Delayed Administration of Tat-HA-NR2B9c Promotes Recovery After Stroke in Rats

Hai-Hui Zhou, PhD; Ying Tang, PhD; Xin-Yong Zhang, PhD; Chun-Xia Luo, PhD; Li-Yan Gao, MS; Hai-Yin Wu, BS; Lei Chang, MS; Dong-Ya Zhu, PhD

Background and Purpose—Previous studies reported that Tat-NR2B9c, a peptide disrupting the N-methyl-D-aspartate receptor–postsynaptic density protein-95 interaction, reduced ischemic damage in the acute phase after stroke. However, its effect in the subacute phase is unknown. The aim of this study is to determine whether disrupting the N-methyl-D-aspartate receptor–postsynaptic density protein-95 interaction in the subacute phase promotes recovery after stroke.

Methods—Studies were performed on Sprague-Dawley rats or nNOS−/− mice, and experimental ischemic stroke was induced by middle cerebral artery occlusion. Animals were treated with drugs starting at day 4 after ischemia. Sensorimotor functions and spatial learning and memory ability were assessed after drug treatment. Then, rats were euthanized for morphological observation and biochemical tests.

Results—Disrupting the N-methyl-D-aspartate receptor–postsynaptic density protein-95 interaction with Tat-HA-NR2B9c significantly ameliorated the ischemia-induced impairments of spatial memory and sensorimotor functions in rats during subacute stage but did not improve stroke outcome in nNOS−/− mice. Consistent with the functional recovery, Tat-HA-NR2B9c substantially increased neurogenesis in the dentate gyrus and dendritic spine density of mature neurons in the motor cortex of rats, meanwhile, reversed the ischemia-induced formation of S-nitrosylation-cyclin-dependent kinase 5 and increased cyclin-dependent kinase 5 activity in ipsilateral hippocampus. However, directly blocking N-methyl-D-aspartate receptors with MK-801 or Ro 25-6981 did not show the beneficial effects above.

Conclusions—Disassociating N-methyl-D-aspartate receptor–postsynaptic density protein-95 coupling by Tat-HA-NR2B9c in the subacute phase after stroke promotes functional recovery, probably because of that it increases neurogenesis and dendritic spine density of mature neurons via regulating cyclin-dependent kinase 5 in the ischemic brain.

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Key Words: cyclin-dependent kinase 5 • nitrosation • regeneration • stroke • Tat-NR2B9c

Stroke is a leading cause of death and adult-acquired disability. Many of the sensory, motor, and cognitive impairments caused by stroke eventually improve, suggesting an ability of brain to rewire itself to restore lost functionalities. The modification of neuronal connections may result from axonal sprouting, neurogenesis, or remodeling of dendritic arbors. These raise the possibility that promoting regenerative repair, including neurogenesis, synaptogenesis, and neural regeneration, may improve stroke outcome even in a delayed phase when options for early interventions have been missed.

Glutamate is the major excitatory transmitter in the mammalian central nervous system. Immediately after ischemia, glutamate accumulates at synapses and leads to excitotoxicity. N-Methyl-D-aspartate receptors (NMDARs) constitute the major subtype of glutamate receptors. Thus, blockade of NMDARs should be logically neuroprotective. Unfortunately, NMDARs play an important role in normal circuit rebuilding.

NMDAR-mediated Ca2+ influx contributes to excitation–neurogenesis coupling. NMDAR activation is critical for neuroblast survival before entering a synaptic network. Deletion of NMDARs leads to defects in adult hippocampal neurogenesis. Moreover, NMDARs contribute to synaptogenesis and synapse stabilization and dendritic complexity of adult-born neurons. Thus, directly inhibiting NMDARs may impair endogenous mechanisms for brain repair.

Postsynaptic density protein-95 (PSD-95) is a scaffolding protein that binds both NMDARs and neuronal nitric oxide synthase (nNOS) at excitatory synapses. This binding couples NMDAR activity to the production of NO, a signaling molecule that mediates NMDAR-dependent excitotoxicity. Interference peptide Tat-NR2B9c, which comprises of the last 9 amino acids of the carboxyl tail of GluN2B, one of the modulatory subunits of NMDARs, and the 11-mer Tat protein transduction domain that renders...

