Erythropoietin Increases Neurogenesis and Oligodendrogliosis of Subventricular Zone Precursor Cells After Neonatal Stroke

Fernando F. Gonzalez, MD; Amara Larthaveesarp, BS; Patrick McQuillen, MD; Nikita Derugin, MA; Michael Wendland, PhD; Ruggero Spadafora, MD; Donna M. Ferriero, MD

Background and Purpose—Stroke is a common cause of neonatal brain injury. The subventricular zone is a lifelong source of newly generated cells in rodents, and erythropoietin (EPO) treatment has shown benefit in different animal models of brain injury. The purpose of this study is to investigate the specific role of exogenous EPO on subventricular zone progenitor cell populations in response to neonatal stroke.

Methods—Intraventricular injections of green fluorescent protein (GFP)–expressing lentivirus to label subventricular zone precursor cells were made in postnatal day 1 (P1) Long-Evans rats, which then underwent transient middle cerebral artery occlusion on P7. Middle cerebral artery occlusion and sham rats were treated with either vehicle or EPO (1000 U/kg) at reperfusion, 24 hours, and 7 days later. The density of double-labeled DCx+/GFP+, NeuN+/GFP+, O4+/GFP+, GFAP+/GFP+, as well as single-labeled GFP+ and Ki67+ cells, was calculated to determine cell fate outcome in the striatum at 72 hours and 2 weeks after stroke.

Results—There was a significant increase in DCx+/GFP+ and NeuN+/GFP+ neurons and O4+/GFP+ oligodendrocyte precursors, with decreased GFAP+/GFP+ astrocytes at both time points in EPO-middle cerebral artery occlusion animals. There was also a significant increase in GFP+ cells and Ki67+ proliferating cells in EPO compared with vehicle-middle cerebral artery occlusion animals.

Conclusions—These data suggest that subventricular zone neural progenitor cells proliferate and migrate to the site of injury after neonatal stroke and multiple doses of EPO, with a shift in cell fate toward neurogenesis and oligodendrogliosis at both early and late time points. The contribution of local cell proliferation and neurogenesis remains to be determined. (Stroke. 2013;44:XXX-XXX.)

Key Words: erythropoietin ■ neonate ■ neurogenesis ■ stroke

Stroke is a major contributor to neonatal morbidity and mortality, making the identification of neuroprotective therapies vital. In rodents, the primary sources of postnatal neurogenesis are the subventricular zone (SVZ) and subgranular zone of the dentate gyrus.1 The SVZ generates immature neurons (type A) that migrate tangentially via the rostral migratory stream to the olfactory bulb where they mature.2 The SVZ also generates astrocytes and oligodendrocytes that migrate radially toward overlying structures during early development.3 Radial glia are stem cells that differentiate into neural stem cells (NSCs; type B), characterized by maintenance of processes that maintain contact with ventricle wall after birth.4 This infects both dividing and nondividing cells and is expressed in their progeny.5

In animal models of hypoxia-ischemia (HI) and stroke, cells originating from the SVZ migrate and differentiate into region-appropriate neurons, but do not seem to survive long term.6 Several methods have been used to quantify the SVZ response to injury, and we have previously injected a lentiviral vector that expresses green fluorescent protein (GFP), directly labeling cells of the NSC lineage that maintain contact with ventricle wall after birth.7 This infects both dividing and nondividing cells and is expressed in their progeny.8 We previously saw decreased cell proliferation and increased astrocytosis of these labeled cells 2 weeks after stroke.9

Erythropoietin (EPO) is cytokine with several roles in addition to erythropoiesis, including cell death inhibition, immunomodulation, and angiogenesis.8 EPO has been shown to preserve brain structure and function in several studies of rodent HI and stroke,9,10 with long-term improvement seen with 3 doses of EPO.11 There is also evidence of increased neurogenesis and oligodendrogliosis in different in vivo and in vitro models, although this may involve both a local and
migratory response. The specific contribution of SVZ NSCs to focal ischemic injury and the effects of exogenous EPO on cell fate outcome in this in vivo model are not clear. The purpose of this study is to elucidate the effect of neonatal stroke and multiple-dose EPO treatment on cell proliferation, differentiation, and migration to the site of injury, specifically in the SVZ NSC population.

