Sonic Hedgehog Signaling Pathway Mediates Cerebrolysin-Improved Neurological Function After Stroke

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Background and Purpose—Cerebrolysin, a mixture of neurotrophic peptides, enhances neurogenesis and improves neurological outcome in experimental neurodegenerative diseases and stroke. The Sonic hedgehog (Shh) signaling pathway stimulates neurogenesis after stroke. The present study tests whether the Shh pathway mediates cerebrolysin-induced neurogenesis and improves neurological outcome after stroke.

Methods—Rats subjected to embolic stroke were treated with cerebrolysin with or without cyclopamine.

Results—Using neural progenitor cells derived from the subventricular zone of the lateral ventricle of adult rats, we found that cerebrolysin significantly increased neural progenitor cells proliferation and their differentiation into neurons and myelinating oligodendrocytes, which were associated with upregulation of Shh and its receptors patched and smoothened. Blockage of the Shh signaling pathway with a pharmacological smoothened inhibitor, cyclopamine, abolished cerebrolysin-induced in vitro neurogenesis and oligodendrogenesis. In the ischemic rats, treatment with cerebrolysin starting 24 hours after stroke significantly increased neural progenitor cell proliferation in the subventricular zone and enhanced neurogenesis, oligodendrogenesis, and axonal remodeling in the peri-infarct area. Moreover, profound neurological function improvements were observed in rats treated with cerebrolysin from week 3 to week 5 after stroke onset compared with vehicle-treated rats. However, in vivo inhibition of the Shh pathway with cyclopamine completely reversed the effects of cerebrolysin on neurorestoration and functional recovery.

Conclusions—These results demonstrate that the Shh pathway mediates cerebrolysin-enhanced neurogenesis and white matter remodeling and improves functional recovery in rats after stroke. (Stroke. 2013;44:1965-1972.)

Key Words: neurogenesis ■ recovery ■ stroke

Stroke results in rapid brain damage as well as long-term functional deficits. Thrombolytic therapy has had limited success in the acute management of stroke, with ≥5% of patients with ischemic stroke treated within the therapeutic window of 4.5 hours. Pharmacological and cell-based therapeutic approaches aimed at promoting brain remodeling and subsequent functional recovery for all patients with stroke, with treatment initiated ≥24 hours after stroke, are therefore under intensive investigation.1 Cerebrolysin, a peptide preparation with neurotrophic like activity, has been tested in the treatment of stroke and other neurodegenerative diseases.2,3 In experimental stroke, we previously demonstrated that treatment with cerebrolysin promotes neurogenesis and concomitantly enhances neurological functional recovery.3 Importantly, potential functional benefits of cerebrolysin have been demonstrated by several randomized clinical trials in patients with stroke and Alzheimer disease.2,4 However, the mechanisms responsible for the beneficial effects of cerebrolysin in the treatment of stroke remain to be elucidated.

Sonic hedgehog (Shh) signaling is a highly conserved signaling pathway that modulates the patterning of the central nervous system.3 On activation, Shh binds to its transmembrane receptor, patched, to relieve the inhibition of Smoothened (Smo). Activated Smo elevates the transcription of Gli genes, which are required for proper cell patterning, proliferation, and differentiation during development.6 In the adult brain, Shh signaling is required for maintaining the neural stem cell niche in the neurogenic regions.5,7 After central nervous system injury, the activation of Shh has been implicated in promoting several aspects of brain remodeling process, including neurogenesis, oligodendrogenesis, and axonal remodeling.8 Given the multifaceted role of the Shh signaling pathway in the regulation of brain remodeling, we hypothesized that the Shh signaling pathway mediates the neurorestorative effects of cerebrolysin on stroke.

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Methods
All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. All outcome measurements were performed by observers blinded to the experimental conditions.

