ORIGINAL RESEARCH

Netrin-1 Preserves Blood-Brain Barrier Integrity Through Deleted in Colorectal Cancer/Focal Adhesion Kinase/RhoA Signaling Pathway Following Subarachnoid Hemorrhage in Rats

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***Background*—**Netrin-1 (NTN-1) has been established to be a novel intrinsic regulator of blood-brain barrier (BBB) maintenance. This study was carried out to investigate the potential roles of exogenous NTN-1 in preserving BBB integrity after experimental subarachnoid hemorrhage (SAH) as well as the underlying mechanisms of its protective effects.

***Methods and Results*—**A total of 309 male Sprague-Dawley rats were subjected to an endovascular perforation model of SAH. Recombinant NTN-1 was administered intravenously 1 hour after SAH induction. NTN-1 small interfering RNA or Deleted in Colorectal Cancer small interfering RNA was administered intracerebroventricular at 48 hours before SAH. Focal adhesion kinase inhibitor was administered by intraperitoneal injection at 1 hour prior to SAH. Neurological scores, brain water content, BBB permeability, RhoA activity, Western blot, and immunofluorescence staining were evaluated. The expression of endogenous NTN-1 and its receptor Deleted in Colorectal Cancer were increased after SAH. Administration of exogenous NTN-1 significantly reduced brain water content and BBB permeability and ameliorated neurological deficits at 24 and 72 hours after SAH. Exogenous NTN-1 treatment significantly promoted phosphorylated focal adhesion kinase activation and inhibited RhoA activity, as well as upregulated the expression of ZO-1 and Occludin. Conversely, depletion of endogenous NTN-1 aggravated BBB breakdown and neurological impairments at 24 hours after SAH. The protective effects of NTN-1 at 24 hours after SAH were also abolished by pretreatment with Deleted in Colorectal Cancer small interfering RNA and focal adhesion kinase inhibitor.

***Conclusions*—**NTN-1 treatment preserved BBB integrity and improved neurological functions through a Deleted in Colorectal Cancer/focal adhesion kinase/RhoA signaling pathway after SAH. Thus, NTN-1 may serve as a promising treatment to alleviate early brain injury following SAH. **(*J Am Heart Assoc*. 2017;6:e005198. DOI: 10.1161/JAHA.116.005198.)**

Key Words: blood-brain barrier • brain edema • early brain injury • netrin-1 • subarachnoid hemorrhage

A burgeoning body of research suggests that blood-brain barrier (BBB) disruption significantly results in the early brain injury (EBI) progression after subarachnoid hemorrhage (SAH).[1] Moreover, BBB disruption and subsequent vasogenic brain edema have been identified as accurate predictors of poor outcomes in SAH patients.[2] Therefore, a therapeutic strategy targeting BBB disruption would be beneficial for attenuating EBI and improving neurological outcomes after SAH.

Netrin-1 (NTN-1), a multifunctional protein, is involved in both physiological and pathological processes such as apoptosis, inflammation, neurogenesis, and tumorigenesis in the nervous system as well as in the lung, heart, and kidneys.[3-8] In the adult brain, NTN-1 was identified as a survival factor for endothelial cells and induced neovascularization and vessel remodeling.[9-11] Overexpression of NTN-1 promoted angiogenesis and improved long-term neurological functions following ischemic stroke.[12] Recent studies indicated that NTN-1 preserved BBB integrity in model of traumatic brain injury and experimental autoimmune encephalomyelitis.[13,14] However, the effects of NTN-1 on BBB integrity in EBI after SAH have not been investigated.

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Accompanying Table S1 and Figures S1 through S3 are available at http://jaha.ahajournals.org/content/6/5/e005198/DC1/embed/inline-supplementary-material-1.pdf

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Deleted in Colorectal Cancer (DCC) is a receptor for NTN-1.[5] Focal adhesion kinase (FAK), a cytoplasmic protein tyrosine kinase, is involved in the regulation of the vascular morphogenesis and endothelial barrier integrity.[15,16] FAK is also one of the key proteins that negatively regulates RhoA activity.[16] In the present study we hypothesized that (1) exogenous NTN-1 preserves BBB integrity and attenuates EBI after SAH and (2) the protective function of NTN-1 on BBB is mediated through a DCC/FAK/RhoA-related signaling pathway.

