Netrin-1 Hyperexpression in Mouse Brain Promotes Angiogenesis and Long-Term Neurological Recovery After Transient Focal Ischemia

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Background and Purpose—Netrin-1 (NT-1) stimulates endothelial cell proliferation and migration in vitro and promotes focal neovascularization in the adult brain in vivo. This in vivo study in mice investigated the effect of NT-1 hyperexpression on focal angiogenesis and long-term functional outcome after transient middle cerebral artery occlusion (tMCAO).

Methods—Adeno-associated viral vectors carrying either the NT-1 gene (AAV–NT-1) or GFP (AAV-GFP) were generated and injected into the brains of separate groups of 93 mice. Seven days later, tMCAO followed by 7–28 days of reperfusion were carried out. Histological outcomes and behavioral deficits were quantified 7–28 days after tMCAO. Small cerebral vessel network and angiogenesis were assessed 28 days after tMCAO, using synchrotron radiation microangiography and immunohistochemistry.

Results—Western blot and immunohistochemistry showed that on the day of tMCAO, NT-1 hyperexpression had been achieved in both normal and ischemic hemispheres. Immunofluorescence imaging showed that NT-1 expression was primarily in neurons and astrocytes. Ischemia-induced infarction in the NT-1 hyperexpression group was attenuated in comparison to saline or AAV-GFP–treated groups (P<0.01). Similarly, neurological deficits were greatly improved in AAV–NT-1–treated mice compared with mice in saline or AAV-GFP–treated groups (P<0.05). In addition, angiogenesis was increased in AAV–NT-1–treated mice compared with the other 2 groups (P<0.05). In vivo synchrotron radiation microangiography 28 days after tMCAO revealed more branches in AAV–NT-1–treated mice than in other groups.

Conclusions—AAV–NT-1 induced NT-1 hyperexpression before tMCAO reduced infarct size, enhanced neovascularization, and improved long-term functional recovery. (Stroke. 2012;43:838-843.)

Key Words: adeno-associated virus ■ angiogenesis ■ gene transfer ■ ischemia ■ middle cerebral artery occlusion ■ netrin-1

Ischemic stroke is the leading cause of disability and a significant burden on public health worldwide. Because many drugs that showed neuroprotection in animal stroke models failed to show benefits in clinical trials, new strategies are needed in developing approaches to stroke therapy. An ideal restorative approach to enhancing long-term functional recovery after stroke should promote both postischemic neuronal regeneration and vascular perfusion in ischemic regions.

Netrin-1 (NT-1), a protein conserved during evolution and initially purified from chick embryos, was found to provide key guidance cues for the development of commissural axons.1 NT-1 receptors include the DCC (Deleted in Colorectal Cancer) and UNC5 (uncoordinated-5) families, both of which belong to the transmembrane immunoglobulin superfamily.2 NT-1 can either attract or repel axonal growth cones through binding to its receptors.3,4 Furthermore, NT-1 is crucial to maintain the survival of DCC-expressed and UNC5H-expressed neurons.5 Because vascular and neural systems share similar growth characteristics, there has been speculation that NT-1’s role in axon guidance and survival suggests it might similarly assist vascular network formation.6 NT-1, a potent vascular mitogen that stimulates in vitro proliferation and adhesion of endothelial and vascular smooth muscle cells,7 also acts as a survival factor for endothelial cells through the blocking of its UNC5H2 receptor. Such silencing of NT-1 during zebrafish development leads to vascular defects.8 In mammalian models, NT-1 promotion of neovascularization improves limb perfusion in hind limb ischemia and reverses vascular and neural pathology in diabetic mice.9 Previous in vivo studies found that NT-1
hyperexpression promotes neovascularization in the adult mouse brain; and earlier in vitro studies demonstrated that NT-1 hyperexpression induced proliferation, migration, and tube formation of human cerebral endothelial cells and human aortic smooth muscle cells. However, roles for NT-1 in long-term neurological recovery after ischemic brain are largely unexplored. We suggest that exogenous hyperstimulation of NT-1 expression can promote neovascularization after stroke from transient middle cerebral artery occlusion (tMCAO) and improve subsequent functional outcomes. If so, stimulation of NT-1 expression might serve as a potential therapeutic candidate for the treatment of cerebral ischemia.