Isolation and Culture of Neural Progenitor Cells
Neural progenitor cells (NPCs) were isolated from the subventricular zone (SVZ) of the lateral ventricle of adult rats (n=4) and were cultured, according to published protocols (Methods in the online-only Data Supplement). To examine whether cerebrolysin upregulates Shh and its receptors in NPCs, NPCs in the growth medium were treated with different concentrations of cerebrolysin (0, 5, 10, and 20 µL/mL), and mRNA levels of these genes were measured with quantitative real-time polymerase chain reaction assay8,10 (Methods in the online-only Data Supplement). To examine whether the Shh signaling pathway is involved in the effects of cerebrolysin on NPCs proliferation and differentiation, NPCs were treated with cerebrolysin (0 and 20 µL/mL) with or without cyclopamine (50 µM/L). Cell proliferation and phenotypes of NPCs were treated with cerebrolysin (0 and 20 µL/mL) and vehicle treatment with or without administration of cerebrolysin (IP) and vehicle (45% 2-hydroxypropyl-cyclodextrin in sterile PBS, intraventricularly infused) for 28 days; (4) cerebrolysin+vehicle, cerebrolysin (IP) and vehicle (45% 2-hydroxypropyl-cyclodextrin in sterile PBS, intraventricularly infused) for 28 days; (5) cerebrolysin alone (intraventricularly infused) for 28 days; and (6) vehicle alone (intraventricularly infused) for 28 days (Methods in the online-only Data Supplement).

Animal Model
Male Wistar rats weighing 350 to 400 g were subjected to embolism middle cerebral artery occlusion (MCAo), as previously described11 (Methods in the online-only Data Supplement).

Experimental Protocols
Twenty-four hours after MCAo, rats were randomly assigned to the following groups: (1) cerebrolysin alone, cerebrolysin (2.5 mL/kg) was intraperitoneally injected daily for 28 days; (2) saline, the same volume of saline was administered; (3) cerebrolysin+cyclopamine, cerebrolysin (IP) and cyclopamine at a dose of 0.11 µg/mL was intraventricularly infused with an osmotic pump for 28 days; (4) cerebrolysin+vehicle, cerebrolysin (IP) and vehicle (45% 2-hydroxypropyl-cyclodextrin in sterile PBS, intraventricularly infused) for 28 days; (5) cyclopamine alone (intraventricularly infused) for 28 days; and (6) vehicle alone (intraventricularly infused) for 28 days (Methods in the online-only Data Supplement).

For labeling proliferating cells, BrdU (100 mg/kg) was administered (IP) daily for 7 consecutive days starting at 24 hours after stroke. All rats were euthanized 35 days after MCAo.

Behavioral Tests
A battery of behavioral tests, including adhesive removal test, modified neurological severity score, and foot-fault test, were performed weekly starting 1 day after onset of MCAo (Methods in the online-only Data Supplement).

Lesion Volume Measurement
Thirty-five days after MCAo, infarct volume was measured as previously described11 (Methods in the online-only Data Supplement).

Immunohistochemistry
All immunohistochemistry analyses were performed 35 days after MCAo in rats subjected to intraventricular infusion of cyclopamine or vehicle treatment with or without administration of cerebrolysin (Methods in the online-only Data Supplement). Following primary antibodies were used: mouse anti-BrdU (Dako) goat anti-double-cortin (DCX, Santa Cruz), mouse anti-βIII-tubulin III (Tuj1, Covance), chicken anti-neurofilament heavy chain (NFH, Affinity Bioreagents), mouse anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase, Millipore), and rabbit anti-NG2 (Millipore).

Statistical Analysis
Data were evaluated for normality, and the data transformation was performed if data were not normal. As a result, ranked data were used for the analysis because the behavior data were not normally distributed. The global test using generalized estimating equation was used to test the group difference on functional recovery from 3 behavior tests.12 The global test on multiple outcomes is more efficient than a single outcome, when the group effects are consistent on all the outcomes.12 The 1-way ANOVA was used to test the treatment effect on histological measurements. The interaction between cerebrolysin and cyclopamine was tested with the critical level 0.05. If interaction (multiplicative effect) was significant at 0.05 level, the effect of treatment was further studied for subadditivity (the combined cerebrolysin and cyclopamine effect is worse than the combined effect of each treatment alone) or superadditivity (vice-versa). Data are presented as means±SE.