**Methods**

All animal research was approved by the University of California, San Francisco Institutional Animal Care and Use Committee, with all measures taken to reduce animal number and suffering.

**GFP-Lentiviral Production**

Packaging plasmid pCMV-dR8.91 and expressing envelope plasmid vesicular stomatitis virus-G protein were transfected with reporter plasmid PG12 into 293FT cells (online-only Data Supplement Methods). A lentivirus titer of 1×10⁷ colony forming units/mL was used.

**Intraventricular Injections**

Timed-pregnant Long-Evans rats were obtained from Simonsen Labs (Gilroy, CA). Postnatal day 1 (P1, day of birth designated P0) rats were anesthetized by hypothermia and placed into a neonatal rodent stereotactic frame (Stoelting, Wood Dale, IL). The right lateral ventricle was targeted at the following coordinates from bregma: 1.0 mm anterior, 1.2 mm lateral, and 2 mm depth. Injections were made with a beveled pulled glass micropipette (Wiretrol 5 μL; Drummonds Scientific Company, Broomall, PA) with a 50-μm diameter tip. Two microliters of lentivirus was injected at a rate of 0.5 μL/min. Animals were returned to the dam and monitored until they resumed nursing.

**Middle Cerebral Artery Occlusion**

Term-equivalent P7 pups underwent middle cerebral artery occlusion (MCAO) or sham surgery. Two pups died after the procedure and 4 did not satisfy injury criteria by diffusion-weighted MRI, leaving 18 animals that underwent successful MCAO surgery. Surgery was performed on spontaneously breathing pups anesthetized with 1.75% isoflurane in a mixture of 70% N₂O/30% O₂. Rectal temperature was monitored on spontaneously breathing pups anesthetized with 1.75% isoflurane in saline. Animals were anesthetized by hypothermia and placed into a neonatal rodent stereotactic frame (Stoelting, Wood Dale, IL). The right lateral ventricle was targeted at the following coordinates from bregma: 1.0 mm anterior, 1.2 mm lateral, and 2 mm depth. Injections were made with a beveled pulled glass micropipette (Wiretrol 5 μL; Drummonds Scientific Company, Broomall, PA) with a 50-μm diameter tip. Two microliters of lentivirus was injected at a rate of 0.5 μL/min. Animals were returned to the dam and monitored until they resumed nursing.

6-0 Dermalon filament was inserted and advanced 7.5 to 8.5 mm, depending on the animal’s weight, and secured with a temporary suture. Animals were reperfused after 90 minutes of occlusion by removing both sutures and the filament and covering the arteriotomy site with Surgicel. We previously demonstrated blood flow restoration in this model by contrast study. Sham animals were anesthetized and the internal carotid artery exposed, then the skin incision was sutured closed.

**Magnetic Resonance Imaging**

MRI was performed using a 2-Tesla magnet (online-only Data Supplement methods). Injury involving ipsilateral striatum and parieto-temporal cortex was verified during occlusion by diffusion-weighted MRI (Figure 1B). Animals with subcortical injury were excluded.

**EPO Treatment**

Immediately upon reperfusion or after sham surgery, vehicle (0.1% BSA, Sigma, St. Louis, MO) in saline or recombinant human EPO (R&D Systems, Minneapolis, MN) at a dose of 1000 U/kg was injected intraperitoneally, as previously described. Doses were repeated at 24 hours and 7 days after injury (Figure 1A). After surgery, animals were returned to their dam, with daily weights measured for the first week to ensure adequate weight gain. For each group and time point (vehicle-sham, EPO-sham, vehicle-occluded, EPO-occluded), n=4, except for EPO-MCAO group at P21 (n=6). Animal sex was equally distributed between groups.

