Agonistic analog of growth hormone–releasing hormone promotes neurofunctional recovery and neural regeneration in ischemic stroke

Yueyang Liu[a,b,1], Jingyu Yang[b,1], Xiaohang Che[b,1], Jianhua Huang[c], Xianyang Zhang[d,e] Xiaoxiao Fu[b], Jialing Cai[b], Yang Yao[a], Haotian Zhang[b], Ruiping Cai[a], Xiaomin Su[a], Qian Xu[a], Fu Ren[f], Renzhi Cai[d,g], Andrew V. Schally[d,g,h,i,j,k,2], and Ming-Sheng Zhou[a,2]

[a]Department of Physiology, Shenyang Medical College, Shenyang 110034, China; [b]Department of Pharmacology, Shenyang Pharmaceutical University, Shenyang 110016, China; [c]The First Affiliated Hospital, Jinzhou Medical University, Jinzhou 121000, China; [d]Endocrine, Polypeptide, and Cancer Institute, Veterans Affairs Medical Center, Miami, FL 33125; [e]Interdisciplinary Stem Cell Institute, Miller School of Medicine, University of Miami, Miami, FL 33136; [f]Department of Anatomy, Shenyang Medical College, Shenyang 110034, China; [g]South Florida VA Foundation for Research and Education, Veterans Affairs Medical Center, Miami, FL 33125; [h]Division of Medical Oncology, Department of Medicine, Miller School of Medicine, University of Miami, Miami, FL 33136; [i]Division of Endocrinology, Diabetes & Metabolism, Department of Medicine, Miller School of Medicine, University of Miami, Miami, FL 33136; [j]Department of Pathology & Laboratory Medicine, Miller School of Medicine, University of Miami, Miami, FL 33136; and [k]Sylvester Comprehensive Cancer Center, Miller School of Medicine, University of Miami, Miami, FL 33136

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Ischemic stroke can induce neurogenesis. However, most stroke-generated newborn neurons cannot survive. It has been shown that MR-409, a potent synthetic agonistic analog of growth hormone–releasing hormone (GHRH), can protect against some life-threatening pathological conditions by promoting cell proliferation and survival. The present study shows that long-term treatment with MR-409 (5 or 10 μg/mouse/d) by subcutaneous (s.c.) injection significantly reduces the mortality, ischemic insult, and hippocampal atrophy, and improves neurological functional recovery in mice operated on for transient middle cerebral artery occlusion (tMCAO). Besides, MR-409 can stimulate endogenous neurogenesis and improve the tMCAO-induced loss of neuroplasticity. MR-409 also enhances the proliferation and inhibits apoptosis of neural stem cells treated with oxygen and glucose deprivation–reperfusion. The neuroprotective effects of MR-409 are closely related to the activation of AKT/CREB and BDNF/TrkB pathways. In conclusion, the present study demonstrates that GHRH agonist MR-409 has remarkable neuroprotective effects through enhancing endogenous neurogenesis in cerebral ischemic mice.

GHRH agonists | ischemic stroke | neural stem cells | neurogenesis | neuroplasticity

Stroke is a leading cause of mortality and permanent disability in adults worldwide (1). Although most pharmacological neuroprotectants have beneficial effects in experimental studies, they fail in clinical trials. The current therapies are inadequate to improve the sequelae of neuromotor dysfunction and clinical outcomes of ischemic stroke. The development of novel therapies for ischemic stroke is an urgent need and of top priority.

It has been demonstrated that ischemic stroke can markedly induce endogenous neurogenesis in the subventricular zone (SVZ) and subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. These areas are also most concentrated locations of neuronal precursor cells (NPCs) in the adult brain (2). The recovery of neurological function after cerebral ischemia mainly depends on the migration of newly formed neurons to severe ischemic lesions, such as the striatum and granular layer of the hippocampus, to replace necrotic neurons. However, only a very small portion of newly generated neurons can differentiate into mature neurons (2, 3). In addition to neurogenesis, the recovery of neurological function after a stroke depends on the neuroplasticity of the established networks in the ipsilateral tissue, including axonal sprouting, dendritic remodeling, and synapse strengthening (4, 5). Therefore, pharmacological neuroprotection and neuronal replacement by facilitating endogenous neurogenesis and neuroplasticity to maximize functional outcome are considered as promising strategies for the treatment of ischemic stroke.

Growth hormone–releasing hormone (GHRH) is a hypothalamic neuropeptide. Beside being expressed in the pituitary cells, GHRH and its receptors (GHRH-Rs) are also found in various extrapituitary cells or tissues, such as fibroblast, cardiomyocyte, ocular tissue, and mesenchymal stem cells (6–9). In the past few decades, many potent GHRH agonistic analogs of JI and MR class have been synthesized by our group (10, 11). Compared with native GHRH peptides, synthetic agonist of GHRH agonists have a long half-life and high stability and potency without the side effects of stimulating GH axis–induced tumor growth (12, 13). Notably, it has been demonstrated that MR-409 exhibits high

Significance

Stroke is a leading cause of mortality and permanent disability in adults worldwide. Although most pharmacological neuroprotectants have beneficial effects in experimental studies, they fail in clinical trials. Here, we report that MR-409, a synthetic analog of growth hormone–releasing hormone, can reduce mortality and improve neurological functional recovery in mice operated on for tMCAO. Mechanistically, MR-409 stimulates endogenous neurogenesis and improves tMCAO-induced loss of neuroplasticity by activation of AKT/CREB and BDNF/TrkB pathways. Our preclinical studies support the notion that MR-409 has remarkable neuroprotective effects through enhancing endogenous neurogenesis and may represent a potential new therapy for cerebral ischemic stroke.

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Competing interest statement: A.V.S. and R.C. are co-inventors on the patents for GHRH agonists, assigned to the University of Miami and Veterans Affairs Medical Center.

[1]Y.L., J.Y., and X.C. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: Andrew.Schally@va.gov or zhoums1963@163.com.

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potency on the activation of tissue repair and self-renewal and the promotion of cell proliferation and survival in various tissues by binding to GHRH-Rs. A large body of work has shown that MR-409 promotes the repair of cardiac tissue and function and reduces myocardial infarct size in experimental myocardial infarct in animal models (14). MR-409 also improves metabolic function and the survival of pancreatic islets after transplantation into experimental diabetic animals (15) and exerts neurovascular protective effects in diabetic retinopathy (16). These studies indicate that GHRH agonists, particularly MR-409, should have therapeutic applications for tissue repair by promoting cell proliferation, which may apply to cerebral ischemic conditions.

In the present study, we investigated the protective effects of long-term MR-409 treatment on neurological function, neurogenesis, and neuroplasticity in the mice operated on for tMCAO. The results demonstrate that MR-409 significantly improves neurological functional outcomes and promotes neurogenesis and neuroplasticity in ischemic damage areas.

