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Cerebrolysin enhances neurogenesis in the ischemic brain and improves functional outcome after stroke

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Abstract

Cerebrolysin is a peptide preparation mimicking the action of neurotrophic factors and has beneficial effects on neurodegenerative diseases and stroke. The present study investigated the effect of Cerebrolysin on neurogenesis in a rat model of embolic middle cerebral artery occlusion (MCAo). Treatment with Cerebrolysin at doses of 2.5 and 5 ml/kg significantly increased the number of bromodeoxyuridine positive ($BrdU^+$) subventricular zone (SVZ) neural progenitor cells and doublecortin (DCX) immunoreactivity (migrating neuroblasts) in the ipsilateral SVZ and striatal ischemic boundary 28 days after stroke when the treatment was initiated 24h after stroke. The treatment also reduced $TUNEL^+$ cells by ~50% in the ischemic boundary. However, treatment with Cerebrolysin at a dose of 2.5 ml/kg initiated at 24 and 48h did not significantly reduce infarct volume, but substantially improved neurological outcomes measured by an array of behavioral tests 21 and 28 days after stroke. Incubation of SVZ neural progenitor cells from ischemic rats with Cerebrolysin dose dependently augmented $BrdU^+$ cells and increased the number of $Tuj1^+$ cells (a marker of immature neurons). Blockage of the PI3K/Akt pathway abolished Cerebrolysin-increased $BrdU^+$ cells. Moreover, Cerebrolysin treatment promoted neural progenitor cell migration. Collectively, these data indicate that Cerebrolysin treatment when initiated 24 and 48h after stroke enhances neurogenesis in the ischemic brain and improves functional outcome and that Cerebrolysin-augmented proliferation, differentiation, and migration of adult SVZ neural progenitor cells contribute to Cerebrolysin-induced neurogenesis, which may be related to improvement of neurological outcome. The PI3K/Akt pathway mediates Cerebrolysin-induced progenitor cell proliferation.

Keywords

Cerebrolysin; neurogenesis; MCAO; rats

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Introduction

Cerebrolysin is a peptide preparation, which exerts beneficial effects on neurodegenerative diseases and stroke (Tatebayashi et al., 2003; Rockenstein et al., 2007)}. Preclinical studies have shown that acute treatment with Cerebrolysin reduces cerebral infarction in rats after transient ischemia while delayed administration of Cerebrolysin promotes neurological functional recovery without reducing lesion volume (Ren et al., 2007; Hanson et al., 2009). These data suggest that in addition to the neuroprotective effect, Cerebrolysin has a capacity to promote brain repair after stroke.

In adult rodent brain, new neurons are generated in the subgranular zone of the dentate gyrus and the subventricular zone (SVZ) of the lateral ventricle (Alvarez-Buylla et al., 2000; Gage, 2002). Cerebrolysin enhances dentate gyrus neurogenesis in normal rat and in mouse models of Alzheimer's disease (AD) (Tatebayashi et al., 2003; Rockenstein et al., 2007) and promotes adult hippocampal progenitor cells to differentiate into neurons in vitro(Chen et al., 2007). Neurogenesis enhanced by Cerebrolysin is related to improvement of behavioral performance and spatial memory (Tatebayashi et al., 2003). Stroke induces neurogenesis in the SVZ and newly generated neuroblasts in the SVZ migrate to the ischemic boundary to replace damaged neurons (Jin et al., 2001; Zhang et al., 2001; Arvidsson et al., 2002). Enhancement of neurogenesis in the ischemic brain promotes brain remodeling and is correlated to neurological outcome (Zhang and Chopp, 2009). However, the effect of Cerebrolysin on neurogenesis in ischemic brain has not been fully investigated. In the present study, we examined whether Cerebrolysin affects stroke-induced neurogenesis in a rat model of embolic middle cerebral artery occlusion (MCAo) and we directly measured the effect of Cerebrolysin on SVZ neural progenitor cells in vitro.

Materials and Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Animal model

Male Wistar rats weighing 350-450g were employed. The middle cerebral artery (MCA) was occluded by placement of an embolus at the origin of the MCA (Zhang et al., 1997).

