albumin (BSA; Millipore), 2 μ g/ μ l CCL5 antibody (Roche), or 20 ng/ μ l recombinant mouse CCL5 (Absin). The injection site was at the following coordinates: 1.0 mm anterior to bregma, 1.0 mm lateral (right) to the sagittal suture, and 1.0-1.5 mm from the surface of the skull. After injection, the syringe was kept in the brain tissue for at least five minutes to achieve good absorption.

For intraperitoneal Maraviroc (MVC) injection, 1 ml of 5 μ M MVC(MCE, HY-13004) was injected at 4 hours, 24 hours, and 48 hours after MCAO surgery.

Behavioral testing

Neurological deficits were assessed and scored according to Longa's five-point scale method. Briefly, the following grading system was applied: grade 0, no observable neurological deficit; grade 1, failure to extend left forepaw fully; grade 2, circling to left; grade 3, falling to left; grade 4, unable to walk spontaneously. The average of neurological score for each group was used to express the severity of neurological deficits. The higher scores reflect the severer function deficits.

For the Rotarod test, mice were placed on an accelerating Rotarod cylinder, and the time the mice remained on the Rotarod was measured. The speed was slowly increased from 4 to 40 rpm within 5 minutes, then kept at 40 rpm for another 5 minutes. A trial ended if the animal fell off the rungs

or gripped the device and spun around for 2 consecutive revolutions without attempting to walk on the rungs. The animals were trained 3 days continuously before MCAO. The mean latency-to-fall (in seconds) on the device was recorded with 3 Rotarod measurements.

qRT-PCR analysis

Total RNA was prepared using RNAiso Plus (TaKaRa) according to the manufacturer's protocols. Reverse transcription of mRNA was performed using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa), and reverse transcription of miRNA was performed using the Mir-X miRNA First-Strand Synthesis Kit (TaKaRa). RT-PCR amplification of mRNA was performed using the TB Green Premix Ex Taq II (Takara), and RT-PCR amplification of miRNA was performed using the TB Green Advantage qPCR Premix (TaKaRa). mRNA levels and miRNA levels were normalized to Gapdh and U6, respectively. The sequences of the mRNA primers used were as follows: *Ccl5*, forward 5'-GGA GTA TTT CTA CAC CAG CAG CAA G-3' and reverse 5'-GGC TAG GAC TAG AGC AAG CAA TGA C-3'. *Gapdh*, forward 5'-GGT GAA GGT CGG TGT GAA CG-3' and reverse 5'-CTC GCT CCT GGA AGA TGG TG-3'. MiRNA-324-5p primers were purchased from Genecopoeia (Cat# MmiRQP0412). RT-PCR amplifications and real-time detection were performed using an Applied Biosystems 7500 Real-Time PCR System. All assays were performed in triplicate. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method ⁴¹ [41](#).

Immunofluorescence staining

35 μ m thick coronal brain sections or co-cultured cells on coverslips were fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized and blocked with 3% donkey serum in PBS containing 0.3% Triton X-100. Samples were then incubated with primary antibody at 4 °C overnight, followed by incubation with appropriate secondary antibody conjugated to Alexa Fluor (Molecular Probes). Primary antibodies used were mouse anti-GFAP (Cell Signaling Technology, 3670), rabbit anti-IBA1 (FUJIFILM, 019-19741), rabbit anti-MAP2 (Synaptic Systems, 188002), mouse anti-SYN1 (Synaptic Systems, 106011), and mouse anti-NEUN (Millipore, MAB377). Nuclei were counterstained with DAPI (Roche). Images were collected on Olympus BX53 microscope.

Golgi staining

Mice were sacrificed and whole brains were impregnated in the impregnation solution provided in the FD Rapid Golgi staining kit (FD Neurotechnologies) according to the manufacturer's instructions. Coronal sections of 160 μ m were prepared using a cryostat. After staining, images were captured on Leica confocal laser scanning SP5 microscope with bright field settings. For spine density analysis, segments of basal dendrites ranging from 25-100 μ m were randomly selected in either the peri-infarct cortex or the contralateral cortex. Based on the head diameter and spine length, dendritic spines were categorized into three types for quantification: mushroom, thin, and stubby ⁴² [42](#).