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the peptide plasma membrane permeable, disrupts the NMDAR–PSD-95 interaction and thereby protects neurons against NMDAR-mediated excitotoxicity without affecting NMDAR current, calcium loading,11,12 NMDAR-dependent synaptic plasticity, or neuronal survival signaling.13 In 2008, it was reported that intravenously using Tat-NR2B9c 1 to 3 hours after stroke in rats reduced infarct volume and improved neurological outcome.14 Moreover, in 2012, treatment with Tat-NR2B9c 1 hour after stroke in nonhuman primates reduced infarct volume.15 Most importantly, it was reported in 2012 that Tat-NR2B9c reduced the number of iatrogenic ischemic infarcts in patients receiving endovascular repair for intracranial aneurysm.16 These findings suggest that Tat-NR2B9c could be valuable for first aid of stroke. However, it remains unknown whether Tat-NR2B9c improves stroke outcome even administrated in a delayed time frame when options for early interventions have been missed. Recently, we prepared a recombinant chimeric peptide Tat-HA-NR2B9c containing Tat-NR2B9c and an influenza virus hemagglutinin epitope-tag and found that treatment with Tat-HA-NR2B9c 1 hour after reperfusion reduced infarct volume in rats subjected to middle cerebral artery occlusion (MCAO).17 Here, we show that a delayed treatment with Tat-HA-NR2B9c after stroke substantially ameliorated the ischemia-induced loss of dendrite spine in the motor cortex, increased neurogenesis in the hippocampal dentate gyrus, and significantly improved sensorimotor functions and cognitive abilities.

Materials and Methods

Drugs

Tat-HA-NR2B9c was prepared in our laboratory.17 MK-801 and RO 25-6981 were purchased from Sigma-Aldrich.

Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. In this study, adult male Sprague-Dawley rats (250–300 g; B&K Universal Group Limited, Shanghai) and nNOS-deficient (nNOS−/−) mice (B6; 129S4-Nos1tm1Plh; stock number: 002633; Jackson Laboratories; maintained at Model Animal Research Center of Nanjing University, Nanjing, China) were used. An experimenter labeled all animals before allocation. Experiments were performed by investigators who were blinded to group allocation.

Surgical Preparation

Focal cerebral ischemia was induced by intraluminal MCAO as described previously.8,15 Before experiments, we did correlation analysis between cerebral blood flow decline and neuroscore after MCAO to control transient MCAO efficacy. There was a positive correlation between neuroscore and the percentage of cerebral blood flow decline after stroke (n=14; R=0.931; P<0.001; Figure 1 in the online-only Data Supplement). Only animals with a certain degree of neurological deficits were used (more details are available in Methods in the online-only Data Supplement).

Behavioral Assessment

Modified Neurological Severity Score test,19 Morris water maze task, Barnes circular maze, grid-walking task, and cylinder task18 were performed as previously reported (more details are available in Methods in the online-only Data Supplement).

Immunofluorescence

Immunofluorescence was performed as we previously reported18 (more details are available in Methods in the online-only Data Supplement).

Biotin-Switch Assay

S-Nitrosylation (SNO) level of tissue protein was detected by biotin-switch assay as previously described9 (more details are available in Methods in the online-only Data Supplement).

Western Blot Analysis

Western bolt analysis was performed as before.4 Primary antibodies were rabbit anti–cyclin-dependent kinase 5 (Cdk5; 1:200; Santa Cruz), rabbit anti-p55 (1:200; Santa Cruz), mouse anti–histone H1 (1:200; Abcam), and mouse anti–phosphohistone H1 (1:1000; Millipore). Internal control was mouse anti–β-actin (1:1000; Sigma-Aldrich). Appropriate horseradish peroxidase–linked secondary antibodies were used for detection by enhanced chemiluminescence (Pierce).

Golgi-Cox Staining

Golgi-Cox staining was performed to show subtle morphological alterations in neuronal dendrites and dendritic spines.10 For morphological analysis, 8 neurons randomly for each sample were measured and the average was regarded as the final value of one sample (more details are available in Methods in the online-only Data Supplement).

Data Analysis

Comparisons among multiple groups were made with 1-way ANOVA (1 factor) followed by Scheffe post hoc test. Data collected at repeating time points were analyzed by 2-way repeated measures ANOVA, followed by Bonferroni post hoc test. Data were presented as mean±SD; P<0.05 was considered statistically significant.

Results

Disrupting the NMDAR–PSD-95 Interaction but Not Directly Blocking NMDARs Improves Stroke Outcome in the Delayed Phase