Experimental protocols

Cerebrolysin or vehicle was intraperitoneally (i.p) injected daily for 21 days, starting at 24h after MCAo. To label mitotic cells, bromodeoxyuridine (BrdU, 100 mg/kg, Sigma) was administered (i.p) daily for 7 consecutive days starting at 24h after stroke. 1) To examine the dose responses of Cerebrolysin, 24h after MCAo, rats were randomly assigned to 1.0, 2.5. and 5.0 ml/kg of Cerebrolysin groups or vehicle group (n=10/group). 2) To examine the therapeutic window of Cerebrolysin, ischemic rats were randomly treated with Cerebrolysin at a dose of 2.5 ml/kg starting at 48, 72, or 96h after stroke onset (n=10/group). These animals were killed 28 days after MCAo. 3) To examine the direct effect of Cerebrolysin on neural progenitor cells, SVZ neural progenitor cells were isolated from non-ischemic rats (n=5) or rats subjected to 7 days of MCAo (n=5).

Behavioral tests

All behavioral tests were performed by observers blinded to the treatments 1, 7, 14, 21, and 28 days after onset of MCAo.

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Foot-fault test

A modified foot-fault test was employed to measure forelimb placement dysfunction(Zhang et al., 2002). The total number of steps (movement of each forelimb) that the rat used to cross the grid and the total numbers of foot faults for left forelimb were recorded.

Adhesive Removal Test

An adhesive removal test was employed to measure somatosensory deficits (Schallert et al., 1982; Zhang et al., 2002). The mean time required to remove both stimuli from limbs was recorded.

Modified neurological severity score (mNSS)

Rats were tested for motor, sensory, reflex, and balance dysfunctions with the mNSS (Chen et al., 2001). Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18).

Histopathology and immunohistochemistry

Infarct volume was measured on 7 hematoxylin and eosin (H&E) stained coronal sections using the microcomputer imaging device (MCID) system (Imaging Research, St. Catharines, Ontario, Canada), as previously described (Zhang et al., 1997).

For immunostaining, a series of coronal sections at bregma −0.4 to −1.4 mm were prepared. The following antibodies were used: mouse anti-BrdU (1: 100, Boehringer Mannheim Indianapolis, IN), goat anti-doublecortin (DCX) (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-β-tubulin III (TuJ-1, 1:500, Covance the Development Services Company, MI), rabbit anti-glial fibrillary acidic protein (GFAP, 1:500, Dako Cytomation California Inc. Carpinteria, CA)**.** The terminal deoxynucleotidyl transferase (TdT) – mediated dUTP-biotin nick end labeling (TUNEL) method (in situ Apoptosis Detection Kit, Chemicon) was used to assess apoptotic cells.

SVZ neural progenitor cell culture

Neural progenitor cells were isolated from the SVZ, as previously described (Wang et al., 2006). To generate neurospheres, SVZ cells were plated at a density of 2×10^4 cells/ml in the presence growth medium (Wang et al., 2006). The growth medium contains Dulbecco's modified Eagle's (DMEM)-F12 medium (Invitrogen Corporation, Carlsbad, California), 20 ng/ml of epidermal growth factor (EGF, R&D System, Minneapolis, MN) and 20 ng/ml basic fibroblast growth factor (bFGF, R&D System, Minneapolis, MN). DMEM-F-12 medium contains L-glutamine (2mM), glucose (0.6%), putrescine (9.6μg/ml), insulin (0.025mg/ml), progesterone (6.3ng/ml), apo-transferrin (0.1mg/ml), and sodium selenite (5.2ng/ml). To examine the effect of Cerebrolysin on neural progenitor cell proliferation, the generated neurospheres (primary sphere) were passaged by mechanical dissociation and then plated directly onto laminin-coated glass coverslips in growth medium with different doses (0, 5, 10, and 20 μl/ml) of Cerebrolysin for 7 days. BrdU (30 μg/ml) was added 24 h before the termination of incubation. To identify cell proliferation, cultured cells were immunostained with an antibody against BrdU, and were counterstained with 4′, 6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA).

To examine the effect of Cerebrolysin on neural progenitor cell differentiation, neurospheres were mechanically dissociated, and then plated directly onto laminin-coated glass coverslips in DMEM-F-12 medium containing 2% FBS and Cerebrolysin at concentrations of 0, 5, 10, and 20 ml/ml medium, but without the growth factors for 7 days. To determine cell

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phenotypes, cultured cells were immunostained with antibodies against TuJ1 (a marker of immature neurons) and GFAP (a marker of astrocytes).