Cell culture

Primary astrocytes were prepared from cerebral cortices of newborn C57BL/6 mice according to previously published method ²¹ [21](#). To prepare astrocyte-neuron coculture coverslips, primary cortical astrocytes were passaged onto 12-well plates. When astrocytic bed reached 90% confluence, primary cortical neurons from newborn C57BL/6 mice were isolated and dissociated with papain as described previously ⁴³ [43](#). Neurons were plated as 5×10^5 cells/well. After 9 days of co-culture, OGD treatment was performed by placing the plates in 37 °C anaerobic chamber (1% O₂, 5% CO₂, 94% N₂) in glucose-free and serum-free medium for 1hr. After OGD, cells were cultured back in neuronal maintenance medium supplemented with proteins (20 ng/ml BSA, 200 ng/ml CCL5 antibody, or 20ng/ml recombinant mouse CCL5 in final concentration) or miRNAs (20nM NC agomir, miR-324-5p agomir or antagomir in final concentration), and incubated under normoxic condition. The cocultured coverslips were fixed with 4% PFA after 3 days or 6 days following OGD. To prepare astrocyte-conditioned medium (ACM), primary cortical astrocytes were

passaged onto T75 flask. After 1 hr OGD, culture medium was changed into neuronal maintenance medium (without B27, or glutamine) supplemented with proteins or miRNAs as mentioned above. ACM was collected after 3 days and 6 days, combined, centrifuged at 1000g for 5 minutes to remove cell debris, and stored at -80°C until further use. Control ACM was collected from astrocytes cultured under normal conditions without OGD.

To prepare neuronal protein samples, primary cortical neurons were plated onto 6-well plate at 3×10^6 cells/well. Cytosine arabinoside was added from DIV4 to limit glial proliferation. OGD treatment were performed at DIV9. Then medium was changed to 50% neuronal maintenance medium + 50% ACM. Neurons were lysed using RIPA buffer 3 days after OGD.

SDS-PAGE and Western blotting

Protein samples of brain tissue or cultured neurons were prepared using RIPA buffer (ThermoFisher Scientific) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (ThermoFisher Scientific). After measuring protein concentration with the BCA protein assay kit (Pierce), protein samples were loaded to 10% SDS-polyacrylamide gels. After separation, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked with 5% BSA and probed with primary antibodies (phospho-ERK1/2, Abcam, ab76299; ERK1/2, Abcam, ab184699; phospho-CREB, Abcam, ab32096; CREB, Cell Signaling Technology, 9197) at 4°C overnight. Membranes were then incubated with peroxidase-conjugated secondary antibody for 1 hour. Detection was performed using West Pico PLUS chemiluminescent substrate (ThermoFisher Scientific) and scanned with Clinx ChemiScope 6000 Imaging System. Densitometry analysis was done with ImageJ.

Statistical analyses

All data are expressed as mean $\pm$ SEM. Each experiment included at least three replicates per condition. All statistical analysis was performed using GraphPad Prism. $P < 0.05$ was considered statistically significant.

Declaration of competing interest

The authors declare no competing interests.

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Reviewer #1 (Public review):

Summary:

Here, the authors attempt to show that CCL5 is increased after stroke, possibly due to decreased miR-324, and that this is a modifiable system to decrease stroke damage. By bidirectionally manipulating CCL5 levels through direct injection of CCL5; a CCL5 blocking antibody; miR324; miR324 antagomir; or CCR5-blocking Maraviroc, they broadly show improvement with lower CCL5 levels. This includes infarct size, behavioral analysis, and immunohistochemical analysis of astrocytes, microglia, and neurons. They further try to mechanistically tie miR324 and CCL5 in astrocytes specifically to stroke-induced changes using a neuronal/astrocytic coculture system. They argue that decreasing CCL5 leads to increased ERK and CREB phosphorylation as a potential neuroprotective mechanism. CCL5 is one potential ligand for CCR5, and recent work identified CCR5 as a targetable mechanism by clinically-approved drug Maraviroc to enhance stroke recovery. Particularly given the high level of interest in CCR5 in stroke recovery, the focus on CCL5 - one of CCR5's potential ligands - and its miR regulation is an exciting expansion of this area of stroke biology.