Results
Shh Signaling Pathway Mediates Cerebrolysin- Increased In Vitro Neurogenesis and Oligodendrogenesis
Reverse transcription polymerase chain reaction analysis revealed that incubation of NPCs with cerebrolysin at a concentration of 20 µL/mL significantly increased mRNA levels of Shh and its receptors patched and Smo, whereas cerebrolysin at a concentration of 5 µL/mL substantially increased Gli1 mRNA levels (Figure 1A). Cerebrolysin significantly increased proliferating cells measured by BrdU positive cells (Figure 1B). Moreover, cerebrolysin considerably increased the numbers of Tuj1, a marker of neuroblasts, and CNPase, a specific inhibitor of Smo, completely suppressed cerebrolysin-increased Tuj1, CNPase, and BrdU positive cells (Figure 1). Collectively, these data indicate that cerebrolysin promotes in vitro neurogenesis and oligodendrogenesis via the Shh signaling pathway.

Shh Signaling Pathway Mediates the Therapeutic Effect of Cerebrolysin in the Ischemic Brain
Behavioral test analysis showed that all rats exhibited severe neurological deficits measured by adhesive removal test, foot-fault, and modified neurological severity score 24 hours after stroke onset with no significant differences among the groups (Figure 2). Ischemic rats treated with cerebrolysin exhibited significant functional improvements starting at day 14 and the improved outcomes persisted up to day 35 after stroke compared with rats treated with saline (Figure 2), which is consistent with our previous findings.3 However, when cyclopamine was coadministered along with cerebrolysin, it abolished cerebrolysin-improved neurological function, whereas coadministration of vehicle and cerebrolysin did not affect the beneficial effect of cerebrolysin (Figure 2). The effect of cyclopamine on cerebrolysin-improved functional outcome seems specific because ischemic rats treated with cyclopamine alone did not exhibit a significant difference of the behavioral tests compared with the rats treated with vehicle and saline (Figure 2). Additional statistical analysis revealed that there was a subadditive interaction (antagonistic effect) of cyclopamine and cerebrolysin on neurological
A functional outcome starting day 14 and persisting up to day 35 after stroke. These data strongly indicate that blocking Shh signaling with cyclopamine abolishes cerebrolysin-improved functional recovery.

There were no significant differences of infarct volume among the rats treated with saline (33.8±2.7% of contralateral), cerebrolysin (32.1±3.5%), saline+vehicle (34.7±3.5%), cerebrolysin+vehicle (34.4±3.5%), saline+cerebrolysin (33.3±3.7%), and cerebrolysin+cyclopamine (33.1±3.0%).

Treatment with cerebrolysin significantly increased the density of BrdU+ SVZ cells in the ipsilateral SVZ compared with the saline control groups (Figure 3A). However, coadministration of cerebrolysin and cyclopamine substantially reduced the number of BrdU+ cells compared...

Figure 1. Effects of cerebrolysin (CB) on neural progenitor cells (NPCs). A. Real-time reverse transcription polymerase chain reaction analyses of Sonic hedgehog (Shh), patched (PTCH), smoothened (Smo), and Gli1 mRNA levels on NPCs treated with different concentrations of cerebrolysin, respectively. B. Immunofluorescent stainings of BrdU, Tuj1, or 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase; red) with 4′,6-diamidino-2-phenylindole (blue) on primary NPCs treated with CB in the presence and absence of cyclopamine (CPM). C. Quantitative data of BrdU, Tuj1, or CNPase immunoreactive cells. Bar=50 µm.

Figure 2. Neurological functional outcome. Graphs showing outcomes from adhesive removal test (A), foot-fault test (B), and modified neurological severity score (C). Significant differences (P<0.05) were detected between following groups by the global test: saline vs cerebrolysin from day 14 to 35; saline+vehicle vs cerebrolysin+vehicle from day 14 to 35; cerebrolysin+vehicle vs cerebrolysin+cyclopamine from day 21 to 35. MCAo indicates middle cerebral artery occlusion.
with the cerebrolysin-treated rats (Figure 3A). Also, cyclopamine alone significantly reduced the density of BrdU+ cells compared with saline-treated rats (Figure 3A). These data suggest that the Shh pathway regulates SVZ NPC proliferation in the ischemic brain of rats.