To measure neural progenitor cell motility, a single neurosphere (~200 μm diameter) derived from non-ischemic rats was seeded in reduced-growth factor medium containing Matrigel (40 μl; BD Biosciences, Franklin Lakes, NJ), in the presence or absence of Cerebrolysin at doses of 5, 10, and 20 μg/ml. The distance of cells that migrate out of the neurosphere was recorded 72h after incubation (Robin et al., 2006; Wang et al., 2006).

To examine whether the PI3K/Akt signaling pathway is involved in the effect of Cerebrolysin on neurogenesis, neural progenitor cells were left untreated or pretreated with LY294002 (10 μM), a PI3K-Akt inhibitor, for 1 h followed by treatment with or without Cerebrolysin (10 ml/ml) for 1h and 7 days for measurements of Akt and cell proliferation, respectively. Levels of Akt were measured using Western blot analysis.

Statistical analysis

Data were evaluated for normality and the data transformation was performed if data were not normal. As a result, the behavior tests were not normal, and ranked data were used for the analysis. The global test using Generalize Estimating Equation (GEE) was employed to test the group difference on functional recovery measured from multiple behavior tests(Lu et al., 2003). The analysis began testing the overall dose effect, followed by pair wise dose group comparisons if the overall dose effect was detected at the 0.05 level. Data are presented as means ± SE.

Results

The effect of Cerebrolysin on neurological outcome

To examine the effect of Cerebrolysin on neurological outcome, an array of behavioral tests were performed. All rats exhibited severe neurological deficits measured by mNSS, foot-fault, and adhesive removal tests 24h after stroke onset, and there were no significant differences among the groups (Fig. 1). Treatment with Cerebrolysin initiated 24h post stroke with doses of 2.5 and 5ml/kg significantly improved performance on mNSS, foot-fault, and adhesive removal test starting 21d and persisting to at least 28 days after stroke compared with vehicle treated rats (Fig. 1). Interestingly, rats treated with Cerebrolysin at the dose of 1 ml/kg exhibited a significant improvement of foot-fault score 28 days after stroke (Fig. 1). These data indicate that Cerebrolysin dose dependently improves neurological outcome. To examine whether further delayed treatment with Cerebrolysin has an effect on neurological outcome, we treated ischemic rats with Cerebrolysin (2.5 ml/kg) 48, 72 and 96h after stroke. Delayed treatment with Cerebrolysin starting at 48h but not at 72, or 96h post stroke significantly improved performance on foot-fault and adhesive removal tests at 21 and 28d after stroke and mNSS at 28d compared with the saline treated rats (Fig. 1). These data suggest that Cerebrolysin at a dose of 2.5 ml/kg has a therapeutic window of 48h in this model. Cerebrolysin treatment did not significantly reduce infarct volume among experimental groups (Table 1).

The effect of Cerebrolysin on neurogenesis in vivo

The results that Cerebrolysin improved functional outcome without a significant reduction in ischemic lesion volume suggest that mechanisms other than the neuroprotection may contribute to observed functional improvement. Cerebrolysin induces neurogenesis in the dentate gyrus of adult rodent brain (Alvarez-Buylla et al., 2000; Gage, 2002) and neurogenesis is related to neurological function (van Praag et al., 1999). To examine the effect of Cerebrolysin on neurogenesis in the SVZ which contains neural stem

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cells(Alvarez-Buylla et al., 2000), proliferated SVZ cells identified by BrdU positive cells and neuroblasts detected by DCX immunoreactive cells were measured 28 d after stroke in animals treated with different doses of Cerebrolysin starting 24h after stroke. Treatment with Cerebrolysin at doses of 2.5 and 5ml/kg, but not 1 ml/kg, significantly increased the number of BrdU positive cells (Fig. 2I) and the DCX immunoreactive area in the ipsilateral SVZ (Fig. 2A to 2J) compared with saline treated rats when the treatment was initiated at 24h after stroke onset. In addition, Cerebrolysin substantially increased the number of DCX positive cells in the striatal ischemic boundary region (Fig. 2D and 2J). Double immunostaining revealed that DCX positive cells in the ischemic boundary were BrdU positive (Fig. 2G and H), indicating neurogenesis. The majority of new neurons die in the ischemic boundary (Arvidsson et al., 2002). To examine whether the increase of neuroblasts by Cerebrolysin results from the effect of Cerebrolysin on the reduction of apoptotic cell death, we measured the number of TUNEL positive cells in the SVZ and ischemic boundary region. Treatment with Cerebrolysin at 1, 2.5 and 5 ml/kg reduced the number of TUNEL positive cells by ~50% in the ischemic boundary ($p<0.05$, Fig. 2K), but did not significantly reduce the number of TUNEL positive cells in the SVZ (Fig. 2K). Collectively, these data indicate that Cerebrolysin enhances neurogenesis in the ischemic brain.