**Immunohistochemistry**

Animals were anesthetized with sodium pentobarbital (100 mg/kg; Abbott Labs, Abbot Park, IL), and brains were perfused, postfixed, and sectioned coronally at 50-μm intervals (online-only Data Supplement Methods). Free-floating sections were immunolabeled with the following antibodies: anti-doublecortin (DCX; rabbit polyclonal, 1:200; Cell Signaling, Beverly, MA), anti-neuronal nuclei (NeuN; mouse monoclonal, 1:500; Chemicon, Temecula, CA), anti-Ki67 (Ki67; rabbit monoclonal, 1:500; Abcam, Cambridge, MA), anti-GFP (chicken polyclonal, 1:500; Abcam), anti-GFAP (mouse monoclonal, 1:500; Chemicon), anti-oligodendrocyte marker O4 (O4; mouse monoclonal, 1:250; Millipore, Billerica, MA), and anti-cleaved caspase-3 (CC3; rabbit polyclonal, 1:400; Cell Signaling). Secondary antibodies were purchased from Jackson (1:500; Bar Harbor, ME). Slides were coverslipped using Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories; Burlingame, CA) to counterstain nuclei.

**Cell Quantification**

Fluorescently immunolabeled sections were analyzed on a Zeiss Axiolab Imager Z.2 with Apotome (Zeiss, Inc, Thornwood, NY).
Images were acquired and reconstructed using AxioVision Rel. 4.9 software. Quantitative analysis of DCx+/GFP+, NeuN+/GFP+, O4+/GFP+, GFAP+/GFP+, GFAP+, Ki67+, and CC3+ cells was performed systematically in every 12th section after random selection of initial section. The striatum was analyzed from bregma +2.28 mm to –0.36 mm (plates 14–30 of Paxinos Rat Brain Atlas) and defined by the corpus callosum, lateral ventricle, and accumbens core. Double-immunostained sections were imaged by randomly placing the ×20 objective (Plan-Apo lens, NA 0.8, camera resolution 1344×1024 pixels, field dimension 400×400×20 μm) within the striatum. After imaging of full-thickness z-stack (1 μm steps) of the ×20 field, the field was manually moved a fixed distance of 600 μm in the horizontal and then vertical axis, resulting in 6 to 8 counting images per striatum per section. Single- and double-labeled cells were quantified using Metamorph Offline (6.0, Universal Imaging Corporation, Downingtown, PA). Cell density was calculated as the average number of cells per 20x high powered field, and cell proportion was calculated as specific double-labeled cells per total GFP+ cells quantified.

Statistical Analysis
Results from cell counting were analyzed using ANOVA with Fisher PLSD post hoc testing. All data are presented as means and SD from the mean. Comparisons were interpreted as significant if \( P < 0.05 \).

Results
EPO Increases Cells of SVZ NSC Lineage in Injured Striatum After Neonatal MCAO
After MCAO, GFP-expressing cells were observed moving away from the SVZ toward the injured striatum, representing core and penumbra (Figure 2A and 2B). To examine the effect

![Figure 2. Intraventricular injection of green fluorescent protein (GFP) lentivirus labels subventricular zone neural stem cells. Coronal sections of P21 rat forebrain (2 weeks after middle cerebral artery occlusion [MCAO]) in erythropoietin (EPO)-MCAO (A) and vehicle-MCAO (B) animal (tiled, ×10 images). GFP+ cell density in striatum was increased in EPO-MCAO vs vehicle-MCAO animals at P10 (\( P < 0.05 \)) and P21 (\( P < 0.05; \) **\( P < 0.04; \) (C). Striatal density of Ki67+ cells also increased in EPO-MCAO vs vehicle-MCAO animals (\( P < 0.05; \) **\( P < 0.04; \) D). Scale bars=250 μm. CTX indicates cortex; EO, EPO-MCAO; ES, EPO-sham; LV, lateral ventricle; STR, striatum; VO, vehicle-MCAO; and VS, vehicle-sham.](http://stroke.ahajournals.org/)