Materials and Methods

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University. The study followed the Guide for the Care and the Use of Laboratory Animals (National Research Council) and complied with the Animal Research: Reporting of In Vivo Experiments guidelines for reporting in vivo experiments.

SAH Model

A total of 309 male Sprague-Dawley rats (280 to 320 g; Harlan, Indianapolis, IN) were used. The endovascular perforation model of SAH was performed as previously described.[17] Briefly, rats were anesthetized and kept on a ventilator during surgery with 2% to 3% isoflurane in air (isoflurane was decreased to 1.5% at the time of puncture). Rodents were placed in a supine position, and the neck was opened with a sharp scalpel in the midline. After localization of the appropriate vessels, a sharpened 3-cm, 4-0 nylon suture was inserted gently into the left internal carotid artery from the external carotid artery stump to the bifurcation of the anterior and middle cerebral arteries. The suture was advanced until resistance was reached, further advanced in order to puncture the vessel, and then immediately withdrawn after artery perforation. For the sham group, the suture was inserted into the left internal carotid artery without perforation. After removal of the suture, the incision was closed, and rodents were placed in heated cages and observed until recovery.

Experimental Design

Five separate experiments were designed as follows (Figure S1).

The numbers of animals used in this study are presented in Table S1.

Experiment 1

The time course of endogenous NTN-1 and DCC receptors in ipsilateral/left hemisphere was measured by Western blot analysis. Double immunofluorescence staining was performed to characterize the cellular localization of NTN-1 and DCC at 24 hours after SAH.

Experiment 2

Three doses of exogenous recombinant NTN-1 (5, 15, or 45 μg/kg; R&D Systems, Minneapolis, MN) dissolved in phosphate-buffered saline (PBS) were administered through tail vein with a total volume of 200 μL at 1 hour after SAH induction. SAH grade was examined 24 hours after SAH. Neurobehavioral scores and brain water content were measured at 24 and 72 hours after SAH. Evans blue (EB) extravasation was evaluated at 24 hours after SAH in sham, SAH+vehicle, and SAH+NTN-1 (45 μg/kg) groups.

Experiment 3

NTN-1 small interfering RNA (siRNA) was administered by intracerebroventricular (ICV) injection at 48 hours before SAH induction. Neurobehavioral scores, brain water content, and Western blot were examined at 24 hours after SAH. EB extravasation was evaluated at 24 hours after SAH in SAH+scramble siRNA and SAH+NTN-1 siRNA groups.

Experiment 4

DCC-1 siRNA was administered by ICV injection at 48 hours before SAH induction and then followed with NTN-1 (45 μg/kg) treatment. Neurobehavioral scores, brain water content, and Western blot were evaluated at 24 hours after SAH.

Experiment 5

FAK inhibitor 14 (Fib-14) (30 mg/kg; Tocris Bioscience, Bristol, UK)[18] dissolved in PBS was administered by intraperitoneal injection with a total volume of 200 μL at 1 hour before SAH induction and then followed with NTN-1 (45 μg/kg) treatment. Neurobehavioral scores, brain water content, and Western blot were detected at 24 hours after SAH.

Intracerebroventricular Drug Administration

For NTN-1 and DCC in vivo knockdown, 3 different rat NTN-1 siRNA duplexes or DCC siRNA duplexes were mixed to enhance the knockdown effect; these were administered by ICV injection as previously described.[19] Following the manufacturer's instructions, rat NTN-1 siRNA (Thermo Fisher Scientific, Waltham, MA), DCC siRNA (OriGene Technologies, Rockville, MD), or negative control scramble siRNA (OriGene Technologies) was dissolved in transfection reagent (OriGene Technologies). ICV injection was conducted. Briefly, rats were placed in a stereotaxic apparatus under 2% isoflurane anesthesia. A scalp incision was made along the midline, and a burr hole (1.0 mm) was drilled on the right side of the skull according to the following coordinates relative to

bregma: 1.5 mm posterior, 1.0 mm lateral. The needle of a 10-μL Hamilton syringe (Microliter 701; Hamilton Company, Reno, NV) was inserted through the burr hole into the right lateral ventricle 3.2 mm beneath the dural surface. NTN-1 siRNA, DCC siRNA, or scramble siRNA (0.5 nmol/5 μL) was delivered into the right ventricle with a Hamilton syringe by a pump at a rate of 0.5 μL/min at 48 hours before SAH induction. The needle was left in place for an additional 5 minutes after injection to prevent possible leakage and was slowly withdrawn within 5 minutes. After the needle was removed, the burr hole was sealed with bone wax, the incision was closed with sutures, and the rats were allowed to recover.