Results

GHRH-Rs Are Expressed in Mouse Brain and Cultured Neural Stem Cells. We determined the protein expression of GHRH-R in the mouse SVZ and SGZ and in cultured neural stem cells (NSCs) isolated from adult mouse hippocampus (17, 18). The SVZ and SGZ have rich endogenous neuronal stem cells. GHRH-R was strongly expressed in the SVZ and SGZ of mouse brain tissues, which was comparable to that of positive control pituitary tissue (Fig. 1*A*). The expression of GHRH-R in cultured NSCs was also comparable to that of positive HeLa cells. The expression of GHRH-R was not detectable in the skeletal muscle and MCF7 cells of negative controls (Fig. 1*B*). Furthermore, the expression of GHRH-R in primary cultured neurons from mouse cortex and hippocampal was comparable to that of positive HeLa cells (*SI Appendix*, Fig. S1).

MR-409 Reduces Mortality and Ischemic Insult in tMCAO Mice. To determine whether MR-409 improves cerebral ischemic insults, the tMCAO mice were treated with MR-409 at the dose of 5 (low) or 10 (high) μg/mouse/d with subcutaneous (s.c.) injection from 1 wk prior to the induction of ischemic stroke to the 28th day of postischemia stroke. The dosage of MR-409 was chosen based on previous studies that show MR-409 at 10 μg/mouse/d has therapeutic effects in mice (19). Treatment with MR-409 dose-dependently reduced mortality in tMCAO mice: 22 of 50 mice died in the untreated tMCAO group, 17 of 50 mice in the low dose of MR-409–treated group, and only 10 of 50 mice in the high dose of MR-409–treated tMCAO group (Fig. 1*C*). The cerebral cortical expansion was examined on the 28th day of

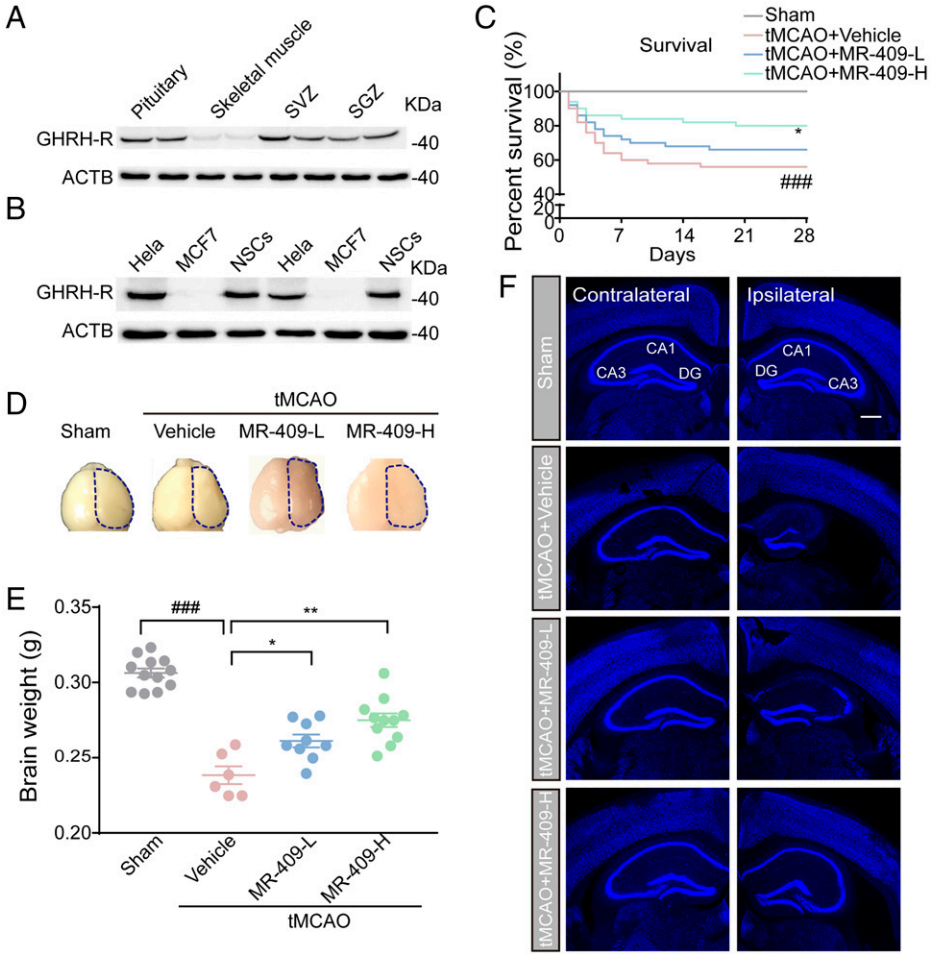

Fig. 1. MR-409 reduces mortality and cerebral ischemic insult in tMCAO mice. GHRH receptors were expressed in the SVG and SGZ zones in vivo (*A*) and in neural stem cells in vitro (*B*). Mouse pituitary was used as a positive control and skeletal muscle as a negative control in vivo. HeLa cells were used as a positive control and MCF7 cells as a negative control in vitro. ACTB was used as a loading control. (*C*) MR-409 increased the survival rate of mice during the 28 d of tMCAO; $n = 50$. Treatment with MR-409 prevented tMCAO-induced cortical cavitation (*D*), loss of brain weight (*E*), and hippocampal neuron loss in tMCAO mice (*F*) ($n = 6$ to 12). (Scale bar, 200 μm.) Data are presented as mean ± SEM $^{###}P < 0.001$ vs. sham group; $^{**}P < 0.01$, $^{*}P < 0.05$ vs. tMCAO group.

postischemic stroke by using cortical width index. tMCAO mice showed cortical cavitation and brain weight loss, which was prevented by MR-409 treatment (Fig. 1 *D* and *E*). Furthermore, the microphotographs of brain sections revealed that tMCAO induced a substantial neuronal loss in the hippocampal CA1, CA3, and DG areas, and MR-409 was also able to prevent this loss (Fig. 1*F*). In addition, we found that MR-409 treatment had begun after the onset of tMCAO. MR-409 treatment at the onset of tMCAO at low dose did not significantly reduce mortality; MR-409 at high dose had a tendency but without statistically significant reduction in the molarity of tMCAO mice ($P < 0.1$; *SI Appendix*, Fig. S2*A*). Notably, MR-409 significantly improved cerebral ischemia injuries, including the prevention of brain atrophy (*SI Appendix*, Fig. S2*B*) and hippocampal neuron loss (*SI Appendix*, Fig. S2*D*) and increased brain weight (*SI Appendix*, Fig. S2*C*) in the late stage of tMCAO mice. These results suggest that MR-409 has a long-term neuroprotective effect on ischemic stroke–induced brain injury.

MR-409 Improves Cognitive Impairment in tMCAO Mice. To determine whether MR-409 improves ischemia-induced cognitive impairment, we performed the novel objective recognition (NOR) test and the Morris water maze (MWM) test. MR-409 treatment begun after the onset of stroke did not significantly change the exploration time both in the acquisition test and recognition test of tMCAO mice (*SI Appendix*, Fig. S3 *A* and *C*) and the discrimination index in the acquisition test (*SI Appendix*, Fig. S3*B*). However, the discrimination index in the recognition test of tMCAO mice was significantly lower than that of the sham-operated ones, which can be reversed by treatment with MR-409 (*SI Appendix*, Fig. S3*D*). In the MWM test, tMCAO mice exhibited a reduction in the crossing number and the time spent in the targeted quadrant in the probe test and a significant increase in the path length and escape latency in the navigation test; treatment with MR-409 1 wk prior to tMCAO significantly increased the crossing number and time spent in the target quadrant and reduced path length and escape latency (Fig. 2 *A* and *B*; *SI Appendix*, Fig. S4 *A* and *B*). These results indicate that MR-409 can improve stroke-induced cognitive impairment.