![Figure 3. Neuronal cell fate of green fluorescent protein (GFP)-labeled neural stem cells and progeny, Analysis of ipsilateral striatum in erythropoietin (EPO)-middle cerebral artery occlusion (MCAO) animal demonstrates coexpression of doublecortin (DCx; A, red, ×20) and NeuN (B, red, ×20) at P21. 4′,6-diamidino-2-phenylindole (DAPI; blue) is used as a nuclear counterstain, double-labeled cells marked with arrows. There was an increased density of DCx+/GFP+ colabeled cells (\( P < 0.02, \) **\( P < 0.05, \) ***\( P < 0.01 \) at P10; \( P < 0.04, **\( P < 0.05 \) at P21; C) and NeuN+/GFP+ cells (\( P < 0.05; \) D) in EPO-MCAO vs vehicle-MCAO animals. The y axis shows proportion of GFP+ cells that colabeled with specified marker. Scale bars=100 μm.](http://stroke.ahajournals.org/)
of stroke on SVZ NSC progeny in the striatum, the striatal density of GFP+ cells was determined at 72 hours (P10) and 2 weeks (P21) after MCAO. In the injured striatum, the majority of GFP+ cells were located near the ventricle at P10 (not shown), with increased migration at 2 weeks after stroke. At both time points, there were more GFP+ cells in the striatum in EPO-MCAO compared with vehicle-MCAO animals (13.0±2.9 vs 10.4±0.83 at P10; 13.9±1.4 vs 7.2±2.0 at P21; Figure 2C), with fewer cells remaining in SVZ of EPO-MCAO animals at P21 (Figure 2A and 2B; not quantified).

To determine whether EPO treatment affects cell proliferation, the density of proliferation marker Ki67 was calculated in the striatum at P10. There was an increased striatal density of Ki67+ cells in EPO-MCAO relative to vehicle-MCAO animals (75±5.9 vs 53±12; Figure 2D). To determine whether EPO also affects cell death, we examined CC3 expression, an apoptotic marker, in the striatum. EPO treatment reduced CC3 expression compared with vehicle-MCAO animals (Figure I in the online-only Data Supplement).

SVZ NSC-Derived Neurogenic Activity Increases With EPO Treatment After Neonatal Stroke

To examine the effects of injury and treatment on neural cell fate commitment of SVZ NSC progeny, we first calculated the proportion of GFP+ cells in the striatum that colabeled with DCx, a marker of immature neurons. There were more colabeled DCx+/GFP+ cells at both time points in EPO-MCAO compared with vehicle-MCAO animals (0.12±0.028 vs 0.09±0.014 at P10; 0.14±0.02 vs 0.08±0.01 at P21; Figure 3A and 3C). As expected from the DCx data, there were more colabeled NeuN+/GFP+ mature neurons in the EPO-MCAO group at P21 (0.085±0.01 vs 0.056±0.01; Figure 3B and 3D), consistent with neuronal survival and maturation.