To test this hypothesis in mice, we used an adeno-associated viral vector with NT-1 (AAV–NT-1) to induce brain NT-1 hyperexpression at a later time, when we then caused experimental stroke from tMCAO. During and after recovery we investigated associations between NT-1 hyperexpression, attenuated neural cell death, neovascularization, and improved neurobehavioral outcomes.

Methods

**AAV–NT-1 Viral Vector Production, Purification, and Titer Determination**

pAAV–NT-1 vector was generated by inserting the chicken NT-1 cDNA between 2 ITRs of pAAV-MCS plasmid (Invitrogen, Carlsbad, CA). pAAV–NT-1 was cotransfected with pHelper and pAAV-RC plasmids into AAV293 cells by calcium phosphate precipitation. The viruses were further purified by CsCl density gradient ultracentrifugation. Viral titer was determined by RT-PCR analysis of the gene content. Adeno-associated virus-IRES-hrGFP (AAV–IRES-hrGFP) was simultaneously prepared as a control.

**AAV-Mediated NT-1 Injection**

Animal procedures were carried out according to a protocol approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University, Shanghai, China. Adult male CD-1 mice weighing 25 g to 30 g were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg, Sigma, San Louis, MO) intraperitoneally. A burr hole was drilled to the left pericranium 2 mm lateral to sagittal suture and 1 mm posterior to coronal suture. A 10-µL syringe (WPI Inc, Sarasota, FL) was slowly inserted into the brain 3 mm under the dura. AAV suspension (2.5 µL) with 3.5×10^9 pAAV–NT-1 particles was injected stereotactically at a rate of 0.2 µL/min. After half amount of suspension was injected, the needle was slowly withdrawn to 2 mm under the dura to finish injection of the remaining AAV–NT-1 vector. Ten minutes after the completion of injection, the needle was withdrawn from the animal over a course of 15 minutes. The bone hole was sealed with bone wax and the wound was closed. After sufficient awakening from anesthesia, animals were returned to their cages for long-term recovery.

**Transient MCAO Model**

Seven days after the AAV–NT-1 injection, animals were anesthetized with 1.5% isoflurane in 70/30 nitrogen/oxygen gas for MCAO. Body temperature was maintained at 37±0.5°C throughout the surgical procedure, using a thermal blanket. The procedure of tMCAO was performed as described previously with some modification. Briefly, after isolation of the common carotid artery, external and internal carotid arteries, left MCA was occluded by a 6–0 nylon suture coated with silica gel. Reperfusion was achieved by partially withdrawing the suture from the internal carotid artery to the common carotid artery after 60 minutes of occlusion. The reperfusion was maintained for up to 28 days.

**Infarct Volume Measurement**

Brains were removed and frozen immediately 7 days after tMCAO. A series of 20-µm-thick coronal sections from anterior commissure to hippocampus were cut and mounted on slides. The sections were fixed and stained, using cresyl violet. Sections were imaged and digitized, and the border between infarct and noninfarct tissue was outlined using Image J (National Institutes of Health). Infarct volume was calculated by subtracting the volume of intact area in the ipsilateral hemisphere from the whole volume of the contralateral hemisphere.