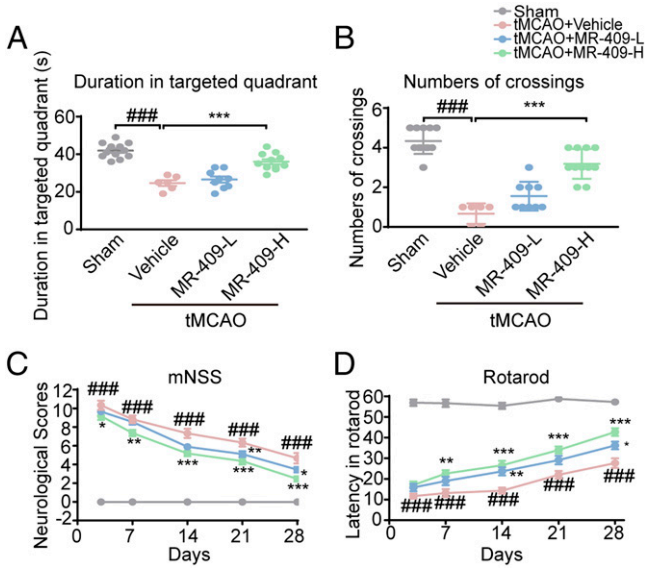

Fig. 2. MR-409 improves long-term neurological functional outcomes in tMCAO mice. MR-409 improved the performance of recognition memory in tMCAO mice, including increases of time spent in targeted quadrant (*A*) and crossing number (*B*) in MWM probe phase of tMCAO mice. Treatment with MR-409 also markedly improved mNSS (*C*) and motor function (rotarod test, *D*) in tMCAO mice; $n = 6$ to 12. $^{###}P < 0.001$ vs. sham group; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. tMCAO group.

MR-409 Improves Long-Term Sensorimotor Dysfunction in tMCAO Mice. To determine whether MR-409 alleviates tMCAO-induced long-term motor disability, mice were subjected to a series of sensorimotor tests, including modified Neurological Severity Score (mNSS) assessments, corner test, and rotarod at the 3rd, 7th, 14th, 21th, and 28th day after tMCAO. The mice from all groups, except the sham control group on the third day of post tMCAO, exhibited similar substantial neurological deficit, and the neurological function gradually recovered within 4 wk after the initial insult. As expected, the mNSS score, an index for comprehensive assessment of sensor and motor function of mice, was significantly higher in tMCAO mice than in sham mice, and treatment with MR-409 significantly reduced the score (Fig. 2*C*). Furthermore, we performed the rotarod test to investigate the motor function of mice. Compared with sham mice, tMCAO mice had a shorter rotation duration at maximal speed. MR-409 significantly increased the rotation duration in tMCAO mice (Fig. 2*D*). In the corner test, compared with sham mice, tMCAO mice had more ipsilateral side turns, which was significantly decreased by MR-409 treatment (*SI Appendix*, Fig. S4*C*). In the grip strength test, compared with the sham group, tMCAO mice had lower grip strength, which was significantly improved by MR-409 treatment (*SI Appendix*, Fig. S4*D*). The improvement of motor function occurred from the 3rd to 7th day after MR-409 treatment until the end of the experiment (28th day). Our results demonstrate that MR-409 can improve stroke-induced long-term sensorimotor dysfunction, which may contribute to lowering the sequelae of permanent motor disability and the improvement of ischemia stroke outcomes.

MR-409 Promotes Proliferation and Migration of Immature Neurons in tMCAO Mice. To determine whether long-term treatment with MR-409 promotes endogenous neurogenesis and improves the outcome of ischemic stroke, we used MCM2 as an endogenous proliferation marker of newly formed neural cells, in conjunction with the NSCs lineage-specific markers (GFAP/Nestin) to label for neural stem cells, Tbr2 for neural progenitor cells and migrating/immature neurons, and DCX for neuroblasts to trace the different differentiation stages of NSCs in the SVZ and SGZ zone of mice at the 14th day after tMCAO. Compared with sham mice, the number of $GFAP^+Nestin^+MCM2^+$ cells in both SGZ (*SI Appendix*, Fig. S5 *A* and *C*) and SVZ (*SI Appendix*, Fig. S6) was markedly increased at the 14th day after tMCAO, which was further augmented by MR-409 treatment in a dose-dependent manner. The number of $Tbr2^+MCM2^+$ in the SGZ (*SI Appendix*, Fig. S5 *B* and *D*) of tMCAO mice was higher than those of sham mice. Treatment with MR-409 doesdependently increased the double-positive cells in tMCAO mice (*SI Appendix*, Fig. S5 *B* and *D*). Next, we determined neuroblast proliferation, using costaining of DCX with exogenous proliferating cell marker BrdU. BrdU (150 mg/kg, intraperitoneally [i.p.]) was injected into the mice on the 14th day after tMCAO and the brain tissues were harvested at 2 h after the injection (Fig. 3*A*). Immunofluorescence staining showed that the number of total DCX^+ and DCX^+BrdU^+ cells was significantly increased in the SGZ and SVZ of tMCAO mice, compared with sham control mice, while MR-409 further increased the number of DCX^+ and DCX^+BrdU^+ cells in tMCAO mice (*SI Appendix*, Fig. S3 *B–D*). Similarly, MR-409 also increased the $MCM2^+DCX^+$ cells in the ipsilateral DG (*SI Appendix*, Fig. S7 *A* and *B*) and SVZ (*SI Appendix*, Fig. S8) of tMCAO mice. In addition, the migration distance of DCX positive (DCX^+) cells was also increased in the ipsilateral SGZ of tMCAO mice, and further enhanced in MR-409–treated mice (*SI Appendix*, Fig. S7*C*). These results suggest that MR-409 is able to promote