The effect of Cerebrolysin on SVZ neural progenitor cells in vitro

To directly examine the effects of Cerebrolysin on neural progenitor cells, we performed a neurosphere assay in which neural progenitor cells were isolated from non-ischemic or ischemic rats (Wang et al., 2006). When they are cultured with bFGF and EGF, the primary neural progenitor cells proliferate, while withdrawing the growth factors, the neural progenitor cells differentiate into neurons and glial cells (Wang et al., 2006). Treatment of non-ischemic or ischemic progenitor cells with Cerebrolysin at 10 and 20 μl/ml medium, respectively, significantly increased the number of BrdU positive cells 7 days after incubation (Fig. 3A to E). In addition, incubation of ischemic progenitor cells with Cerebrolysin at a dose of 20 μl/ml almost doubled the number of Tuj1 positive cells from 9.6% in the control to 18.1% in the Cerebrolysin group (Fig. 3F to J). Although Cerebrolysin at doses of 10 and 20 μl/ml increased the number of Tuj1 positive cells in the no-ischemia derived progenitor cells, it did not reach a statistical significance (Fig. 3J). Cerebrolysin did not significantly increase the number of GFAP positive cells (Fig. 3K to O). These data provide direct evidence that Cerebrolysin dose dependently enhances neurogenesis, which is consistent with in vivo results.

In vivo findings that an increase in DCX positive cells in the ischemic boundary suggest that Cerebrolysin may promote migration of neuroblasts in the SVZ to the ischemic boundary. We, therefore, examine the effect of Cerebrolysin on neural progenitor cell motility. When a single neurosphere is placed into Matrigel, neural progenitor cells migrate out of the neurosphere (Robin et al., 2006). Distances of the cells that migrated out of the neurosphere were recorded 72h after incubation. Cerebrolysin at 20μl/ml significantly increased migration distance compared with the distance in the control group (Fig. 3P to S), indicating that Cerebrolysin promotes progenitor migration.

The PI3K/Akt pathway mediates neurogenesis (Katakowski et al., 2003; Wang et al., 2005; Mairet-Coello et al., 2009). To examine whether Cerebrolysin activates this pathway, we performed Western blots. Incubation of SVZ neural progenitor cells with Cerebrolysin at 10 μl/ml substantially increased phosphorylated Akt, whereas a PI3K/Akt inhibitor, LY294002, suppressed Cerebrolysin-elevated phosphorylated Akt (Fig. 3T). Moreover, although Cerebrolysin significantly increased progenitor cell proliferation (43.6 ±3 % of $BrdU^+$ cells, n=6) compared to the control group (27.1 ±3.3 % of $BrdU^+$ cells, n=6), Cerebrolysin-augmented cell proliferation was abolished by LY294002 (29.7 ± 4 % of $BrdU^+$ cells, n=6).

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Discussion

The present study demonstrates that Cerebrolysin dose-dependently improved neurological outcome when Cerebrolysin was administered 24 and 48 h after stroke. In vivo and in vitro data show that Cerebrolysin substantially augmented neurogenesis in the ischemic brain and did not reduce the ischemic lesion. Therefore, our data suggest that neurogenesis increased by Cerebrolysin may contribute to the improved functional outcomes.