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![Figure 1. Adeno-associated viral vector (AAV)-mediated Netrin-1 (NT-1) hyperexpression in the brain. A. Graphic illustration of a mouse brain coronal section and the distribution of AAV-mediated GFP expression. Asterisks indicate 2 different locations of viral injection in the left hemisphere. The ischemic core is shown as black area, and peri-infarct region is shown as gray area. Intraparenchymal injection of AAV-GFP resulted in an intense GFP signal (green) at 7 days after injection. Bar=50 µm. B. Western blot analysis of NT-1 expression in the brain. Brain samples were extracted from the striatum 7 days after administration of AAV-GFP, normal saline (NS), or AAV–NT-1. L indicates left hemisphere, R, right hemisphere. Histogram shows the quantification of NT-1 level. ∗P<0.05 vs AAV-GFP or saline, n=5 per group. C. Immunohistochemical staining of NT-1 in the brain sections after AAV-GFP (a), saline (b), or AAV–NT-1 (c) injection and the phenotype of NT-1–positive cells in AAV–NT-1–injected brain. NT-1–positive cells (green) expressed GFAP (d) and NeuN (e) but not Glut-1 (f). Bar=50 µm. D. Double immunostaining of NT-1 receptors. DCC (green in a, d, g) and UNC5H2 (green in a, d, g) receptors are colocalized with GFAP (red in b), NeuN (red in e), and CD31/Glut-1 (red in h), as shown in merged images (yellow in c, f, i). Bar=20 µm.]
Behavioral Tests
Mice were trained for 3 days before AAV–NT-1 injection. Baseline values were generated by averaging 3 trials. Animals were tested at 1–4 weeks after tMCAO. Beam-walking and rotarod tests were performed as described previously.13

For the beam-walking test, mice were trained to traverse a horizontally elevated square beam with 5-mm side length to reach an escape platform placed 1 meter away. Mice were placed on one end of the beam, and the latency to traverse 80% of the beam toward the escape platform was recorded. Motor test data were analyzed as mean latency to cross the beam from 3 trials.

For the rotarod test, the task requires the mice to balance on a rotating rod. Animals were allowed a 1 minute adaptation period on the rod at rest, after which the rod was steadily accelerated to 40 revolutions per minute over 2 minutes, and time spent on the rod was recorded. Motor test data were analyzed as mean duration on the rotarod from 3 trials.

Immunohistochemistry and Immunofluorescence
Immunohistochemistry was performed according to the protocol described previously.10 After blocking with 10% bovine serum albumin, brain sections were incubated with primary antibodies at the following dilutions: NT-1 (1:100; Santa Cruz Biotechnology Inc, Santa Cruz, CA), DCC (1:300; Santa Cruz), UNC5H2 (1:300; R&D systems, Tustin, CA), NeuN and GFAP (1:500; Millipore Inc, Billerica, MA), GluT-1 (1:300; Thermo, Waltham, MA), CD31 (1:300; R&D systems), and Proliferating Cell Nuclear Antigen (PCNA, 1:1000; Abcam, Cambridge, England) overnight at 4°C. Sections were then incubated with biotinylated or fluorescence-conjugated secondary antibodies. Each experiment had appropriate positive and negative controls.

Microvessel Counting
Frozen coronal sections (20 μm thick) were fixed with 4% paraformaldehyde at room temperature for 10 minutes and then incubated with fluorescein-lycopersicin esculentum lectin (1:400, Vector Laboratory, Burlingame, CA) overnight at 4°C. Microvessel density was quantified by counting the number of microvessels in 3 microscopic fields at the left, right, and bottom of the peri-infarct area of striatum.

Western Blot Analysis
An equal amount total protein of brain sample was loaded on 10% resolving gel for electrophoresis. Subsequently, proteins were transblotted onto a nitrocellulose membrane (Whatman Inc, Florham Park, NJ). The membrane was placed in 5% nonfat milk in 0.1% TBST for 1 hour to block nonspecific binding and immunoprobed with NT-1 primary antibodies overnight at 4°C. After washing with
TBST, the blots were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature and then reacted with an enhanced ECL substrate (Pierce, Rockford, IL). The result of chemiluminescence was recorded with an imaging system (Bio-Rad, Hercules, CA).