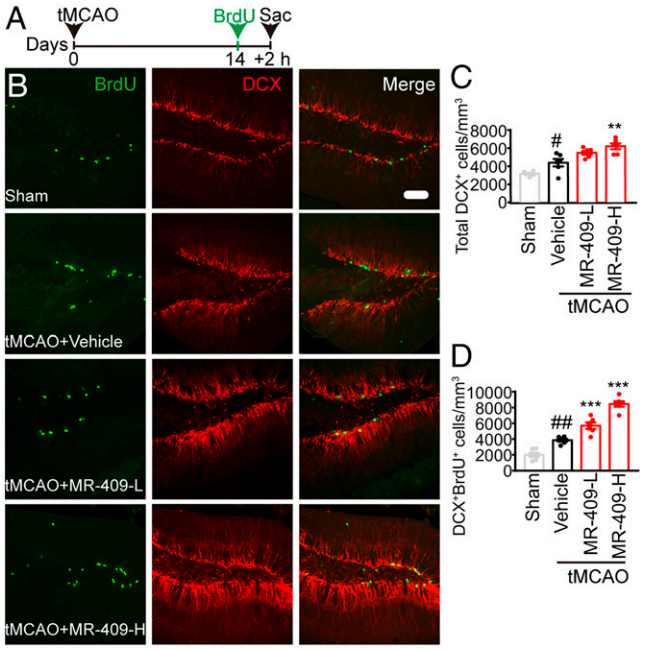

Fig. 3. MR-409 promotes the proliferation and migration of neuroblasts in the ipsilateral DG of tMCAO mice. (*A*) A schematic diagram of experimental design, BrdU (150 mg/kg, i.p.) was injected into mice on day 14 after tMCAO; 2 h later, the brain tissues were harvested. (*B*) Representative confocal images of the colocalization of BrdU (green, an exogenous marker of proliferating cells) and DCX (red, a marker of neuroblasts)-positive cells in the ipsilateral DG. (Scale bar, 40 μm.) DAPI (blue) indicates nucleus. Quantification analysis of migrating neuroblasts (DCX^+, *C*) and proliferating neuroblast (DCX^+BrdU^+, *D*) in the ipsilateral DG ($n = 6$). $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. sham group; $^{***}P < 0.001$, $^{**}P < 0.01$ vs. tMCAO group.

the proliferation and migration of immature neurons in the ipsilateral SGZ and SVZ after ischemic stroke.

MR-409 Promotes the Survival of Newborn Neurons in tMCAO Mice. The therapeutic effect of NSCs depends not only on the ability of NSCs to proliferate, but also on NSCs differentiation into newly formed neurons and engraftment in the ischemic damaged zone. To identify whether MR-409 can increase the survival of newborn neurons in the ipsilateral SGZ and SVZ of tMCAO mice, BrdU (50 mg/kg, i.p.) was injected into mice twice a day for 7 consecutive days from day 1 after tMCAO, and the tissues were obtained on the 28th day (Fig. 4*A*). Immunofluorescence staining showed that the densities of newborn mature neurons ($NeuN^+BrdU^+$) were significantly increased in the ipsilateral SGZ (Fig. 4 *B*–*D*) and SVZ (*SI Appendix*, Fig. S9) of tMCAO mice, while treatment with MR-409 further increased the densities of newborn mature neurons in tMCAO mice. More importantly, in addition to the absolute number of $NeuN^+BrdU^+$ cells, MR-409 also augmented the ratio of $NeuN^+BrdU^+$ double positive cells with the total $BrdU^+$ cells (Fig. 4*E*), suggesting that MR-409 may have important therapeutic effects on promoting the differentiation of newborn NSCs into newly formed neurons and newborn neuron survival in the ischemic cerebral tissues.

MR-409 Increases Hippocampal Synaptic Plasticity in tMCAO Mice. Neuroplasticity-mediated circuit reorganization is closely related to poststroke functional recovery. We assessed the effect of MR-409 on the ultrastructural morphology of synapses in the hippocampal CA1 pyramidal neurons on the 28th day in tMCAO mice, using transmission electron microscopy (TEM). Compared with sham mice, the number of synapses in tMCAO mice was significantly decreased, while treatment with MR-409 prevented tMCAO-induced loss of synapses (Fig. 5*A*). Furthermore, we examined the changes in ultrastructural morphology in MR-409–treated tMCAO mice, including the postsynaptic density (PSD), synaptic cleft, synaptic curvature, and presynaptic vesicles. As shown in Fig. 5*B*, tMCAO mice exhibited an increase in PSD thickness and a decrease in postsynaptic cleft. In addition, tMCAO mice also manifested changes in the synaptic curvature, as indicated by the higher percentage of flat synapses and the loss of synaptic vesicles; treatment with MR-409 reversed all of the above-mentioned morphological changes (Fig. 5*B*). To evaluate the effect of MR-409 on dendritic complexity and synaptic plasticity in tMCAO mice, we further analyzed the number of dendritic branches and spine density with Golgi-Cox staining. As shown in Fig. 5*C*, tMCAO induced a sparse and confused distribution of neurons in the hippocampal CA1 and CA3 regions. Besides, tMCAO significantly reduced dendritic complexity and length in the hippocampus CA1 region. Treatment with MR-409 not only increased the total number of hippocampal neurons and improved the neuron distribution, but also restored the morphology of dendrites. Moreover, we analyzed both the apical and basal dendritic spine density of hippocampal CA1 neurons (Fig. 5 *D* and *E*). The results showed the spine density in tMCAO mice was significantly decreased, and treatment with MR-409 significantly increased the spine density in both apical and basal dendrites (Fig. 5 *F* and *G*). Furthermore, the expressions of major presynaptic scaffold proteins, synaptophysin (SYN) and PSD95, were profoundly decreased by the 28th day in the ischemic hippocampal of tMCAO mice, which were prevented by MR-409 (Fig. 5 *H*–*J*). These results indicate that MR-409 is able to enhance the structural neuroplasticity of the hippocampus after tMCAO.

MR-409 Activates AKT/CREB and BDNF/TrkB Pathways and Inhibits the STAT3 Inflammatory Pathway in Brain Tissue of tMCAO Mice. To investigate the underlying mechanisms by which MR-409 promotes neuroregeneration, we performed a broad-spectrum gene screening in the ipsilateral hippocampal tissues on the 7th day after tMCAO by using RNA-sequencing analysis. Selective Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology and Gene Ontology (GO) function enrichment analysis showed that MR-409 can affect seven major signaling pathways and four types of kinase activities, including the enriched proliferation genes of MAPK/ERK and PI3K/AKT pathways, and JAk-STAT, Toll-like receptor, and chemokine inflammatory signaling pathways (Fig. 6*A*). Moreover, compared to tMCAO mice, MR-409 up-regulated the tyrosine kinase–related gene expressions (Fig. 6*A*). Meanwhile, the top 10 KEGG enrichment pathway analyses showed that PI3K-AKT and MAPK pathways were enriched in MR-409–treated tMCAO mice compared to tMCAO mice (Fig. 6*B*). In addition, multiple neuroprotective genes were significantly up-regulated by MR-409 treatment (Fig. 6*C*). BDNF is a key neurotrophic factor (20, 21), which binds to its specific receptor-tyrosine receptor kinase B (TrkB) (22), resulting in the activation of downstream signaling pathways, including the PI3K/AKT and MAPK/ERK pathways (23). It has been reported that the secreted mature BDNF (m-BDNF, ∼13 kDa) has biological activity, while the BDNF precursor (pro-BDNF, ∼30 kDa) is an inactive form with opposite effects (24). Three major splice variants of the TrkB are as follows: full-length TrkB (TrkB-FL, 145 kDa), TrkB-Shc (95 kDa), and TrkB.T1. The structure of TrkB.T1 and TrkB-Shc does not have a tyrosine kinase domain. The binding of BDNF to TrkB-FL induces receptor dimerization, which leads to kinase activation and autophosphorylation of the receptor (25). Compared with sham mice, the expression of TrkB-FL in the ipsilateral hippocampus of tMCAO mice was lower, while the expression of TrkB was higher, which was reversed by MR-409 treatment (Fig. 6 *D*–*F*). The protein expression of pro-BDNF was up-regulated in tMCAO mice, which was also reversed by MR-409. No significant changes were observed in the