We further examined the effects of cerebrolysin and cyclopamine on neuroblasts by measuring DCX+ cells in the SVZ and peri-infarct striatum. Although cerebrolysin treatment significantly increased the DCX+ cell density in the SVZ and peri-infarct striatum compared with rats treated with the vehicle control, cyclopamine completely suppressed cerebrolysin-increased DCX+ cell density (Figure 3B). Double immunofluorescent staining revealed that treatment with cerebrolysin substantially increased the percentage of DCX+/BrdU+ cells in the SVZ, which was completely abolished by the coadministration of cyclopamine (Figure 3C). There was a subadditive interaction of cyclopamine and cerebrolysin on peri-infarct striatum of DCX+ cell density and the percentage of DCX+/BrdU+ cells in the SVZ, suggesting that cyclopamine blocks cerebrolysin-induced neurogenesis in the rats after stroke.

To examine the effect of cerebrolysin and cyclopamine on oligodendrocytes in the ischemic brain, we measured oligodendrocyte progenitor cells (OPCs) and mature oligodendrocytes identified by phenotype markers of NG2 and CNPase, respectively, in the SVZ and white matter. Pericytes

Figure 3. BrdU and doublecortin (DCX) immunoreactivities in the ischemic brain. A, Representative images of BrdU immunoreactive cells in the ipsilateral subventricular zone (SVZ) acquired from representative rats treated with saline+vehicle, cerebrolysin+vehicle, saline+cyclopamine, and cerebrolysin+cyclopamine. Bar graph (A) shows quantitative data of BrdU immunoreactive cells in the SVZ. B, Representative images of DCX (red) and 4',6-diamidino-2-phenylindole (blue) acquired from representative rats treated with saline+vehicle, cerebrolysin+vehicle, and cerebrolysin+cyclopamine. C, Double immunofluorescent staining of BrdU (green) with DCX (red) in the SVZ (upper) and peri-infarct striatum (lower) from a representative rat treated with cerebrolysin+vehicle. D, Quantitative data of percentage of DCX immunoreactive cells (upper), and DCX+/BrdU+ cells relative to total BrdU positive cells (lower) in the ipsilateral SVZ and peri-infarct striatum. LV indicates lateral ventricle; and Str, striatum. Bars in A and B=50 µm. Bar in C=25 µm.
also express NG2. To avoid counting NG2+ pericytes, only NG2+ cells with typical multiprocess morphology were counted. Treatment with cerebrolysin significantly augmented the density of NG2+ cells in the SVZ and peri-infarct striatum compared with rats treated with the vehicle control (Figure 4A). Double immunofluorescent staining revealed that cerebrolysin substantially increased the percentage of NG2+/BrdU+ (Figure 4B) and CNPase+/BrdU+ (Figure 5A) positive cells in the peri-infarct corpus callosum and striatum compared with the vehicle group (Figures 4 and 5). These data indicate that cerebrolysin enhances oligodendrogenesis in the ischemic brain. However, coadministration of cyclopamine completely blocked the effects of cerebrolysin on oligodendrocytes (Figures 4 and 5). Moreover, cyclopamine alone substantially reduced the percentage of CNPase+/BrdU+ cells in the peri-infarct corpus callosum and striatum, although cyclopamine did not significantly change the density of NG2+ cells and the percentage of NG2+/BrdU+ cells (Figure 4D), suggesting that cyclopamine may inhibit OPCs to differentiate into mature oligodendrocytes. Collectively, our data indicate that cyclopamine abolishes the effects of cerebrolysin on oligodendrogenesis in the ischemic brain.