Synchrotron Radiation Microangiography In Vivo
Synchrotron radiation microangiography was performed at Beam line 13W of the Shanghai Synchrotron Radiation Facility. A PE-10 tube connected with a syringe pump (Longerpump Inc, Baoding, China) was carefully inserted into the proximal external carotid artery of the tMCAO mice after 28 days of reperfusion. The mouse was placed perpendicular to the X-ray beam, lying on its right side. X-ray energy was 33.3 keV, just above the iodine K-edge energy. Nonionic iodine contrast agent (350 mg/mL, Omnipaque, GE, Fairfield, CO) was injected from external carotid artery into internal carotid artery at a rate of 2 mL/min, with a total volume of 100 μL. The CCD camera was placed 650 mm from the animal. Dynamic images were obtained every 172 ms, with a resolution of 13 μm per pixel. Six animals per group were subjected to synchrotron radiation imaging.

Statistical Analysis
Parametric data in different groups were compared using a 1-way ANOVA followed by Student-Newman-Keuls test. All data were presented as mean±SD. A probability value of <5% was considered statistically significant.

Results
Fluorescence Microscopy
Fluorescence microscopy showed many GFP-positive cells in both adjacent and distal to the needle tracks of the ipsilateral hemisphere (Figure 1A). Western blot analysis showed that NT-1 expression was significantly increased in AAV–NT-1–injected mice, compared with AAV-GFP or saline-injected mice (Figure 1B, P<0.05). Immunocytochemistry showed substantial numbers of NT-1–positive cells in the brain 7 days after tMCAO. However, NT-1–positive cells were barely detected in the mouse brain treated with AAV-GFP or saline. Double-labeled immunostaining revealed that NT-1 proteins were expressed primarily in neurons and astrocytes but not endothelial cells (Figure 1C). In addition, expression of NT-1 receptors (DCC and UNC5H2) was found in neurons, astrocytes, and endothelial cells (Figure 1D).
Infarct Size After Stroke

Next, we examined whether AAV-mediated NT-1 hyperexpression affected the outcome after focal ischemia. The modified suture model produced stable infarct that is primarily striatal. As shown in Figure 2, AAV–NT-1–treated ischemia mice reduced infarction volume compared with that observed in saline or AAV-GFP–treated ischemia mice (AAV–NT-1/H11005 = 2.6±1.7, saline = 9.7±2.5, and AAV-GFP = 8.8±2.9 mm³; P<0.01), suggesting that NT-1 affected histological outcome after focal ischemia.

Neurological Outcomes

We then determined whether AAV-mediated NT-1 hyperexpression affected the neurological deficits after focal ischemia. Neurobehavioral outcome was measured at 1–4 weeks after tMCAO. Beam-walking and rotarod tests indicated that there was not significant difference in performance among saline-treated, AAV-GFP–, and AAV–NT-1–treated groups before tMCAO. However, neurological deficits measured by the beam-walking and rotarod tests were significantly improved in AAV–NT-1–treated mice than in saline-treated or AAV-GFP–treated mice at 1, 2, and 4 weeks of reperfusion. (Figure 3, P<0.05).

Neovascularization

We confirmed that the number of microvessels in peri-infarct area was significantly increased in AAV–NT-1–treated mice compared with the saline and AAV-GFP–treated mice (Figure 4, P<0.05). Dual-labeled immunofluorescence showed that PCNA-positive cells expressed CD31, indicating the presence of proliferating endothelial cells. Significantly more PCNA/CD31-positive cells were observed in AAV–NT-1–treated mice than in saline and AAV-GFP–treated groups (Figure 5: AAV–NT-1 = 9.8±2.8, AAV-GFP = 2.8±1.2, and saline = 2.7±1.6 cells/field, P<0.001).

Synchrotron Radiation Microangiography

Using synchrotron radiation angiography in living animals, we also found that branches of left MCA in AAV–NT-1–treated mice were greater than that in the control groups 28 days after tMCAO, suggesting that NT-1 hyperexpression could promote local angiogenesis and new vascular system remodeling after focal ischemia (Figure 6). The number of MCA branches within the control group or the NT-1–treated group was consistent. However, quantification of branches using synchrotron radiation angiography is a challenge same as in the CTA/MRA quantification. We are currently developing software to quantify angiogenesis based on synchrotron radiation angiography.