To examine whether a delayed treatment with Tat-HA-NR2B9c benefits stroke outcome, we subjected rats to MCAO and treated them with Tat-HA-NR2B9c (1.12 mg/kg IV) for 7 days or selective noncompetitive NMDAR antagonist MK-801 (1 mg/kg IP) for 4 days starting at day 4 after MCAO. We used MK-801 only for 4 days because treatment with MK-801 (1 mg/kg) for >4 days after stroke caused abnormal behaviors, such as convulsion, salivation, and hypoactivity (data not shown). Tat-HA-NR2B9c-, MK-801- or vehicle-treated rats displayed similar infarct volume (31±7.1%; 33.6±7.6% versus 31.8±8.8%), suggesting no decreased damage when given in the delayed manner. Sensorimotor functions were assessed at days 11, 18, 32, and 46 after MCAO by forelimb foot faults in grid-walking task and forelimb asymmetry in cylinder task. Spatial cognitive performance in Morris water maze was measured during days 33 to 39 after MCAO (Figure 1A). Ischemia significantly increased escape latency (F(2,34)=17.40; P<0.001), decreased time spent in target quadrant (F(3,102)=10.21; P<0.001) and target crossings (F(3,102)=16.40; P<0.001) in Morris water maze (Figure 1B), and increased foot faults in the grid-walking task (F(3,102)=29.92; P<0.001; Figure 1C) and forelimb asymmetry scores in the cylinder task (F(3,102)=144.19; P<0.001; Figure 1D), compared with sham, suggesting impaired spatial
Memory and sensorimotor functions. The delayed treatment with Tat-HA-NR2B9c significantly ameliorated the ischemia-induced impairments of spatial memory (P < 0.002, in latency; P < 0.004, in total time in target quadrant; P < 0.023, in target crossings) and sensorimotor functions (P < 0.001, in grid-walking task; P < 0.001, in cylinder task), compared with vehicle, whereas MK-801 did not (Figure 1B–1D), although both of them significantly reduced infarct size and neurological deficit when immediately given after reperfusion (Figure II–IIC in the online-only Data Supplement). All rats were able to remember the target right at day 39. However, when we assessed the maintenance of the spatial memory 1 week later, we found that Tat-HA-NR2B9c–treated rats still remembered the right target, whereas MK-801–treated rats did not (Figure IIID in the online-only Data Supplement).

MK-801 is an efficient blocker of both synaptic and extrasynaptic NMDARs. Stimulating synaptic NMDARs leads to cAMP response element-binding protein activation and brain-derived neurotrophic factor production. The inefficacy of MK-801 in the delayed phase is likely due to the blockade of synaptic NMDARs. GluN2B-containing NMDARs are enriched at extrasynaptic sites. Next, we investigated whether a delayed treatment with GluN2B antagonist improves stroke outcome. We subjected rats to MCAO and treated them with a selective GluN2B antagonist Ro 25-6981 (3 mg/kg IV, a dose being highly neuroprotective when used immediately after reperfusion in MCAO rats [Figure IV A–IVC in the online-only Data Supplement]) for 7 days starting at day 4 after MCAO. Sensorimotor functions and spatial cognitive performance were assessed at time points shown in Figure 1A. Similar to MK-801, the delayed treatment with Ro 25-6981 did not ameliorate the ischemia-induced impairments of spatial memory (F(2,29) = 10.34 and P = 0.001 in latency; F(2,29) = 5.77 and P = 0.035 in total time in target quadrant; F(2,29) = 3.12 and P = 0.05 in grid-walking task; F(2,29) = 41.08 and P = 0.001 in cylinder task), compared with vehicle (Figure 2A–2C). Taken together, our data strongly suggest that disrupting the NMDAR–PSD-95 interaction but not directly blocking NMDARs improves stroke outcome in the delayed phase.

**Figure 1.** Disrupting the N-methyl-D-aspartate receptor (NMDAR)–postsynaptic density protein-95 (PS-95) interaction but not blocking NMDARs improves stroke outcome in the delayed phase. A, Schematic representation of experimental design for B–D. B, Escape latency measured during days 34 to 38, time spent in target quadrant and target crossings measured at day 39 after middle cerebral artery occlusion (MCAO) in Morris water maze task. C, Functional recovery was assessed with forelimb foot faults in grid-walking task. D, Forelimb asymmetry was assessed in cylinder task (n=9 for sham, vehicle, and MK-801; n=11 for Tat-HA-NR2B9c). Data are means±SD (**P<0.001 vs sham; &P<0.05, ##P<0.01, and ###P<0.001 vs vehicle; &P<0.05 and &P<0.01 vs MK-801).
significantly increased escape latency ($F_{1,26}=19.54; P<0.001$) and decreased target crossings ($F_{1,26}=7.62; P=0.014$) in Morris water maze (Figure 3A), and increased foot faults in the grid-walking task ($F_{1,26}=51.50; P<0.001$; Figure 3B) and forelimb asymmetry scores in the cylinder task ($F_{1,26}=39.87; P<0.001$; Figure 3C) in nNOS−/− mice, compared with sham. However, Tat-HA-NR2B9c did not ameliorate the ischemia-induced impairments in nNOS−/− mice ($P=1.000$ in latency; $P=0.963$ in target crossings; Figure 3A–3C), compared with vehicle.