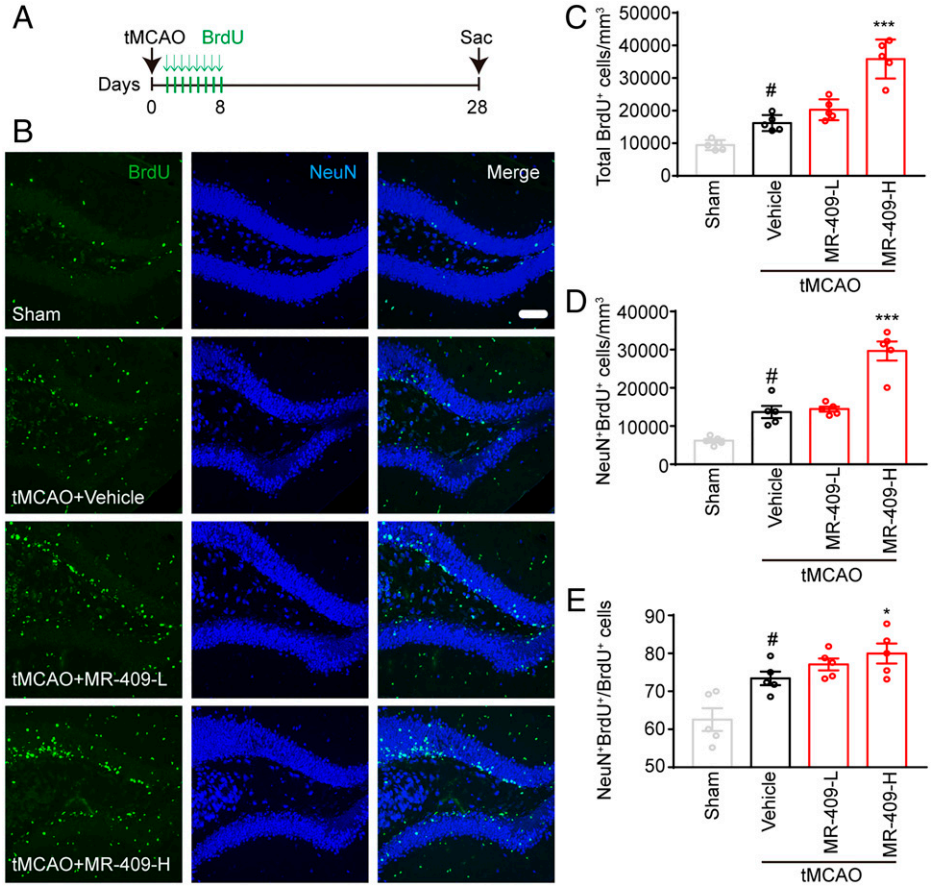

Fig. 4. MR-409 promotes the survival of newborn neurons in the ipsilateral DG of tMCAO mice. (*A*) A schematic diagram of experimental design, the mice were subjected to tMCAO. BrdU (50 mg/kg, i.p.) was administrated twice a day for the first 7 consecutive days after tMCAO surgery; the tissues were harvested on the day 28 post-tMCAO. (*B*) Representative confocal images of the colocalization of BrdU (green) and NeuN (blue, a marker of mature neurons) in the ipsilateral DG. (Scale bar, 40 μm.) Quantification analysis of total newborn neurons ($BrdU^+$, *C*), newborn mature neurons ($NeuN^+BrdU^+$, *D*), and the proportion of newborn neurons ($NeuN^+BrdU^+/BrdU^+$, *E*) in the ipsilateral DG ($n = 5$). $^{\#}P < 0.05$ vs. sham group; $^{***}P < 0.001$, $^{*}P < 0.05$ vs. tMCAO group.

level of m-BDNF between sham and tMCAO mice, and MR-409 remarkably up-regulated the expression of m-BDNF (Fig. 6 *D*, *G*, and *H*). These results suggest that the therapeutic effects of MR-409 on ischemic stroke may be related to its activation of the BDNF/TrkB signaling pathway. The neuroinflammation and neurogenesis after ischemic stroke have complex interaction, which may affect neural stem cell survival and brain repair (26). Western blot analysis showed that tMCAO significantly increased the ratio of NF-κB p-p65/p65 and p-stat3 (Tyr705)/stat3 and the expressions of inflammatory cytokines IL1β and TNFα. Treatment with MR-409 significantly decreased the expression of inflammatory cytokines IL-1β and TNFα and reduced the ratio of p-stat3 (Tyr705)/stat3 after tMCAO; MR-409 had a tendency to inhibit the ratio of p-p65/p65 but did not reach statistical significance in tMCAO mice (*SI Appendix*, Fig. S10). These results suggest that MR-409 may reduce stroke-induced inflammatory response.

MR-409 Promotes the Proliferation of Neural Stem Cells. We performed a series of experiments to investigate the proliferative effect of MR-409 in cultured H9-derived human NPCs (hNPCs) and mice NSCs exposed to OGD/R. Immunofluorescence showed the colocalization of Nestin (green) with Sox2 (red) and Nestin (green) with Pax6 (red), which were used to identify neural stem cells (*SI Appendix*, Fig. S11). TUNEL and Nestin staining were used to determine the effect of MR-409 on the apoptosis and survival of cell exposure to OGD/R, respectively. OGD/R significantly increased the number of TUNEL-positive apoptosis cells and decreased the Nestin-positive cells; both effects were prevented by MR-409 treatment (Fig. 7 *B* and *C*; *SI Appendix*, Fig. S12*A*). Ki67 and BrdU were used as endogenous or exogenous proliferative markers, respectively. OGD/R caused a significant increase in Ki67- and BrdU-positive cells, which was further enhanced by MR-409 treatment (Fig. 7 *B* and *C*; *SI Appendix*, Fig. S12*B*). The 3-(4,5-Dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide (MTT) assay showed that MR-409 dose-dependently increased cell viability both in hNPCs and NSCs after OGD/R (Fig. 7*A*; *SI Appendix*, Fig. S12*C*). RNA-sequencing analysis revealed that MR-409 up-regulated AKT and ERK pathways in vivo. OGD/R significantly decreased the ratios of p-AKT/AKT and p-ERK/ERK in hNPCs and NSCs, both of which were prevented by MR-409 treatment (Fig. 7 *D* and *E*; *SI Appendix*, Fig. S12 *D–F*). The activation of AKT may increase cAMP response element binding protein (CREB) to provide a prosurvival effect (27). OGD/R decreased the expression of p-CREB/CREB, which was reversed by MR-409 treatment (Fig. 7 *D* and *F*; *SI Appendix*, Fig. S12 *D* and *G*). These results suggest that MR-409 is able to promote the proliferation of NSCs' exposure to OGD/R, and the underlying mechanism may involve the activation of ERK and AKT signaling pathways.