Mature oligodendrocytes are essential for axonal myelination.13 To examine whether the aforementioned changes in oligodendrogenesis affect axonal remodeling, we performed double immunofluorescent staining to identify NFH+ axons and CNPase+ oligodendrocyte processes. Cerebrolysin treatment significantly increased the density of NFH+ and CNPase+ processes in the peri-infarct corpus callosum and striatum compared with the saline control. However, administration of cyclopamine completely repressed cerebrolysin-increased NFH+ and CNPase+ density (Figure 5C and 5D). These data suggest that cerebrolysin enhances myelinated axons and that the Shh pathway likely mediates this process.

**Discussion**

In an animal model of focal cerebral ischemia, we demonstrated that cerebrolysin enhances neurogenesis and oligodendrogenesis and promotes improvement of neurological outcome. Cerebrolysin modulates Shh signaling in NPCs, which contributes cerebrolysin-induced neurogenesis and oligodendrogenesis. Moreover, our in vitro and in vivo data showed that inactivation of the Shh signaling pathway by a pharmacological blocker of Smo, cyclopamine, abolished the observed beneficial effects of cerebrolysin. These findings suggest that the Shh pathway plays an

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**Figure 4.** Oligodendrocyte progenitor cells in the ischemic brain. A, Fluorescent microscopic images of NG2+ cells (green) in the ipsilateral subventricular zone (SVZ, upper) and peri-infarct striatum (lower) of representative rats treated with saline+vehicle, cerebrolysin (CB)+vehicle, and CB+cyclopamine (CPM). B, Quantitative data of NG2+ cells. C, Double immunofluorescent staining of BrdU+ (green) with NG2+ (red) cells in the SVZ (upper) and peri-infarct striatum (lower) of a representative rat treated with CB+vehicle. D, Quantitative data of percentage of NG2+/BrdU+ cells relative to total BrdU+ cells. LV indicates lateral ventricle. Bars=25 μm.
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important role in cerebrolysin-mediated neurorestorative processes in ischemic brain.

We previously demonstrated that treatment with cerebrolysin at 24 hours after stroke onset enhances neurogenesis and functional recovery in the ischemic rats. Here, we extend our previous finding by showing that cerebrolysin treatment initiated 24 hours after stroke not only increases neurogenesis, but also profoundly amplifies oligodendrogenesis. The OPCs originating from the SVZ migrate into gray and white matter and some of the OPCs differentiate into mature oligodendrocytes in the adult rodent brain. Cerebrolysin amplified generation of OPCs in the SVZ and mature oligodendrocytes in white matter of the peri-infarct region as measured by BrdU/NG2 and BrdU/CNPase positive cells, respectively. In the present study, proliferating cells were labeled with BrdU for 7 days from day 1 after stroke and these animals were euthanized 35 days after stroke. Mature oligodendrocytes do not proliferate. Thus, we speculate that BrdU/CNPase positive oligodendrocytes observed in the present study likely come from proliferating OPCs. We cannot distinguish whether oligodendrocytes are generated in situ in the white matter or recruited from the SVZ. However, in vitro data indicate that cerebrolysin promotes SVZ NPCs to differentiate into OPCs. Brain injury including stroke induces SVZ NPC migration toward injured white matter, where they differentiate into mature oligodendrocytes. Therefore, SVZ NPCs may also contribute to cerebrolysin-increased oligodendrocytes. However, the effects of cerebrolysin on the migration, survival, and cell fate of newly generated cells after stroke warrant further investigation.

Oligodendrocytes are the only myelin-forming cells in the central nervous system, and proper axonal myelination is required for efficient neuronal signaling transduction. In the ischemic brain, myelinating oligodendrocytes participate in white matter remodeling processes. Our data show that cerebrolysin enhances myelinated axons measured by NFH/CNPase immunoreactive density in the peri-infarct white matter, suggesting that oligodendrocytes increased by cerebrolysin may facilitate axonal remodeling in the ischemic brain. Our findings are in line with a previous study, which shows that cerebrolysin not only protects against neuronal network degeneration, but also promotes axonal outgrowth in cultured neurons. During stroke recovery, axonal outgrowth is closely associated with myelinating oligodendrocytes in the peri-infarct regions, and axonal remodeling is correlated to neurological function. Thus, it is likely that the enhancement of axonal remodeling and oligodendrogenesis by cerebrolysin could lead to the improved functional recovery after stroke.