Our data suggest that enhancement of functional recovery by delayed treatment with Tat-HA-NR2B9c is nNOS dependent.

**Tat-HA-NR2B9c Promotes Regenerative Repair After Stroke**

NMDARs mediate neurogenesis and synaptic plasticity, thereby contribute to memory formation.21–26 Accordingly, we investigated whether delayed treatment with Tat-HA-NR2B9c increases neurogenesis and synaptic plasticity. Rats were treated with BrdU (100 mg/kg×2 IV; 24-h intervals) to label proliferating cells during days 4 to 5 after MCAO and euthanized for BrdU/NeuN staining or Golgi-Cox staining at days 46 (Figure 4A). Tat-HA-NR2B9c given from days 4 to 10 after stroke substantially increased neurogenesis in the dentate gyrus ($F_{1,26}=12.19; P=0.027$) and reversed the ischemia-induced dendrite spine loss in the motor cortex ($F_{1,26}=15.11; P=0.007$), but MK-801 did not ($P=0.997$ in neurogenesis; $P=0.999$ in spine density), compared with vehicle (Figure 4B and 4C).

Moreover, neuroblasts migrating from the subventricular zone into the ischemic striatum after stroke contribute to functional recovery.18 We thus examined the effect of Tat-HA-NR2B9c and MK-801 on the number of DCX+ (a marker for immature neurons) cells in the striatum in MCAO rats. Treatment with Tat-HA-NR2B9c but not MK-801 significantly increased the total number and the migration distance.
of DCX+ cells (Figure V in the online-only Data Supplement), indicating substantially increased newborn neurons in the ischemic striatum.

**Tat-HA-NR2B9c Reverses the Ischemia-Induced Alteration of Cdk5**

What is the underlying mechanism by which Tat-HA-NR2B9c improves regenerative repair? It is well accepted that Cdk5 is involved in the regulation of dendritic spine growth, neurogenesis, and synaptic plasticity, and exerts a critical role in neurodegenerative diseases, including stroke. We thus investigated whether Tat-HA-NR2B9c affects Cdk5 after ischemia. We subjected rats to MCAO and measured levels of Cdk5 and histone H1 phosphorylation, which reflects the enzymatic activity of Cdk5, at days 1, 4, 7, and 10 after MCAO in ipsilateral hippocampus. Ischemia caused a significant increase in Cdk5 activity (F4,15=8.47; *P*<0.05, 0.019, 0.003, and 0.048 for days 1, 4, 7, and 10 respectively; Figure 5A), although Cdk5 protein level did not change significantly (F4,15=1.89; *P*=0.916, 0.998, 0.987, and 0.642 for days 1, 4, 7, and 10 respectively; Figure 5B). Moreover, ischemia did not change the level of p35 (F4,15=2.55; *P*=0.672, 0.350, 0.806, and 0.989 for days 1, 4, 7, and 10 respectively; Figure 5C), a protein regulating Cdk5 activity, compared with sham. To know how ischemia activates Cdk5, we measured SNO of Cdk5, another way to activate Cdk5. Indeed, ischemia led to remarkable increase in SNO of Cdk5 in ipsilateral hippocampus (Figure 5D). Therefore, ischemia stimulates Cdk5 activity via SNO after stroke. Finally, we investigated the effect of Tat-HA-NR2B9c on Cdk5 activation. As expected, treatment with Tat-HA-NR2B9c for 7 days starting at day 4 after MCAO reversed the ischemia-induced formation of SNO-Cdk5 and increase in Cdk5 activity in ipsilateral hippocampus, but MK-801 did not (F4,15=8.78, *P*=0.026, Tat-HA-NR2B9c versus vehicle; *P*=0.986, MK-801 versus vehicle; Figure 6A and 6D). Moreover, neither Tat-HA-NR2B9c nor MK-801 changed Cdk5 and p35 protein levels (Figure 6B and 6C).

**Discussion**

Although regenerative processes after stroke are activated over months leading to a certain degree of functional recovery, they are not enough to fundamentally change the life quality of patients. New therapeutics may emerge from understanding how to overcome inhibitory mechanisms that block regeneration. It is reported that dampening tonic inhibition by an α5-GABAAR antagonist from day 3 after stroke showed reduced cell death. However, studies concerned with delayed treatment are deficient.