Discussion

Ischemic stroke is still one of the leading causes of mortality and disability in adults worldwide. Consistent with high

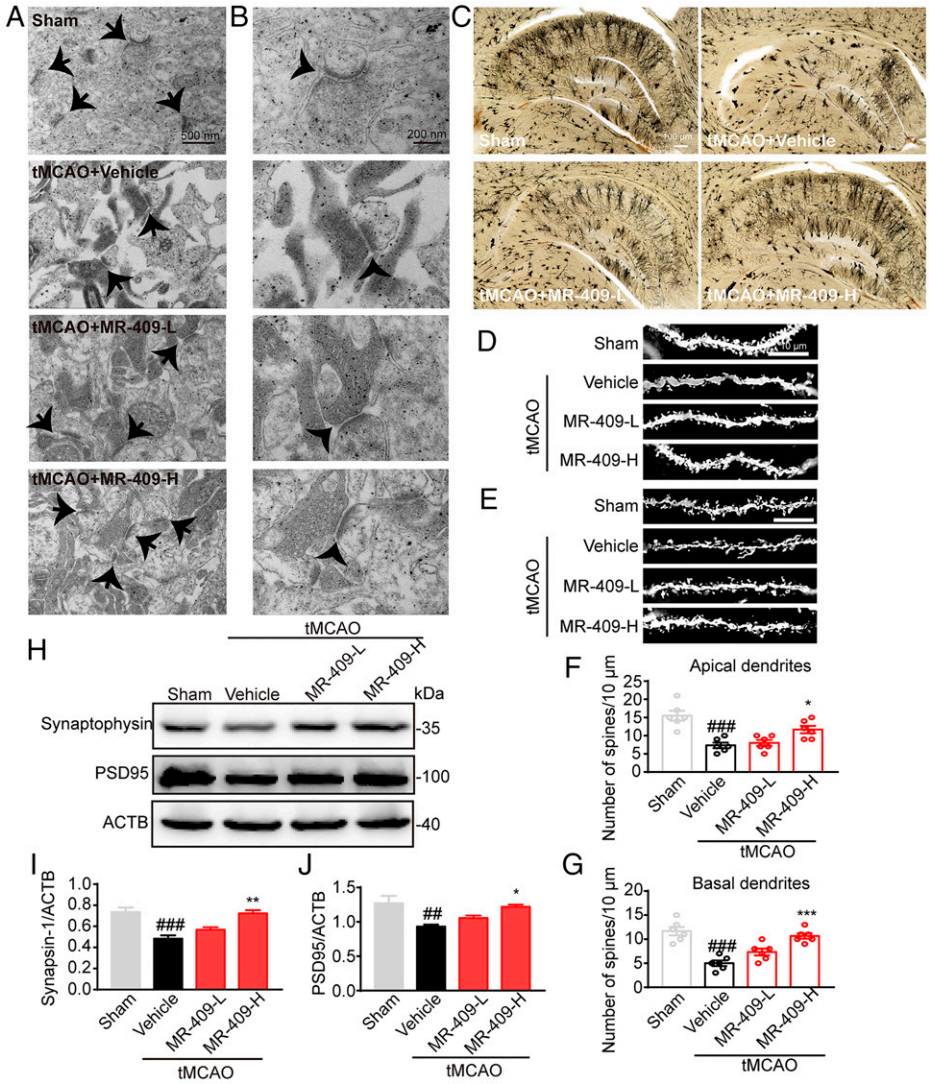

Fig. 5. MR-409 increases the hippocampal synaptic plasticity in tMCAO mice. Representative images of the ultrastructural morphology of synapses in the hippocampal CA1 pyramidal neuron assessed by TEM (*A*), a higher magnification view of ultrastructural morphology of synapses (*B*), and the number and morphology of dendritic branches in the ipsilateral hippocampal CA1 region, determined by Golgi-Cox staining (*C*). The representative images of apical (*D*) and basal dendritic spine (*E*) density of hippocampal CA1 neurons. Quantification analysis of spine density in the apical (*F*) and the basal (*G*) dendritic spine density of the hippocampal CA1 neurons. MR-409 prevented tMCAO-induced loss of synapses, changes in ultrastructural morphology, and decrease in the number of spines ($n = 6$, *F* and *G*). (*H*) Representative immunoblot bands of Synaptophysin (SYN) and PSD95 expressions in the ipsilateral hippocampus on the 28th day of tMCAO mice. Quantitative analysis of the immunoblotted proteins showing MR-409 significantly increased the SYN (*I*) and PSD95 (*J*) levels in tMCAO-treated mice ($n = 5$). ACTB was used as a loading control. $^{###}P < 0.001$, $^{##}P < 0.01$ vs. sham group; $***P < 0.001$ $**P < 0.01$, $*P < 0.05$ vs. tMCAO group. (Scale bars: 500 nm in *A*, 200 nm in *B*, and 100 μm in *C*.)

mortality in stroke patients, we showed that survival rate of tMCAO mice was lower especially in the first 7 d poststroke. To our surprise, treatment with MR-409 markedly lowered mortality and protected against ischemic-induced hippocampal loss of neurons and neurological dysfunction. Moreover, our results demonstrated that either treatment with MR-409 started 1 wk before (preventive regiment) or after tMCAO (therapeutic regiment) can increase long-term survival of neurons. In terms of reducing mortality of tMCAO mice, it seems that MR-409 treatment started before tMCAO is better than treatment after tMCAO. The neuroprotective effects of MR-409 may be attributed to its improvement of NSCs' niche and up-regulation of the BDNF/TrkB and AKT/CREB pathways (*SI Appendix*, Fig. S13).

Previous work has shown that systemic administration of GHRH agonists can stimulate cell proliferation in the peripheral tissues (15, 17, 19, 28–30); this leads to reducing myocardial infarction (17) and improves cardiac remodeling (30), wound healing (28), as well as islet transplantation (15, 31). In the present study, we found that MR-490 can improve neuron survival through the enhancement of endogenous neural stem cell–derived neurogenesis after tMCAO. Stroke can promote neurogenesis in SVZ and SGZ; it has been reported that stroke may induce the production of new neurons to replace dead ones in nonneurogenic niches, such as striatum and neocortex (2, 32, 33). Ischemic stroke can increase proliferation of NPCs and subsequent neuroblast migration, but only these newly generated neurons, which can differentiate into neuronal subtypes and functionally integrate into the adherent structure and reconnect to the correct pathways, are potential sources for neuronal replacement after stroke. In our work, treatment with MR-409 improves the ultrastructural morphology of synapses and the number of dendritic branches and spine density, indicating that MR-409 is able to enhance the