SAH Grading

Evaluation of the severity of SAH was done blindly through a grading system immediately after euthanasia as previously described.[20] The basal cistern was divided into 6 segments that were scored from 0 to 3 according to the amounts of subarachnoid blood. SAH rats with a score <8 at 24 hours were excluded from this study.

Neurological Score

Neurological score was assessed using modified Garcia test and Beam balance test at 24 or 72 hours after SAH by an investigator (C.R.) blind to group information, which included the following.[19]

Brain Water Content

Brains were collected at 24 or 72 hours after surgery and separated into left hemisphere, right hemisphere, cerebellum, and brain stem. Each part was weighed immediately after removal (wet weight) and then dried in an oven at 105°C for 72 hours (dry weight). After that, the percentage of brain water content was calculated as [(wet weight−dry weight)/wet weight]×100%.[21]

Blood Brain Barrier Permeability

BBB permeability was evaluated by EB extravasation using spectrophotometry and fluorescence microscopy as previously described.[22,23] At 24 hours after surgery, EB dye (2%; 5 mL/kg; Sigma-Aldrich, St. Louis, MO) was injected into the right femoral vein over a period of 2 minutes, thus allowing the dye to circulate for 60 minutes. The rodents were anesthetized under isoflurane anesthesia and then subjected to transcranial perfusion with 120 mL of PBS. Brains were subsequently removed and divided into left and right hemispheres. Brain specimens were weighed, homogenized in PBS (1 mL PBS for each 300 mg of tissue), and then centrifuged at 15 000 g for 30 minutes; 0.5 mL of the supernatant was added to an equal volume of trichloroacetic acid (T6399, Sigma, St. Louis, MO). The samples were incubated overnight at 4°C and centrifuged at 15 000 g for 30 minutes. An amount of 0.8 mL of the supernatant was used for spectrophotometric quantification of extravasated EB dye at 610 nm.

For EB fluorescence, rats were subject to intracardial perfusion with 60 mL PBS followed by 60 mL 10% paraformaldehyde. Their brains were removed, fixed in 10% paraformaldehyde for 24 hours, and then in 30% sucrose for 72 hours. Coronal brain sections (15 μm) were cut, and the red autofluorescence of EB dye was visualized using excitation and emission filters for rhodamine fluorescence (Leica Microsystems, Wetzlar, Germany). Intensity of EB fluorescence was analyzed in each slide of 5 random sections for the ipsilateral cortex using ImageJ software (ImageJ 1.5, National Institutes of Health, Bethesda, MD).

Immunofluorescence Staining

Double fluorescence staining was conducted as described previously.[24] At 24 hours following SAH, rats were transcardially perfused under deep anesthesia with 60 mL of cold PBS (pH 7.4) and then perfused with 60 mL 10% paraformaldehyde through the upper part of the body. Brains were removed and fixed in 10% paraformaldehyde for 24 hours and then in 30% sucrose for 72 hours. Frozen coronal slices (10 μm) were sectioned in a cryostat (CM3050S; Leica Microsystems, Wetzlar, Germany). Sections were blocked with 5% donkey serum for 1 hour and incubated at 4°C overnight with primary antibodies: rabbit anti-NTN-1 (1:500, Abcam, Cambridge, UK), rabbit anti-DCC (1:200, Thermo Fisher Scientific, Waltham, MA), and mouse anti-CD31 (1:100, Abcam) followed by incubation with appropriate fluorescence-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) for 2 hours at room temperature. Negative control staining was performed by omitting the primary antibody. LASX software enabled slide viewing and pictures taken in a fluorescence microscope (Leica DMi8; Leica Microsystems, Wetzlar, Germany).