More importantly, the present study indicates that the Shh pathway mediates the beneficial effect of cerebrolysin on stroke. In vivo, blocking of the Shh pathway with cyclopamine completely suppressed cerebrolysin-enhanced neurogenesis, oligodendrogenesis, and white matter remodeling, as well as cerebrolysin-improved functional outcome. Cyclopamine seems to ablate the cerebrolysin effect specifically because cyclopamine alone did not exacerbate ischemic damage and neurological functional deficits in ischemic rats. The specific effect of cyclopamine is further supported by our statistical analysis showing a subadditive effect in which the
combined cerebrolysin and cyclopamine effect was worse than the combined effect of each treatment alone. Molecular mechanisms underlying interaction between cerebrolysin and the Shh pathway are not known. Shh signaling interacts with neurotrophic factors, such as epidermal growth factor, to coordinate neurogenesis.5 Our in vitro data show that cerebrolysin upregulated Shh and its receptors, patched and Smo, and a transcription factor, Gli1, on NPCs and that blocking Shh pathway with cyclopamine abolished cerebrolysin-enhanced NPCs proliferation and differentiation, suggesting a direct effect of cerebrolysin on activation of the Shh signaling pathway. Moreover, it has been suggested that Shh regulates the patterning of the ventral neural tube through a Smo-independent noncanonical pathway.21 Together, these data suggest that cerebrolysin, a mixture of neurotrophic peptides, interacts with the Shh pathway. However, we previously demonstrated that cerebrolysin promotes neurogenesis via the PI3K/Akt pathway,5 suggesting that cerebrolysin-induced neurogenesis could be independent of the Shh signaling pathway. The phosphatidylinositol 3-kinases/Akt pathway has also been shown to act synergistically with Shh signaling to regulate neural precursor proliferation and patterning.22 Thus, the effects of cyclopamine on Shh signaling should be interpreted with caution. Additional in vivo studies to investigate the interaction between cerebrolysin and the Shh pathway are warranted.

The Shh signaling pathway regulates neurogenesis.5,7,8 Systemic administration of cyclopamine at a dose of 10 mg/kg per day reduces SVZ NPC proliferation in adult mice.3 In experimental stroke, intracerebroventricular delivery of a low dose cyclopamine (60 pmol/d) reduces hippocampus NPC proliferation in adult mice,23 whereas intraventricular injection of cyclopamine at a dose of 1.8 mg/kg immediately after MCAo downregulated Gli1, patched1 level, and exacerbated brain damage and behavioral deficits 24 hours after stroke onset.24 These studies suggest that cyclopamine may regulate adult neurogenesis and stroke outcome in a dose-dependent and tissue-specific manner. Our data show that intraventricular infusion of cyclopamine at a dose of 2.5 µg/d for 28 days substantially reduced SVZ NPC proliferation and did not exacerbate ischemic cell damage. Moreover, compared with ischemic rats treated with the vehicle, cyclopamine treatment did not reduce DCX-positive neuroblasts and CNPase-positive oligodendrocytes, which may account for why we did not detect aggravation of neurological outcome in these rats. Our data support the role of the Shh signaling pathway in regulating SVZ NPCs proliferation. Among many factors, the dose of cyclopamine may be responsible for the discrepancy of the effect of cyclopamine on ischemic brain damage reported by Ji et al.21 The dosing regimen of cyclopamine used for the intraventricular infusion in the present study is ≈50 µmol/L in cerebrospinal fluid, which was calculated on the basis of ≈90 to 120 µL of cerebrospinal fluid in the adult rat.25 Accordingly, this dose mimics the concentration of cyclopamine used in our vitro study.

Conclusion Our data demonstrate that cerebrolysin treatment enhances neurogenesis, oligodendrogenesis, and axonal remodeling in the ischemic brain and that the Shh signaling pathway mediates these processes. Accumulated evidence implies the interdependent interaction of multiple neurorestorative events, including neurogenesis, oligodendrogenesis, and axonal remodeling, which in concert promote brain remodeling and neurological functional recovery after brain injury. Thus, it is likely that the observed cerebrolysin-boasted brain repair processes contribute to cerebrolysin-improved neurological function.