Stroke stimulates neurogenesis in the SVZ and newly generated neuroblasts in the SVZ migrate to the ischemic boundary region (Jin et al., 2001; Zhang et al., 2001; Arvidsson et al., 2002). The present study indicates that Cerebrolysin augments neurogenesis in the ischemic SVZ and striatal boundary region. Neurogenesis enhanced by Cerebrolysin is likely involved in multiple mechanisms. Cerebrolysin treatment substantially reduced the TUNEL positive cells in the ischemic boundary but not in the SVZ, suggesting that Cerebrolysin attenuates neuroblast apoptosis. Cerebrolysin can enhance hippocampal neurogenesis by augmenting survival of the hippocampal neural progenitor cells (Tatebayashi et al., 2003; Rockenstein et al., 2007). In addition, our in vitro data indicate that in the presence of the bFGF and EGF, Cerebrolysin promotes neural progenitor cell proliferation measured by an increase in the number of BrdU cells. The effect of Cerebrolysin on cell proliferation is specific because the non-ischemic and ischemic neural progenitor cells exhibited different dose responses to Cerebrolysin-enhanced cell proliferation. Furthermore, Cerebrolysin enhances the ischemic neural progenitor cell differentiation into neurons. These findings are consistent with studies in adult hippocampal neural progenitor cells, which show that Cerebrolysin increases neural progenitor cell proliferation and neuronal differentiation (Chen et al., 2007). Thus, in addition to augmentation of neural progenitor cell survival, Cerebrolysin can promote neurogenesis by directly acting on enhancement of proliferation and differentiation of SVZ neural progenitor cells.

The present in vivo data show that Cerebrolysin substantially increased DCX positive cells in ischemic boundary region. DCX positive cells are migrating neuroblasts and they are generated in the SVZ (Alvarez-Buylla et al., 2000). Under physiological condition, these neuroblasts migrate to the olfactory bulb where they differentiate into neurons (Alvarez-Buylla et al., 2000). After focal cerebral ischemia, neuroblasts in the SVZ migrate to the ischemic boundary region to replace damaged neurons (Jin et al., 2001; Zhang et al., 2001; Arvidsson et al., 2002). Our in vitro data indicate that Cerebrolysin has a direct effect on promoting neural progenitor cell migration. Together with in vivo results, the present study suggests that in addition to an increase in the number of neuroblasts, Cerebrolysin may promote SVZ neuroblast migration to the ischemic boundary.

The neuropeptide mixture Cerebrolysin has neurotrophic effects in vivo and in vitro (Chen et al., 2007), which could contribute to enhanced neurogenesis observed in the present study. Cerebrolysin contains peptides with ciliary neurotrophic factor (CNTF) and other trophic activities, but not the intact proteins (Chen et al., 2007). Blockage of CNTF abolishes Cerebrolysin-induced neurogenesis in adult hippocampal neural progenitor cells (Chen et al., 2007), indicating that CNTF in Cerebrolysin plays a major role in promoting neurogenesis (Chen et al., 2007). CNTF induces neurogenesis and gliogenesis (Chen et al., 2007; Nagao et al., 2007). In vitro, CNTF along with Notch signals or in the presence of bFGF facilitates generation of astrocytes in the SVZ neural progenitor cells (Nagao et al., 2007). In addition to neurotrophic factors, Cerebrolysin modulates glycogen synthase kinase3 β (GSK3β) and cyclin-dependent kinase-5 (CDK5) dependent phosphorylation (Rockenstein et al., 2007). GSK3β is a well-characterized Akt substrate (Vojtek et al., 2003). The PI3K/Akt pathway regulates cell proliferation, survival, and migration (Vojtek et al.,

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2003; Wang et al., 2005). We previously demonstrated that the activation of the PI3K/Akt pathway promotes SVZ neural progenitor cell proliferation, differentiation, and migration (Katakowski et al., 2003; Wang et al., 2005). The present study shows that Cerebrolysin activated Akt in SVZ neural progenitor cells and blockage of the PI3K/Akt pathway with LY294002 abolished Cerebrolysin-increased cell proliferation. Thus, our data suggest that the PI3K/Akt pathway plays an important role in Cerebrolysin-enhanced neurogenesis.

Our data show that treatment with Cerebrolysin initiated 24 and 48h after stroke significantly improved functional outcome but did not substantially reduce the infarction, which is consistent with previous findings (Ren et al., 2007). Augmentation of proliferation, migration, and surviving of neuroblasts may contribute to the observed improvement of functional outcome.