Western Blot

Western blot analysis was performed as previously described.[25] After sample preparation, equal amounts of a sample protein (50 μg) were loaded onto an SDS-PAGE gel. First, electrophoresis and transfer of the samples to a nitrocellulose membrane were performed. Second, the membrane was blocked and incubated overnight at 4°C with the following primary antibodies: rabbit anti-NTN-1 (1:800, Abcam), rabbit anti-DCC (1:500, Abcam), rabbit anti-FAK

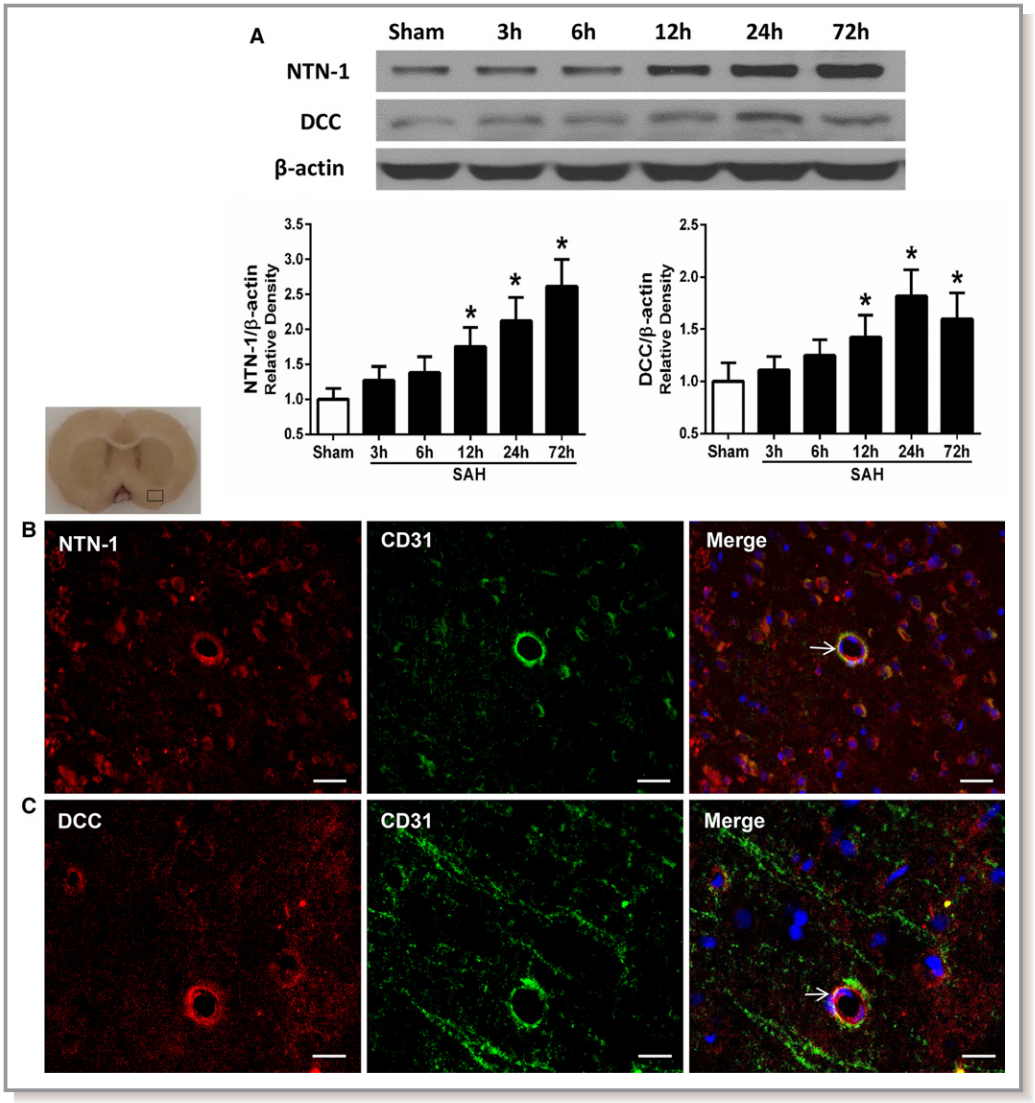

Figure 1. Expression of endogenous Netrin-1 (NTN-1) and Deleted in Colorectal Cancer (DCC) receptor after subarachnoid hemorrhage (SAH). A, Representative Western blot bands and quantitative analyses of NTN-1 and DCC time course from the ipsilateral hemisphere after SAH. Relative densities of each protein have been normalized against the sham group. n=4 for each group per time point. Representative microphotographs of double immunofluorescence staining showed that both NTN-1 (B) and DCC receptor (C) were colocalized with CD31-positive endothelial cells at 24 hours after SAH. n=2 for each group. Arrow indicates that NTN-1 (B) and DCC (C) were expressed in endothelial cells. **P*<0.05 vs sham. Scale bar=50 μm.

(phospho Y397) (1:1000, Abcam), rabbit anti-FAK (1:1000, Abcam), rabbit anti-MMP-9 (1:1000, Abcam), rabbit anti-ZO-1 (1:200, Santa Cruz Biotechnology, Dallas, TX), and rabbit anti-Occludin (1:50 000, Abcam). GTP-RhoA and total-RhoA were detected by using Rho Activation Assay Kits (Millipore, Temecula, CA). β-Actin was used as an internal loading control. The secondary antibodies were all from Santa Cruz Biotechnology. Immunoblots were probed with an ECL Plus kit (Amersham Biosciences, Little Chalfont, UK). Blot bands were quantified by densitometry using ImageJ software (ImageJ 1.4; NIH, Bethesda, MD).

Statistical Analysis

All analyses were performed using SigmaPlot 11.0 and GraphPad Prism 6 (GraphPad software, San Diego, CA). Data are represented as a mean±SD. Data normality was first confirmed using the Shapiro-Wilk normality test. For the data that passed the normality test, the statistical differences among groups were further analyzed using one-way ANOVA followed by Tukey multiple comparison post hoc analysis. For the data that failed the normality test, Kruskal-Wallis 1-way ANOVA on Ranks was used, followed by Tukey multiple comparison post hoc analysis. *P* value of <0.05 was considered statistically significant.