Recently, we prepared a recombinant Tat-HA-NR2B9c peptide by genetic engineering techniques. The chimeric peptide blocked the ischemia-induced NMDAR–PSD-95 association and had potent neuroprotection in acute treatment of stroke. Here, we found that uncoupling NMDARs and PSD-95 by Tat-HA-NR2B9c without directly blocking NMDARs in the subacute phase could improve ischemia-induced impairments of spatial memory and sensorimotor functions, and neurofunctional deficient (Figure VI in the online-only Data Supplement). NMDAR activation mediates calcium influx, cAMP response element-binding protein phosphorylation, and brain-derived neurotrophic factor production, which are necessary to neurogenesis, dendritic plasticity, and synaptogenesis. Therefore, directly inhibiting NMDARs might be deleterious to animals and human. NMDAR activity is unaffected by genetically deleting PSD-95 in vivo or by suppressing its expression in vitro. NMDAR-mediated EPSCs are also unaffected when the nNOS–PSD-95 interaction is disrupted. These findings may explain why treatments with Tat-HA-NR2B9c and NMDAR antagonists in the subacute phase had substantially different outcome for stroke.
NO donor and neuron-derived nNOS inhibit neural stem cell proliferation and neuronal differentiation.\textsuperscript{34} Dissociating the NMDAR–PSD-95 interaction by Tat-HA-NR2B9c decreases the production of NO.\textsuperscript{11} Thus, enhancement of neurogenesis and, moreover, had similar swimming speed to locate the hidden platform trial, suggesting normal cued behavior of ischemia induced the formation of SNO-Cdk5 and increased spine density in ischemic brain. However, NMDAR antagonist MK-801 had no effect on Cdk5 activity. It is reported that stimulation of synaptic NMDARs leads to a decrease in Cdk5 activity,\textsuperscript{15} NO production, and in turn, Cdk5 SNO activate Cdk5. MK-801 blocks both synaptic NMDARs and NMDAR–PSD-95–nNOS–NO pathway. These complex effects of MK-801 may counteract each other and cause a null-effect on Cdk5. In this study, animals had similar latency to reach platform in the visible platform trial, suggesting normal cued behavior and, moreover, had similar swimming speed to locate the hidden platform, indicating no disturbances in gross motor skills (Figure VII in the online-only Data Supplement).

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**Disclosures**

None.

**References**


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Supplementary Methods

Surgical preparation

Focal cerebral ischemia was induced by intraluminal MCAO as described previously.\textsuperscript{1,2} Under chloralhydrate anesthesia (350 mg/kg, intraperitoneally), a 4/0 (for rats) surgical nylon monofilament with rounded tip was introduced into the internal carotid artery (ICA) through the external carotid artery advanced 20-21 mm past the carotid bifurcation until a slight resistance was felt, effectively occluding the middle cerebral artery, then the filament was withdrawn after 120 min and the external carotid artery was ligated. Body temperature was maintained at 37 ± 0.5 °C with a thermostatically controlled infrared lamp. Animals were then returned to their cages and closely monitored until they recovered from anesthesia. Only animals with a certain degree of neurological deficits were used. In sham-operated rats, the ICA was surgically prepared for insertion of the filament, but the filament was not inserted.

Infarct volume measurement and neuroscore assessment

The neuroscore assessment and infarct volume measurement were performed 24 and 24.5 h after MCAO respectively as described previously.\textsuperscript{1} In brief, brains were removed rapidly and frozen at -20 °C for 5 min. Coronal slices were made at 1-2 mm from the frontal tips, and sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) at 37 °C for 20 min. The presence or absence of infarction was determined by examining TTC-stained sections for the areas on the side of infarction that did not stain with TTC. Infarct volume was expressed as a percentage area of the coronal section in the infarct hemisphere. Neuroscore assessment was performed by an experimenter blinded to the experimental groups (Rating scale: 0 = no deficit, 1 = failure to extend left forepaw, 2 = decreased grip strength of left forepaw, 3 = circling to left by pulling the tail, and 4 = spontaneous circling).

Modified Neurological Severity Score (NSS) test

Measurement of neurological functional outcome was determined by the NSS test\textsuperscript{3}. The score was graded from 0 to 18 (normal score, 0; maximal deficit score, 18). Severe injury is indicated by a score of 13 to 18, moderate injury 7 to 12, and mild injury 1 to 6. In the severity scores of impairment, one point is scored for the inability to perform the task or lacking proper response for a given reflex.

Correlation analysis between CBF decline and neuroscore

To determine whether MCAO is successful, regional CBF was monitored. We measured baseline CBF (before ischemia) and CBF after insertion of the occluding filament (during ischemia) and calculated the percentage of CBF decline (during ischemia/before ischemia). Neuroscore assessment was performed after MCAO. Strength of association between the CBF decline and neuroscore was estimated with Pearson correlation coefficient (\(R\)). The overall correlation was \(R = -0.931\) (\(P < 0.001\)). Our data showed that neuroscore correlated well with the decline of CBF after MCAO. Thus, those animals whose neuroscore was lower than 1 were excluded at d 1 after MCAO. Animals were randomly allocated to groups at d 4
(before drug administration) by computer-generated randomization schedules.