Discussion

Increased AAV-mediated NT-1 protein in the mouse brain significantly reduced infarct volume and improved neurobehavioral outcome compared with the saline and AAV-GFP–treated mice. Immunohistochemistry and synchrotron radiation angiography demonstrated that microvessel density and collateral vessels were increased in AAV–NT-1–treated mice, suggesting that NT-1 promoted local neovascularization and vessel remodeling in injured brain. In the present study, we achieved NT-1 hyperexpression in neurons and astrocytes in the ischemic brain using AAV–NT-1 virus. NT-1 receptors including DCC and UNC5H2 were expressed in neurons, astrocytes, and endothelial cells of the brain after tMCAO. AAV–NT-1–mediated gene transfer is a reliable tool for long-term and steady expression of NT-1 protein. We chose the AAV–NT-1 vector for delivery into mouse brains because it has higher transduction ability with less toxicity.14,15 Our previous study showed that NT-1 expression was significantly increased at mRNA and protein levels in AAV–NT-1–transfected HEK293 cells.10 In the present study, we proved that AAV–NT-1 can effectively transfer into the mouse brain neurons and astrocytes.

Netrins are highly conserved laminin-associated secret proteins.1 As one of the family members in mammals, NT-1 shows several functions in neural system development. NT-1 would induce mouse mammary epithelial cell invasion and migration, mediate pancreatic epithelial cell adhesion, and promote tumor growth.
epithelial cell survival. In ex vivo experiments, NT-1 induced the aortic endothelial cells proliferation and migration. NT-1 hyperexpression increased the number of neovessels in ischemic muscles and reversed neural and vascular pathology in a diabetic rodent model. In cardiac ischemia and reperfusion, NT-1 reduced myocardial infarct volume by DCC/ERK1/2 feedback mechanism in cardiac endothelial cells and myocardial cells. NT-1 also protected renal tubular epithelial cells against ischemia reperfusion-induced injury by increasing proliferation and suppressing apoptosis of the cells. NT-1 hyperexpression induced angiogenesis in adult mouse brains. Consistently, our data showed that AAV–NT-1 can induce angiogenesis in the ipsilateral hemisphere of an adult mouse model of focal ischemia. Our data indicated that NT-1 hyperexpression reduced infarct volume, which was associated with increased functional microvessels in the peri-infarct area. We further demonstrated that NT-1 hyperexpression effectively improved motor function as assessed by beam walking and rotarod tests at different time points after MCAO. These findings suggest that NT-1 can participate in neovascularization and vessel remodeling process, which may contribute to long-term functional outcome after ischemic cerebral injury. Further studies are needed to access the rescue effects of NT-1 gene or protein transferred after cerebral ischemia, which would be more valuable in translation to clinical practice. With the development of safer virus-mediated gene therapy techniques, NT-1 gene therapy might provide a promising strategy for stroke treatment.

NT-1 receptors include DCC and UNC5 family members. NT-1 attracts axons through interaction with DCC and repels axons by UNC5 homodimer or UNC5-DCC heterodimer. The involvement of UNC5H2 and DCC in NT-1–induced angiogenesis is still a topic of debate. NT-1 receptors include DCC and UNC5 family members. The ligand-receptor complexes and the control of axon growth and guidance of DCC family receptors convert netrin-induced growth cone attraction to repulsion. Consistently, our data showed that AAV–NT-1 can induce angiogenesis in the ipsilateral hemisphere of an adult mouse model of focal ischemia. Our data indicated that NT-1 hyperexpression reduced infarct volume, which was associated with increased functional microvessels in the peri-infarct area. We further demonstrated that NT-1 hyperexpression effectively improved motor function as assessed by beam walking and rotarod tests at different time points after MCAO. These findings suggest that NT-1 can participate in neovascularization and vessel remodeling process, which may contribute to long-term functional outcome after ischemic cerebral injury. Further studies are needed to access the rescue effects of NT-1 gene or protein transferred after cerebral ischemia, which would be more valuable in translation to clinical practice. With the development of safer virus-mediated gene therapy techniques, NT-1 gene therapy might provide a promising strategy for stroke treatment.

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Disclosures

None.

References

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