Altered Gliogenesis of SVZ-derived NSCs With EPO Treatment After Neonatal Stroke

In previous studies, we identified increased generation of astrocytes after MCAO consistent with astrogliosis and a glial scar. To determine whether EPO treatment resulted in a favorable effect on the generation of oligodendrocytes versus astrocytes, we calculated the density of GFP+ cells that colabeled with markers for oligodendrocyte precursors (O4+) and astrocytes (GFAP+) in the striatum. There was an increased striatal density of colabeled O4+/GFP+ oligodendrocytes at both time points in both EPO-sham and EPO-MCAO animals (EPO-MCAO 0.36±0.01 vs vehicle-MCAO 0.21±0.09 at P10; EPO-MCAO 0.33±0.03 vs vehicle-MCAO 0.2±0.06 at P21; Figure 4A and 4C). There were more colabeled GFAP+/GFP+ astrocytes in the striatum in the vehicle-MCAO compared with vehicle-sham group (0.6±0.08 vs 0.37±0.03 at P10; 0.62±0.07 vs 0.39±0.08 at P21; Figure 4B and 4D), which was significantly reduced in EPO-MCAO animals (0.4±0.07 at P10; 0.45±0.04 at P21) and also reduced in EPO-sham animals at both time points (0.27±0.03 at P10; 0.29±0.03 at P21).

Discussion

This study demonstrates that in this model of neonatal stroke, multiple-dose EPO treatment results in a shift in SVZ NSC cell fate that favors production of neurons and oligodendrocytes in injured tissue, with less astrogliosis, at both early (72 hours) and late (2 weeks) time points. We previously reported decreased labeled neural precursor cells and progeny, with increased astrocyte production, after MCAO. This change in neuronal, oligodendrocyte, and astrocytic cell number originating from the SVZ to the site of focal ischemia after exogenous EPO treatment may result from increased progenitor proliferation, decreased precursor cell death, or a change in cell fate choice, and we observed evidence for each of these pathways. The effects of EPO on SVZ cell fate and number in the current study may play a significant role in the long-term histological and functional improvement previously reported with this protocol of prolonged EPO treatment.

In rodents, neurogenesis is thought to primarily occur during embryogenesis, except in the SVZ and subgranular zone, where neurogenesis peaks during the first 2 postnatal weeks.
Previous studies have found SVZ hypertrophy in response to brain injury, with an increase in progenitor cells and neuroblast migration, which do not persist and drop to baseline levels 2 to 4 weeks later. In this study, we used a high-titer, highly efficient GFP lentivirus that infects both rapidly and slowly dividing cells in contact with the lateral ventricle wall when administered by intraventricular injection, including type B NSCs. We previously demonstrated labeled cells in the SVZ 2 days after injection that exhibit radial glia morphology, coexpress vimentin, and coexpress DCX and NeuN in the rostral migratory stream and olfactory bulb. Here, we see labeled striatal glia and neurons, with robust labeling of all cells known to emerge from SVZ type B cell lineage.

This model of MCAO is distinct from HI, or the Vannucci model, in that there is transient focal ischemia without systemic hypoxia, followed by a reperfusion phase when the obstruction is removed and blood flow is restored. Reperfusion is an important part of injury progression in stroke, with increased excitotoxicity, free radical formation, and nitric oxide production leading to delayed cell death. This is also similar to the cause and pattern of injury seen in human neonatal stroke. Although others have described increased cell proliferation and DCX expression in the short term after neonatal HI, we did not see increased proliferation of SVZ NSCs by GFP+ staining or increased colabeled DCX+ immature neurons in vehicle-MCAO animals. This may be secondary to the more focal nature of injury with MCAO and our examination of the core as opposed to more mildly injured tissue. In addition, although the lentivirus is efficient in labeling cells in contact with the lateral ventricle at P1, including anterior and posterior SVZ cells and ependymal cells, it still represents a subset of progenitor cells, some of which no longer maintain contact with the ventricle at the time of injection.