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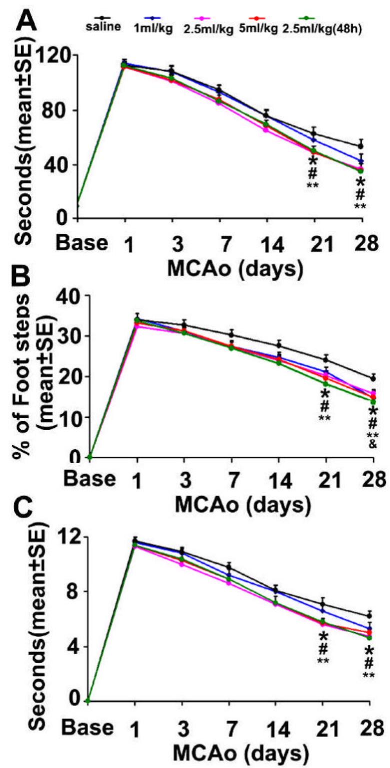

Fig. 1.

The effects of Cerebrolysin treatment on neurological outcome. Panels A to C show temporal profiles of adhesive-removal test (A), foot-fault test (B), and mNSS (C) during 28 days after MCAo. & (1ml/kg, 24h), * (2.5ml/kg, 24h), # (5ml/kg, 24h), ** (2.5ml/kg, 48h) $p<0.05$ versus the vehicle group. (n=10/group).

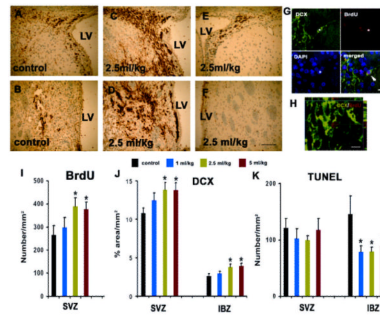

Fig. 2.

The effect of Cerebrolysin on neurogenesis and apoptotic cells. Panels A to F show DCX positive cells in the ipsilateral (A, B, C, and D) and contralateral (E and F) SVZ (A and C) and the ischemic (B and D) and contralateral (F) striatum. Double immunostaining (G) shows that a DCX (green) positive cell in the ischemic striatum was BrdU (red) positive cells (merged, arrow). Panel H shows an orthogonal view of a DCX (green) and BrdU (red) positive cell. Panels I to K show quantitative data of BrdU (I), DCX (J) and TUNEL (K) cells in different experimental groups (n=10/group). Bar = 100 μm in F and 10 μm in G. *$p<0.05$, versus the vehicle group. Control = saline and LV=lateral ventricle.

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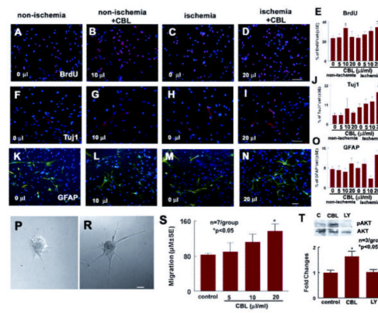

Fig. 3.

The effect of different concentrations of Cerebrolysin on proliferation, differentiation and migration of neural progenitor cells. SVZ neural progenitor cells were harvested from non-ischemic (A, B, F, G, K, L) and ischemic (C, D, H, I, M, N) rats. Panels A to D show representative BrdU immunoreactive cells (red) and DAPI positive nuclei (blue) in the control (A and C) and Cerebrolysin (10 and 20 µl/ml in B and D) groups. Panels F to I and K to N show representative Tuj1 (red) and GFAP (green), respectively, immunoreactive cells in the control (F, H, K, M) and Cerebrolysin (10 µl/ml in G, L and 20 µl/ml in I, N) groups. Panels E, J, and O are quantitative data of BrdU, Tuj1, and GFAP positive cells. Panels P and R show representative migration of neural progenitor cells out of a neurosphere in the control (P) and Cerebrolysin (20 µl/ml, R) groups. Panel S shows quantitative migration data. Panel T shows Western blots and quantitative data. *$P<0.05$ vs the control group. Bar = 20 µm. n=6/group. C = control, CBL= Cerebrolysin, and LY = LY294002.

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Table 1

Infarct Volume 28 days after MCAo

Groups (n=10/group)	% of Contralateral Hemisphere (Mean ± S.E.)
Saline (24h)	32.55±1.82
1 ml/kg (24h)	30.40±2.91
2.5 ml/kg (24h)	28.77±2.25
5 ml/kg (24h)	28.69±2.34
Saline (48h)	31.52±2.23
2.5 ml/kg (48h)	27.54±1.27
2.5 ml/kg (72h)	29.81±2.03
2.5 ml/kg (96h)	30.90±1.92