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Disclosures
None.

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Supplemental Methods

**Culture of neural progenitor cells:** Neural progenitor cells isolated from the SVZ were plated at a density of $2 \times 10^4$ cells/ml in the presence of growth medium that contains Dulbecco's modified Eagle's (DMEM)-F12 medium, 20ng/ml of epidermal growth factor (R&D Systems, Minneapolis, MN) and 20ng/ml of basic fibroblast growth factor (R&D Systems). The generated neurospheres (primary sphere) were dissociated and reseeded as single cells at a density of 20cells/µl. Passages 2 and 3 neural progenitor cells were used in the present study.

To examine whether the Shh signaling pathway is involved in the effects of cerebrolysin on NPCs proliferation, NPCs were plated directly onto laminin-coated glass coverslips in the growth medium, and were treated with cerebrolysin (0 and 20µl/ml) with or without cyclopamine (50µM) for 7 days. To analyze cell proliferation, bromodeoxyuridine (BrdU, 20µg/ml, Sigma), was added 18 h prior to termination of incubation. BrdU-positive cells were measured.

To examine whether the Shh signaling pathway is involved in the effects of cerebrolysin on cell differentiation, NPCs were cultured with differentiation medium that contains DMEM-F12 medium, 2% fetal bovine serum, but without the growth factors. The cells were treated with cerebrolysin with or without cyclopamine (50µM) for 14 days. Immunocytochemistry with antibodies against β-tubulin III (Tuj-1), and 2',3'-cyclic nucleotide 3’-phosphodiesterase (CNPase) were performed to identify neuroblasts and oligodendrocytes, respectively.
SUPPLEMENTAL MATERIAL

Real-time PCR: To examine whether cerebrolysin upregulates Shh and its receptors in NPCs Quantitative real-time PCR assay for mRNA levels of Shh, PTCH, Smo, and Gli1 were measured with Taqman primers specific to these genes using a quantitative real-time PCR assay according to published methods. Each sample was tested in triplicate, and data obtained from three independent experiments were used to quantify the relative gene expression by the $2^{-\Delta\Delta C_T}$ method.

Animal model: Male Wistar rats were subjected to embolic middle cerebral artery occlusion (MCAo). Briefly, blood clots from a donor rat were obtained 24h prior to MCAo. Under the operating microscope (Carl Zeiss, Inc), the right common carotid arteries (CCA), the right external carotid artery (ECA) and the internal carotid artery (ICA) were isolated via a midline incision. A modified PE-50 catheter with a 0.3 mm outer diameter was introduced into the ECA through a small puncture. A 18-19 mm length of catheter was gently advanced from the ECA into the ICA until its tip positioned at the origin of MCA. A single blood clot was delivered through the catheter. The catheter was withdrawn immediately after injection.

For intraventricular infusion, osmotic minipumps designed to deliver 0.11 μl/h for 28 d (Alzet 1004, Durect Corp) were preloaded with 90 μl of cyclopamine at the concentration of 1mg/ml or vehicle (45% 2-hydroxypropyl-cyclodextrin in sterile PBS), and were subcutaneously implanted 24h after MCAo. The terminal cannula attached to the osmotic minipump was placed in the right lateral cerebral ventricle at the coordinates: 0 mm anteroposterior relative to bregma, 1.5 mm lateral to midline, 3.5 mm vertical depth to the pial surface based on the atlas by Paxinos and Watson (1998). The cannula was cemented in place, and the incision was sutured.
Lesion volume measurement: Rats were sacrificed 35 days after MCAo, and infarct volume was measured on hematoxylin and eosin (H&E) stained seven coronal sections using the MicroComputer Imaging Device (MCID; Imaging Research, St. Catharines, Canada) system. Briefly, the area of lesion, ipsilateral hemisphere, and contralateral hemisphere were measured. The intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, which is defined as the indirect infarct area for each slice. A volume was determined by multiplying the appropriate area by the section interval thickness. The ischemic volume is presented as the percentage of infarct volume of the contralateral hemisphere (indirect volume calculation).