This model enables us to evaluate the effects of neuroprotective therapies, specifically multiple dose EPO treatment, on cell fate outcome, primarily in the SVZ NSC population. EPO has shown promise as a trophic factor that may support cell differentiation, survival, and incorporation into neural networks. EPO has previously been shown to preserve brain volume after neonatal HI and stroke. Both in vitro and in vivo models have also demonstrated shifts in cell fate with EPO treatment after brain injury but not in the SVZ NSC population after neonatal focal ischemic injury. We previously reported increased neurogenesis and decreased astrogliosis in the injured striatum 6 weeks after stroke, but we could not determine whether this represented migration from the SVZ or local neurogenesis and repair. The purpose of this study was to determine the specific fate of NSCs in injured tissue that originate from the SVZ after MCAO and EPO treatment. In this study, an earlier (72 hours) time point after stroke was chosen to examine the early proliferative response to injury and treatment, as well as production of immature neurons and oligodendrocyte precursors that migrate to the injured area. The later time point (2 weeks after stroke) was examined to verify survival and maturation of immature neurons at the later time point and to evaluate the proportion of astrocytes and oligodendrocytes that was previously shown to be altered 2 weeks after neonatal stroke.

Although there were increased colabeled neurons in EPO-MCAO animals, there were many single-labeled DCX+, NeuN+, and O4+ cells that did not colabel with GFP. Although a subset of NSCs is labeled in this model and cells in the outer SVZ or that have previously translocated to more superficial regions of the brain may not be labeled, it is also possible that there is a significant local response to injury. It is known that some reactive astrocytes and progeny of radial glia may retain neurogenic potential, and local production of neurotrophic factors by astrocytes and other cell types may influence cell survival and fate in the penumbra. The role of local neurogenesis and repair still needs to be clarified.

We did not previously see long-term improvement with single-dose EPO after MCAO, but 3 doses administered during a 1-week period did result in long-term histological and behavioral improvement. This may indicate the need for additional booster doses, such as the 7-day dose in this protocol, for long-term improvement. Interestingly, delayed initiation of prolonged EPO treatment after neonatal HI resulted in increased oligodendrogliosis and short-term behavioral improvement, despite lack of change in gross histology. This may be secondary to the cell-specific effects of EPO, including increased or improved myelination. Although there was an increased density of immature neurons in EPO-MCAO animals, others have seen maturational delay or block of cell types after injury. For this reason, we examined neuronal maturation of GFP+ cells in the striatum by calculating the density of NeuN+/GFP+ mature neurons at P21. In this study, there were increased colabeled mature neurons in EPO- versus vehicle-MCAO animals, with these cells originating from the SVZ.

EPO and EPO receptor expression is elevated during gestation but declines rapidly after birth, with cell-specific increased expression after injury. After hypoxia, neuronal transcription factors hypoxia-inducible factor-1 and hypoxia-inducible factor-2 are stabilized, with increased expression of downstream targets (including EPO and vascular endothelial growth factor) that initiate pathways for neuroprotection, angiogenesis, and repair. These pathways have been shown to have antiapoptotic, anti-inflammatory, and proangiogenic effects, as well as shifting cell fate toward a neurogenic outcome in adult brain injury models. Postinjury angiogenesis may also be necessary for long-term survival of injured or newly generated cells, which may be enhanced by EPO and its interaction with VEGF. For example, EPO-treated mature rats have increased VEGF and brain-derived neurotrophic factor levels after stroke, as well as enhanced angiogenesis and neuroblast proliferation/migration to these regions. However, the time course of activation of these pathways in the immature brain after stroke and the role of early versus delayed EPO therapy on different signaling pathways are not clear.

To help clarify the effects of stroke and prolonged EPO treatment on cell proliferation and cell survival in the injured striatum, we calculated the relative density of single-labeled Ki67+ in the striatum. Ki67 is expressed in all phases of the cell cycle, with rapidly decreasing expression after exiting the cell cycle. There were more newly generated Ki67+ cells at 72 hours in EPO-MCAO compared with vehicle-MCAO animals,
consistent with an overall increase in cell proliferation after injury. In addition, there were fewer CC3+ apoptotic cells in the injured striatum at 72 hours in EPO-MCAO animals. It was not possible to quantify colabeled CC3+/GFP+ cells because there were not relatively few double-labeled cells at this time point. CC3 expression peaks at 18 to 24 hours after injury,29 and 72 hours may represent a relatively late time point for the initial wave of apoptotic cell death. More definitive quantification of apoptosis and cell death will require double labeling at earlier time points in both the SVZ and the striatum. In addition, the role of early EPO on cell death can be further clarified by initiating EPO therapy 24 hours, or later, after stroke.