**Morris water maze task**

The protocol of Morris water maze task for rats was similar to that for mice, which has been detailedly described in our previous study. During the visible platform trials, rats were placed in the opaque water of the circular swimming pool (180 cm in diameter, Jiliang Neuroscience Inc.), the platform was elevated 0.5 cm above the water level and marked by a yellow label. The location of the visible platform varied for each trial. Four trials were administered. The latency to reach the visible platform and swimming speed were measured. Then, rats were trained to locate the hidden platform 1.2 cm under the surface of the water. The room contained numerous cues on the walls, which remained constant throughout the experiment, in order to aid animals in this spatial memory task. In the training to find the hidden platform, rats were allowed to swim for maximum of 60 s in the pool for each trial. One block of four trials per day was given for 5 consecutive days. Each trial was videotaped via a ceiling-mounted video camera and the animal’s movement was tracked using Ethovision software (Noldus Information Technology), which allows the calculation of various measures such as latency (time to reach the platform). Next day, rats were given one 60-s retention probe test in which the platform was removed from the pool. During retention, the number of crossings of the platform location and the time spent in the target quadrant were measured.

**Barnes circular maze**

Spatial task performance was tested in the Barnes circular maze. This test was performed from d 33 to d 39 and d 46 after MCAO. The test was conducted on a blue circular surface, 1.22 m in diameter, with 18 holes equally spaced around the perimeter. The circular open field was elevated 75 cm from the floor. A blue Plexiglas escape box, which had paper cage bedding on its bottom, was located under one of the holes. The hole above the escape box represented the target, analogous to the hidden platform in the Morris task. The location of the target was consistent for a given rat, but was randomized across rat. The maze was rotated daily, with the spatial location of the target unchanged with respect to the distal visual room cues, to prevent a bias based on olfactory or proximal cues within the maze. Two trials per day were conducted for 6 successive days. At d 39, a probe trial was conducted without the escape box, to confirm that this spatial task was acquired based on navigation using distal environment room cues. One trial was conducted immediately after the probe test and 7 days later another probe trial was conducted. The number of errors to reach the target hole and the time spent around each hole were recorded by video tracking software.

**Grid-walking task**

The protocol of grid-walking task for rats was similar to that for mice, which has been detailedly described in previous reports. Rat ran one trial per day at approximately the same time each day. The grid area was 60 cm×60 cm×50 cm (length×width×height) with 30×30 mm diameter openings. Behavior was recorded using a camera that was placed underneath the grid, in order to assess the animals’ stepping errors (i.e. ‘footfaults’). Animals were given 5 min to walk atop the elevated wire surface. Footfaults for each limb were counted and
compared to the overall step number made by that limb. Thus, % footfaults was calculated by: (#footfaults/#overall steps)×100%. A step was considered a footfault if it was not providing support and the foot went through the grid hole.

**Cylinder task**

The cylinder task encourages use of the forelimbs for vertical wall exploration in a cylinder. When placed in the cylinder, the animal rears to a standing position on the cylinder wall, supporting its weight with either one or both of its forelimbs. A Plexiglas cylinder 30 cm in height with a diameter of 20 cm was used. Animals were placed inside the cylinder and videotaped for 5 min. The top of the cylinder was covered to prevent animals from jumping out. A mirror was placed behind the cylinder at an angle to enable the rater to record forelimb movements when the animal was turned away from the camera. Videotape of animals in the cylinder was evaluated quantitatively in order to determine forelimb preference during vertical exploratory movements. Behavior was scored by observers who were blind to the treatment group of the animals in the study. While the videotape was played in slow motion (1/5th real time speed), the total time (sec) each animal spent on the right forelimb, the left forelimb, and simultaneous use of both forelimbs was assessed. Only wall placements that could be clearly seen (both forelimbs in view) were timed. From these three measures, the amount of time spent on either limb independently as well as the time the animal spent rearing with both limbs on the wall, was derived. The percentage of time spent on each limb was calculated, and these data were used to derive an asymmetry index (% ipsilateral use-% contralateral use).