Behavioral tests:

Adhesive Removal Test: Rats were tested for somatosensory deficits with the adhesive removal test. The mean time required to remove both stimuli from limbs was recorded.

Modified neurological severity score (mNSS): mNSS is a composite of motor, sensory, reflex, and balance tests. Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18).

Foot-fault test: Rats were tested for forelimb placement dysfunction with a modified foot-fault test. The total number of steps (movement of each forelimb) that the rat used to cross the test apparatus and the total numbers of errors (foot faults) for left forelimb were recorded.

Immunohistochemistry: Coronal sections (8 µm thickness) at bregma -0.4 to -1.4 mm were used for single and double immunofluorescent staining. Monoclonal antibody (mAb) against BrdU (Dako) was used at a titer of 1:100 for identifying proliferated cells. The
SUPPLEMENTAL MATERIAL

Following antibodies were used as phenotype markers of neuroblasts and oligodendrocytes: goat anti-doublecortin (DCX) polyclonal antibody (pAb) at a titer of 1:200 (Santa Cruz Biotechnology), mouse anti-β-tubulin III mAb (Tuj-1, Covance) at a titer of 1:1000, chicken anti-neurofilament heavy chain pAb (NFH, Affinity Bioreagents) at a titer of 1:300, mouse anti-cyclic nucleotide phosphodiesterase mAb (CNPase, Millipore) at a titer of 1:200, and rabbit anti-NG2 pAb (Millipore) at a titer of 1:300. 4′,6′-diamidino-2-phenylindole Vector Laboratories) at a titer of 1:10000 were used for nuclei counterstaining.

For quantification, three coronal sections/rat/immunostaining were used. Each coronal section was digitized using a 40x objective via the MCID system. We defined the peri-infarct striatum and corpus callosum areas as striatal and corpus callosum tissues that were 500μm from the infarct border. The BrdU, NG2, BrdU/DCX, BrdU/NG2 immunoreactive cells were measured throughout SVZ adjacent to the ipsilateral lateral ventricle wall and on 8 fields of peri-infarct striatum. The NFH, CNPase, and BrdU/CNPase immunoreactivity was measured within 8 fields of peri-infarct striatum and corpus callosum. To quantify SVZ cell proliferation, the numbers of BrdU immunoreactive cells within the ipsilateral and contralateral SVZ were counted and divided by the lateral ventricle wall area (mm\(^2\)) to determine the cell density. To assess the treatment effects on neuroblasts, DCX immunoreactive cells and DCX and BrdU double immunoreactive cells in the ipsilateral lateral ventricle wall and peri-infarct striatum were digitized. The density of DCX immunoreactive cells was determined by dividing the cell number by the respective area (mm\(^2\)). A DCX/BrdU positive cell was identified as a cell with a DCX positive cytoplasm and BrdU positive nuclei. DCX and
SUPPLEMENTAL MATERIAL

BrdU double immunoreactive cells were calculated as the percentage of total BrdU positive cells. To assess the treatment effects on oligodendrocyte progenitor cells, NG2 immunoreactive cells with typical multiprocess morphology and BrdU and NG2 double immunoreactive cells in the ipsilateral SVZ and peri-infarct striatum were digitized. The numbers of NG2 immunoreactive cells within the SVZ were counted and presented as cell density relative to the SVZ area (mm²). NG2 and BrdU double immunoreactive cells were calculated as the percentage of total BrdU positive cells. To assess the treatment effects on mature oligodendrocytes, BrdU and CNPase immunoreactive cells in peri-infarct corpus callosum and striatum were measured, and presented as the percentage of total BrdU positive cells. To quantify myelinated axons, NFH immunoreactive axons with the presence of CNPase immunoreactivity in the peri-infarct corpus callosum and striatum, and the contralateral homologous area were digitized. Data are presented as the percentage of immunoreactive area at the peri-infarct corpus callosum and striatum relative to the contralateral homologous regions on the same section.