This study demonstrates the effects of prolonged exogenous EPO administration specifically on SVZ NSC fate outcome in the injured striatum after neonatal stroke. Additional studies are needed to determine the relative importance of neuroprotection and cell survival versus cell proliferation and repair after neonatal stroke, as well as alternative sources of new cells in the developing forebrain.

Acknowledgments

This work was supported by National Institutes of Health (NIH) grant K08 NS064094 (Dr Gonzalez), NIH grant P50 NS33902 (Dr Ferriero), NIH grant NS33997 (Dr Ferriero), and NIH/National Center for Research Resources University of California, San Francisco-Clinical & Translational Science Institute (UL1 RR024131; Dr Gonzalez).

Disclosures

None.

References

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Stroke. published online February 7, 2013;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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Title: Erythropoietin increases neurogenesis and oligodendrogliosis of SVZ precursor cells after neonatal stroke

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Supplemental Methods

GFP-Lentiviral Production

Packaging plasmid pCMV-dR8.91 (8.9) and expressing envelope plasmid VSV-G were transfected with reporter plasmid FG12, containing the gene encoding GFP under the human Ubiquitin-C promoter, into 60-80% confluent 293FT cells with Effectene transfection reagent according to manufacturer’s instructions (Qiagen, Valencia, CA, USA). After 48 h, the viral supernatant was collected and centrifuged at 2000 rpm for 10 min at 4° C to remove cell debris. The virus was concentrated 100-fold by ultracentrifugation at 75,000 rpm for 90 min at 4° C, resuspended in PBS and stored at –80° C until use. To measure titers, serially diluted lentivirus was used to transduce 293FT cells; 72 h later, cells expressing GFP were counted to calculate viral titer. Lentivirus with a titer of 1x10^7 CFU/ml was used.

Magnetic Resonance Imaging

MRI was performed using a 2 Tesla magnet equipped with a Bruker Omega system and actively shielded gradients that provide 200 mTm⁻¹ gradient amplitude. The instrument settings for T2W and DW sequences were previously described. Pups were anesthetized with 1.5–2% isoflurane/100% O₂, placed supine on a plastic support, and water recirculating warming pads were wrapped around the pup below the neck to maintain body temperature. Pups were inserted into a 3.8-cm diameter birdcage imaging coil. DW spin echo MRI was conducted 60 minutes after MCAO to confirm the presence of injury. Eight consecutive 1-mm coronal sections were acquired, and the 6 that best covered the MCA territory beginning at the anterior edge of the forceps minor corpus callosum were used. Animals with subcortical injury were excluded.
Immunohistochemistry

Animals were anesthetized with sodium pentobarbital (100 mg/kg; Nembutal, Abbot Labs, Abbot Park, Ill., USA), and brains were harvested after transcardial perfusion with ice-cold 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were postfixed overnight, equilibrated in 30% sucrose in 0.1 M PBS and left at 4°C for a maximum of 72 h. Immunofluorescent staining was performed on 50-um free-floating coronal sections, which were collected throughout the brain using a sliding microtome. Sections were stored at 4°C in 0.1M phosphate buffer with 0.1% sodium azide for a maximum of 2 weeks.
Supplemental Figure 1. Decreased cleaved caspase-3 (CC3) staining in EPO-MCAO compared to vehicle-MCAO animals. Cleaved caspase-3 (red) staining in ipsilateral striatum in EPO-MCAO (A, 20x) and vehicle-MCAO rats (C, 20x), and merged images [GFP (green), CC3 (red) and DAPI (blue) staining] (B, D; 20x). Scale bars: 100-µm