Strengths:

The authors' findings that decreasing CCL5 acutely after stroke shows behavioral improvement appear robust. This broadly replicates work from other groups, although the finding that miR324 manipulation can phenocopy direct CCL5 manipulation is novel and intriguing. However, many of their other claims are difficult to evaluate based on a combination of missing methodological information, inappropriate statistical testing, and a flawed culture system.

Weaknesses:

Broadly speaking, the manuscript takes a zoomed-out view of what is fundamentally highly localized biology.

(1) miRNA-based regulation, by definition, has to include miR and mRNA in the same cell type; as the authors note, CCL5 is expressed in many cells. It is therefore impossible to propose any interaction on the basis of the tissue-level changes described; any evidence of in vivo cell-type specificity would dramatically improve the claims.

(2) The authors treat an extensive area of ipsilesional cortex uniformly as "IP". Astrocytic and microglial responses to localized injuries such as stroke are highly location-dependent and undoubtedly change dramatically within this area. The presented data cannot be interpreted without confirmation that these were taken at identical distances from the injury, and what that distance was. These do not appear to be adjacent to the injury, where the responses would presumably be the most informative. Similarly, it is difficult to interpret the neuronal Sholl and spine data without more information on where within the large IP region these neurons were found.

The authors attempt to narrow in on cell-type specificity via culture. However, astrocytes are notoriously prone to a dramatic change in culture and require careful methods (immunopanning; see eg doi: 10.1016/j.neuron.2011.07.022) to maintain much resemblance to their in vivo counterpart. It is difficult to conclude much about the role of astrocytes in the CCL5 pathway based on the use of this shaking-based culture system, particularly in the absence of cell-type specific validation in vivo.

There is missing methodological information, including infarct size measurements, TUNEL staining, and statistical testing. The TTC figures look very odd, like a collection of overlapping stars have been placed on the images rather than the natural relatively smooth infarct edges one would expect. It is unclear if the infarct volume measurements accounted for edema, as is standard; there is no description of the protocol used for quantification. It is also unclear if the infarct volume measurement comparisons were also done with t-tests vs ANOVA, as the statistical test used is not listed in the figure legends. In numerous cases where statistical testing is listed, repeated t-tests between subgroups are used vs the more appropriate ANOVA (assuming normality; nonparametric testing as appropriate), making it difficult to have confidence in the results.

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Reviewer #2 (Public review):

The authors presented evidence from various in vivo and in vitro experiments demonstrating the mutual interaction between CCL5 and astrocytic miR-342-5p in the ipsilateral core of cerebral ischemia. However, miR-342-5p was downregulated only late after MCAO (D3-7). Additionally, this downregulation was observed not only in the ipsilateral core but also in the ipsilateral penumbra and contralateral sides. Therefore, it is not convincing that the upregulation of CCL5 in the ipsilateral core at later time points (D3 and D7) is attributable to the decreased expression of miR-342-5p. In particular, infarct injury was already evident within a short time period (say 24 h) following MCAO.

(1) The temporal and spatial expression patterns of miR-324-5p do not match those of CCL-5, especially at D1 and D3 (see Figure 1C, 1D). Despite the inverse relationship between miR-324-5p and CCL-5 expression at D7 after MCAO, what was the purpose of administering miR-324-5p agomir (or antagomir) at D1 post-MCAO? If the connection cannot be clearly established, the conclusion reached at the end will be difficult to accept.

(2) Would administering miR-342-5p or anti-CCL5 at later time points (e.g., after D3) reduce infarct size or improve functional recovery? If this is not the case, the effect of CCL5 on neuronal cell damage (infarct size formation) must occur within a very short time after MCAO. Additionally, if the increased CCL5 expression is due to the downregulation of miR-342-5p, its impact would likely be less significant.

(3) While the study offers valuable insights into the roles of CCL5 and its connection with the regulation of miR-342-5p (though this connection is somewhat weak), it is recommended that the authors explore potential translational applications of these findings.

Overall, given the experimental designs and results, it is difficult to support the conclusions drawn in the manuscript.

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