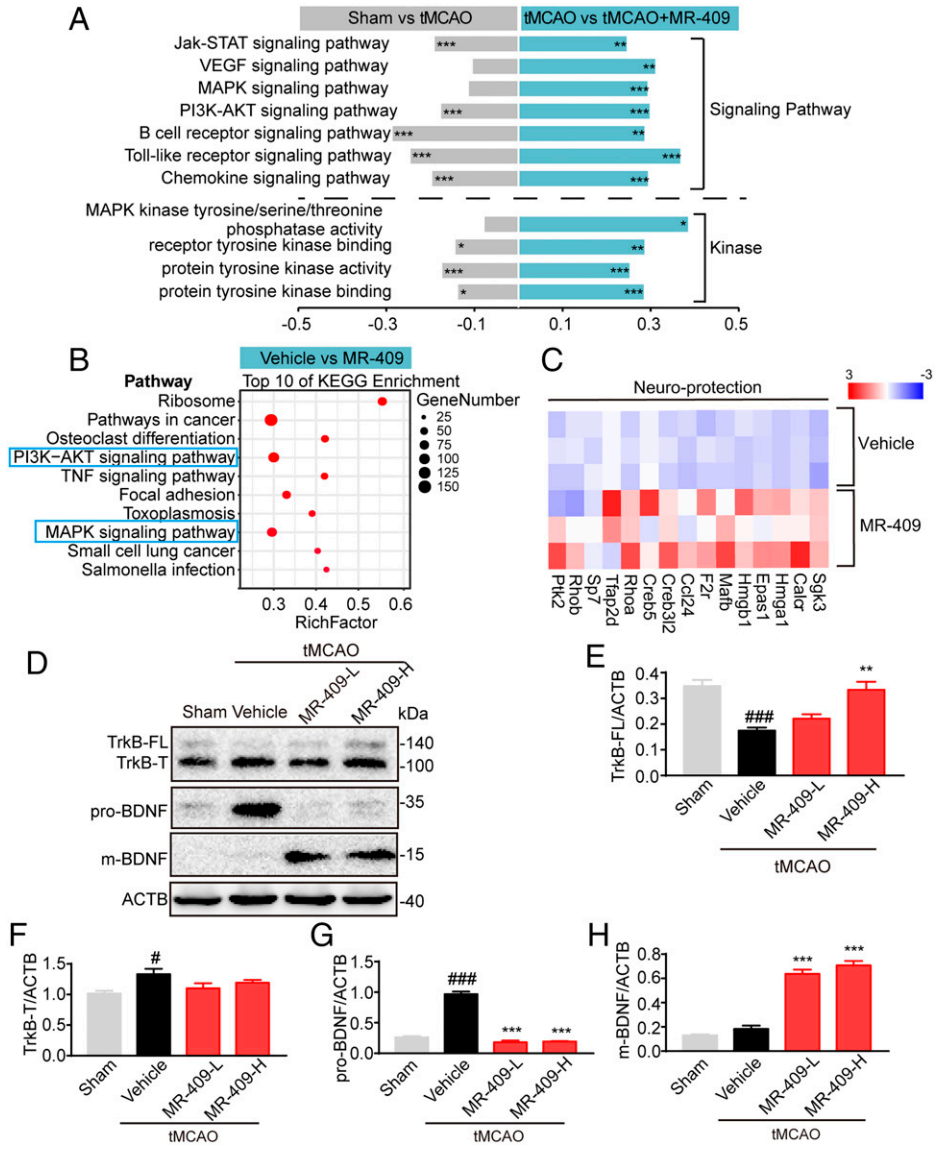

Fig. 6. MR-409 up-regulates cell proliferation genes and neuroprotective genes and activates the BDNF/TrkB pathway in the ipsilateral hippocampus of tMCAO mice. (*A*) Selective KO and GO function enrichment analysis showed that MR-409 mainly affected MAPK/ERK and PI3K/AKT signaling pathways and tyrosine kinase activity. The fold enrichment is expressed from −0.5 to 0.5. The calculated *P* value went through false discovery rate correction, ***FDR-adjusted $P < 0.001$, **FDR-adjusted $P < 0.01$, *FDR-adjusted $P < 0.05$. (*B*) Pathway enrichment analysis showed that MR-409 affected the top 10 enriched pathways in tMCAO mice. (*C*) Heatmaps showed that MR-409 up-regulated the expression of neuroprotective genes in tMCAO mice. Each sample contained hippocampus from three mice. (*D*) Representative images of immunoblotting bands for protein expression of neurotropic factors pro-BDNF, mBDNF, and their receptors TrkB-FL (full length), TrkB-T (truncated) in the ipsilateral hippocampus from the 7th day of tMCAO mice. Quantitative analysis of immunoblotted proteins showed that MR-409 significantly decreased the pro-BDNF and TrkB-T levels, increased the TrkB-FL (*E*) and m-BDNF (*H*) expressions, and decreased the TrkB-T (*F*) and pro-BDNF (*G*) expressions in tMCAO mice ($n = 5$). ACTB was used as a loading control. $^{###}P < 0.001$, $^{#}P < 0.05$ vs. sham group; $^{***}P < 0.001$, $^{**}P < 0.01$ vs. tMCAO group.

structural neuroplasticity after tMCAO as well. It has been shown that GHRH-R is expressed in the cortex and the hippocampus and other parts of the central nervous system (CNS) (34). Here we demonstrate GHRH-R was expressed not only in NSCs but also in primary neurons of mice. Thus it cannot be excluded that MR-409 may exert a direct action on the neurons to promote neuroplasticity, which needs to be further investigated.

It has been shown that the activation of GHRH-R by GHRH can increase the resistance of neuronal cells to oxidative, metabolic, and excitotoxic injury (35, 36), which may play an important role in regulating the function and plasticity of neuronal circuits in the CNS. GHRH-R is a G protein coupled receptor, which is known to activate at least three major signaling pathways: cAMP/PKA, MAPK/ERK, and PI3K/AKT (37). The PI3K/AKT pathway plays a central role in regulating cell growth, proliferation, and survival, and it also participates in axonal sprouting, which is an important mechanism for post-stroke functional recovery (38). Previous studies have reported that both PI3K/AKT and MAPK/ERK pathways are involved in survival effects induced by GHRH agonist (18, 19, 28). Using RNA-sequencing analysis, we found that MR-409 up-regulated PI3K/AKT and MAPK/ERK pathways and its downstream molecular CREB. It has been shown that MR-409 increases the levels of cellular cAMP and the phosphorylation of CREB in INS-1 cells (19). Our in vivo and in vitro data indicate that MR-409 markedly increases the expressions of BDNF and its specific receptor TrkB-FL in ischemic stroke tissue and NSCs. Interestingly, many studies have found that the binding of