**Immunofluorescence**

Immunofluorescence was performed as we previously reported. Animals were perfused transcardially with 0.05 M sodium phosphate (pH 7.4) containing 0.8% NaCl, followed by 4% paraformaldehyde in 0.05 M sodium phosphate (pH 7.4, containing 0.8% NaCl). Brains were removed and postfixed overnight in the same solution. Serial hippocampal sections (40 μm) were made on an oscillating tissue slicer in a bath of physiological saline. BrdU staining has been described previously (Luo et al., 2005). The sections were heated (85 °C for 5 min) in antigen-unmasking solution (Vector Laboratories); incubated in 2 M HCl (37 °C for 30 min); rinsed in 0.1 M boric acid, pH 8.5, for 10 min; and blocked in PBS containing 3% normal goat serum, 0.3% (w/v) Triton X-100, and 0.1% bovine serum albumin (BSA) at room temperature for 1 h, followed by incubation with primary antibodies at 4 °C overnight. The primary antibodies used were as follows: rat anti-BrdU (1:200; Accurate Chemical & Scientific Corporation), mouse anti-NeuN (1:500; Millipore Bioscience Research Reagents), and rabbit anti-DCX (1:1000; Abcam). Subsequently, the sections were incubated with secondary antibodies goat anti-rat Cy3 (1:200; Millipore Bioscience Research Reagents), goat anti-mouse dylight 488 (1:400; Jackson ImmunoResearch) and goat anti-rabbit Cy3 (1:200; Jackson ImmunoResearch) for 2 h at room temperature. An experimenter coded all slides from the experiments before quantitative analysis. All BrdU+/NeuN+ cells and DCX+ cells were counted in each section by another experimenter blinded to the study code. The analysis was conducted on every tenth section in a series of 40-μm coronal sections. To determine the total number of BrdU+/NeuN+ cells or DCX+ cells, the counts from sampled
sections were averaged, and the mean values were multiplied by the total number of sections. We used a confocal laser-scanning microscope (LSM700, Zeiss) to capture images and confirm colocalization of BrdU⁺/NeuN⁺ cells.

**Biotin-Switch Assay**
This assay was performed in the dark as previously described.² Briefly, cells were lysed in HEN buffer (250 mM HEPES, 1 mM EDTA and 100 mM neocuproine) and adjusted concentration as needed (not exceed 0.8 μg per μl). Samples were homogenized and free cysteines were blocked for 1 h at 50 °C in three volumes of blocking buffer (HEN buffer plus 2.5% SDS, HENS) containing methyl methanethiosulfonate (0.1%, Sigma-Aldrich). Proteins were precipitated with three volumes of cold acetone at -20 °C and washed with cold acetone (3×5 ml) and resuspended in 240 μl HENS solution. After adding fresh ascorbic acid (20 mM, Sigma-Aldrich) and biotin-HPDP (1 mM, Sigma-Aldrich), proteins were incubated at room temperature for 1 h. Proteins were precipitated with three volumes of cold acetone at -20 °C and washed with cold acetone (4×1 ml). Alternatively, biotinylated proteins were resuspended in 250 μl HENS buffer plus 750 μl neutralization buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) and precipitated with 30 μl prewashed avidin-affinity resin beads (Sigma-Aldrich) at room temperature for 1 h. The beads were washed three times at room temperature using neutralization buffer containing 600 nM NaCl. Biotinylated proteins were eluted using 30 μl elution buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 100 mM β-mercaptoethanol) and heated at 100 °C for 5 min in reducing SDS-PAGE loading buffer.

**Golgi-Cox Staining**
Golgi-Cox staining was performed with FD Rapid Golgi Stain TM Kit (FD NeuroTechnologies) according to user manual.² The fresh brains without perfusion and fixation were used for Golgi-Cox staining to show subtle morphological alterations in neuronal dendrites and dendritic spines. Large brain specimens should be sliced with a sharp blade into blocks of approximately 10 mm thickness. Immerse brains in the impregnation solution at room temperature for 2 weeks. Transfer tissue into Solution C and store at 4 °C in the dark for at least 48 hours. Then brains were cut into 100 μm coronal sections using a vibratome (World Precision Instruments) and stained. For morphological analysis, eight neurons randomly for each sample were measured and the average was regarded as the final value of one sample.