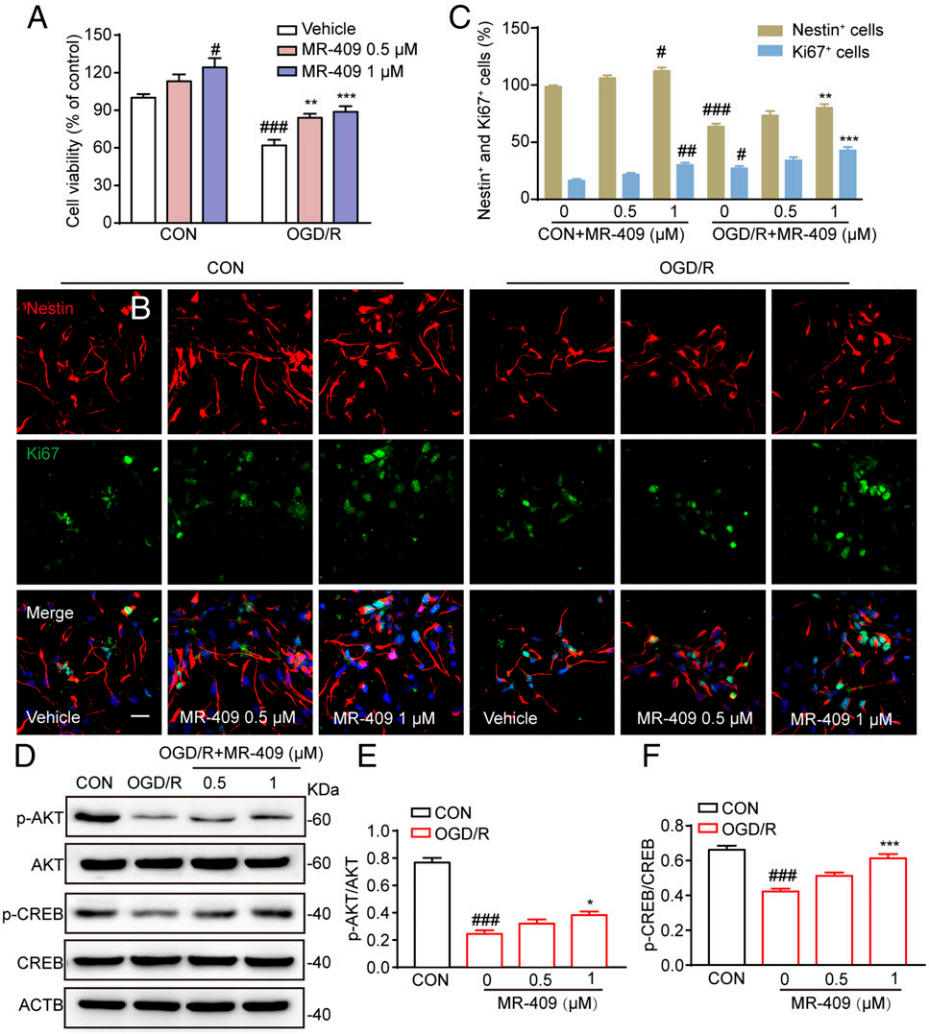

Fig. 7. MR-409 increases the proliferation of human neural progenitor cells and stimulates AKT and ERK signaling pathways in the OGD/R-treated H9-derived neural progenitor cells (hNPCs). (*A*) MTT assay showed that MR-409 enhanced the cells viability in OGD/R-exposed hNPCs ($n = 4$). (*B*) Representative confocal images of the colocalization of Ki67 (green, a marker of proliferating cells) and Nestin (red, a marker of hNPC)-positive cells in the hNPCs after OGD/R. (Scale bar, 10 μm.) (*C*) Quantification analysis of Ki67$^+$ and Nestin$^+$ hNPCs ($n = 8$ to 10). (*D*) Representative immune bands of protein expressions of AKT and CREB in OGD/R-exposed hNPCs. Quantitative analysis showed that MR-409 significantly increased the p-AKT/AKT (*E*) and p-CREB/CREB (*F*) levels in OGD/R-treated NSCs ($n = 5$). $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$ vs. CON group; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. OGD/R group.

BDNF to TrkB receptor can initiate an intracellular signaling cascade, such as the activation of the PI3K/AKT signaling pathway (39), suggesting that AKT/CREB may in turn be a downstream target of BDNF/TrkB (40), MR-409 may activate the BDNF/AKT/CREB pathway to regulate the loop between neurons and NSCs, which promote neuronal survival and synaptic plasticity processes.

Ischemic stroke is often associated with a nonsterile inflammation in the brain tissue. The immune inflammatory response after ischemic stroke can be beneficial and detrimental (26, 41). Stroke-induced immune response and microglia release of inflammatory cytokines may be necessary for neurogenesis (42). However, chronic inflammation after stroke not only impairs basal neurogenesis but also attenuates the increased neurogenesis seen after injury (43). It has been shown that proinflammatory cytokine TNFα can inhibit progenitor cell proliferation and suppress survival of newly formed neurons after brain injury (44, 45). RNA-sequencing analysis demonstrates that ischemic stroke and MR-409 significantly affect multiple inflammatory signaling pathways, including Jak-STAT, inflammatory chemokine, and Toll-like receptor pathways. MR-409 resolution of stroke-induced inflammatory response seen in the present study may be another mechanism to add a beneficial effect on neurogenesis and brain repair.

It should be noted that peripheral administration of MR-409 must pass through the brain blood barrier (BBB) to exert its pharmacological effects on brain tissue. The present study did not provide the direct evidence that MR-409 is able to cross the BBB in tMCAO mice. However, we and others have demonstrated that peripheral administration of GHRH analogs can influence the functions of the CNS, and that native GHRH and sermorelin, a GHRH analog and the parent structure of MR-409, is able to cross the BBB (46–49). Because ischemic stroke can disrupt the BBB to increase permeability of the BBB (50, 51), the present study shows that subcutaneous injection of MR-409 can reduce stroke-induced brain damage and improve brain function; thus these results indicate that MR-409 is able to cross the BBB in ischemic stroke.

In conclusion, we demonstrate that MR-409, a synthetic agonist of GHRH-R, has remarkably neuroprotective and pro-neurogenesis effects, which may contribute to the recovery of neurological function in early-stage and long-term postischemia.

To our knowledge, the present study provides evidence demonstrating that MR-409 is one of the most promising agents for the treatment of chronic ischemic stroke. MR-409 should be treated in clinical trials to confirm its therapeutic effects in patients with cerebral ischemia.

Materials and Methods

Experimental Design. The present study aimed to investigate the therapeutic effects of MR-409 in tMCAO mice and underlying potential mechanisms. We performed a series of experiments to evaluate the effect of long-term treatment with different doses of MR-409 on mouse mortality, cerebral ischemic insult, and neurological function recovery and used different fluorescence markers to trace the process of proliferation and migration and differentiation of NSCs into newly formed neurons in the ipsilateral DG of tMCAO mice. We used TEM and Golgi-Cox staining to evaluate the effect of MR-409 on tMCAO-induced loss of neuroplasticity. In addition, we used broad-spectrum RNA-sequencing analysis to identify the potential signaling pathways of MR-409 neuroprotective effects in the early stage of cerebral ischemia in vivo and further confirmed the protective effects of MR-409 on cultured neural stem cells in vitro. We initially designated 8 to 12 mice per group for various experiments as mentioned; however, because of high mortality caused by tMCAO, the actual experimental number per group was determined by the number of surviving animals ($n = 5$ to 12, as indicated in the figure legends). All histologic samples were run in a blind manner; the reviewers were not aware of the groups to which the mice belonged. We used a total of 50 mice per group for the mortality study; these animals come from different experiments.

Animals. Male 7-wk-old C57BL/6 mice were purchased from Beijing HFK Bioscience Co. All experiments were conducted according to the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were approved by the Experimental Animals Ethics Committee of the Shenyang Medical College (Liaoning, China).

Statistical Analysis. The statistical studies were carried out blindly during data collection and quantification. All data were expressed as mean ± SEM, and statistical analysis was performed using GraphPad Prism 7 software. Statistical analysis was performed with one-way or two-way ANOVA followed by post hoc Tukey test for multiple comparison. The Kaplan–Meier test was used for mouse survival rate. The comparison of mNSS, corner test, and crossing number in probe trials was performed by the nonparametric Kruskal–Wallis test. $P < 0.05$ is considered as statistical significance.

Other Experimental Procedures. Additional methods are described in *SI Appendix*.

Data Availability. All datasets have been deposited in Zenodo at http://doi.org/10.5281/zenodo.5613278 (52). Other data generated from this study are included in the manuscript and *SI Appendix*.

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