**Supplemental References**
Figure I. Scatterplot for correlation between neuroscore and percentage of CBF decline after stroke. (n = 14, R = -0.931, P < 0.001).
Figure II. MK-801 and Tat-HA-NR2B9c prevent cerebral ischemia. (A) Representative of triphenyltetrazolium chloride-stained slices. Infarct size ($F_{3.35} = 49.05, P < 0.001$, MK-801 vs Vehicle; $P = 0.005$, Tat-HA-NR2B9c vs Vehicle. B) and neurological score ($F_{3.35} = 30.94, P < 0.001$, MK-801 vs Vehicle; $P = 0.009$, Tat-HA-NR2B9c vs Vehicle. C) were measured at 24.5 and 24 h after reperfusion. (n=10 for Sham, MK-801 and Tat-HA-NR2B9c, n=9 for Vehicle). Data are mean ± SD (***$P < 0.001$, vs Sham; ##$P < 0.01$, ###$P < 0.001$, vs Vehicle).
Figure III. Tat-HA-NR2B9c improves stroke outcome in the subacute phase. (A) Schematic representation of experiment design for B-D. (B-C) The latency ($F_{3,36} = 5.51, P = 0.007$, Vehicle vs Sham. B) and number of error trials to target ($F_{3,36} = 7.35, P = 0.003$, Vehicle vs Sham. C) during training (d 33-38) were measured. (D) Histogram summarizes statistical data of the latency ($F_{3,36} = 8.55, P = 0.027$, Tat-HA-NR2B9c vs Vehicle at d 46; P = 0.984, MK-801 vs Vehicle at d 46) and error to target ($F_{3,36} = 8.18, P = 0.025$, Tat-HA-NR2B9c vs Vehicle at d 46; P = 0.967, MK-801 vs Vehicle at d 46) and time spent in target quadrant ($F_{3,36} = 8.30, P = 0.049$, Tat-HA-NR2B9c vs Vehicle at d 46; P = 0.980, MK-801 vs Vehicle at d 46) at d 39 and 46. (n=9 for Sham, n=8 for Vehicle, n=11 for MK-801, n=12 for Tat-HA-NR2B9c). Data are mean ± SD (**P < 0.01; *P < 0.05, vs Vehicle; &P < 0.05, &&P < 0.01, vs MK-801).
Figure IV. RO 25-6981 prevent cerebral ischemia. (A) Representative of triphenyltetrazolium chloride-stained slices. Infarct size ($F_{2.28} = 38.08, P = 0.003$, Ro-25-6981 vs Vehicle. B) and neurological score ($F_{2.28} = 35.21, P = 0.007$, Ro 25-6981 vs Vehicle. C) were measured at 24.5 and 24 h after reperfusion. (n=10 for Sham and Vehicle, n=11 for RO 25-6981). Data are mean ± SD (**P < 0.001, vs Sham; ##P < 0.01, vs Vehicle).
Figure V. Tat-HA-NR2B9c increases the total number and the migration distance of DCX⁺ cells in the striatum (STR). We subjected rats to MCAO and treated them with MK-801 for 4 d or Tat-HA-NR2B9c for 7 d starting at d 4 after MCAO. Then, rats were sacrificed to estimate the number of DCX⁺ (a marker for immature neurons) cells at d 18. (A) Representative of DCX⁺ cells in the Striatum. Scale bar, 200 μm. (B-C) Bar graph showing the total number ($F_{3,17} = 16.25, P = 0.031$, Tat-HA-NR2B9c vs Vehicle; $P = 1.000$, MK-801 vs Vehicle, B) and the migration distance ($F_{3,17} = 10.99, P = 0.016$, Tat-HA-NR2B9c vs Vehicle; $P = 1.000$, MK-801 vs Vehicle, >900 μm, C) of DCX⁺ cells in the striatum. (n=5 for Sham and Vehicle, n=6 for MK-801, n=5 for Tat-HA-NR2B9c). Data are mean ± SD ($^#P < 0.05$ vs Vehicle; $^\&P < 0.05$, vs MK-801).
Figure VI. Tat-HA-NR2B9c improves neurofunctional deficient in an nNOS-dependent manner in the subacute phase. (A) NSS score related to Fig. 1. \( F_{3,34}=519.12, P < 0.001, \text{Tat-HA-NR2B9c vs Vehicle} \). (B) NSS score related to Fig. 2. \( F_{2,29}=213.34 \). (C) NSS score related to Fig. 3. \( F_{2,26}=253.50 \). Data are mean ± SD (***P < 0.001, vs Sham).
Figure VII. Drugs have no effect on cued behavior and gross motor skills of animals. The latency to reach platform and swimming speed were measured at d 33 in visible platform trials, and swimming speed was measured during d 34-38 in hidden platform trials after MCAO. (A) The latency to reach platform (F\(_{3,34}=0.81\)), swimming speed in visible platform trials (F\(_{3,34}=0.40\)) and swimming speed in hidden platform trials (F\(_{3,34}=0.24\)) related to Fig. 1. (B) The latency to reach platform (F\(_{2,29}=0.80\)), swimming speed in visible platform trials (F\(_{2,29}=1.18\)) and swimming speed in hidden platform trials (F\(_{2,29}=0.12\)) related to Fig. 2. (C) The latency to reach platform (F\(_{2,26}=1.39\)), swimming speed in visible platform trials (F\(_{2,26}=0.74\)) and swimming speed in hidden platform trials (F\(_{2,26}=0.41\)) related to Fig. 3. (P > 0.05 between groups). Data are mean ± SD.