Results

Mortality and Exclusion

There was no significant difference in SAH grading score at 24 hours in all SAH groups (Figure S2A and S2B). Mortality rates were not significantly different among these operated groups (Figure S2C). No rats died in the sham group. According to the SAH grading score, 24 rats with mild SAH were excluded from this study (Table S1).

Endogenous NTN-1 and DCC Receptor Expression Were Upregulated After SAH

As shown in Figure 1, NTN-1 expression in the left hemisphere was significantly increased from 12 hours and reached its highest level at 72 hours after SAH (Figure 1A). DCC expression was also elevated from 12 hours and peaked at 24 hours but declined at 72 hours after SAH (Figure 1A). Double immunofluorescence staining revealed that NTN-1 and DCC receptor were predominantly expressed in endothelial cells in cerebral cortex at 24 hours after SAH (Figure 1B and 1C).

Administration of Exogenous NTN-1 Improved Neurobehavioral Functions and Reduced Brain Edema and BBB Permeability After SAH

The rats from the vehicle and NTN-1 (5 and 15 μg/kg) groups presented worse neurological deficits (Figure 2A) and higher brain water content (Figure 2C) in both hemispheres at 24 hours post-SAH than those in the sham group. Administration of exogenous NTN-1 at a dose of 45 μg/kg significantly ameliorated neurological deficits (Figure 2A and 2B) and reduced brain water content (Figure 2C and 2D) in both hemispheres at both 24 and 72 hours after SAH, compared with the vehicle and NTN-1 (5 and 15 μg/kg) groups. Based on the outcome study, the optimal dose of NTN-1 was 45 μg/kg, which was used for the rest of the experiments.

BBB permeability was assessed by EB extravasation in both hemispheres. Although EB extravasation in the vehicle group was markedly increased at 24 hours post-SAH, exogenous NTN-1 treatment significantly reduced EB dye leakage in both hemispheres (Figure 2E). The intensity of EB fluorescence in the ipsilateral cortex was consistent with the findings of EB extravasation measured by spectrophotometry (Figure 3D).

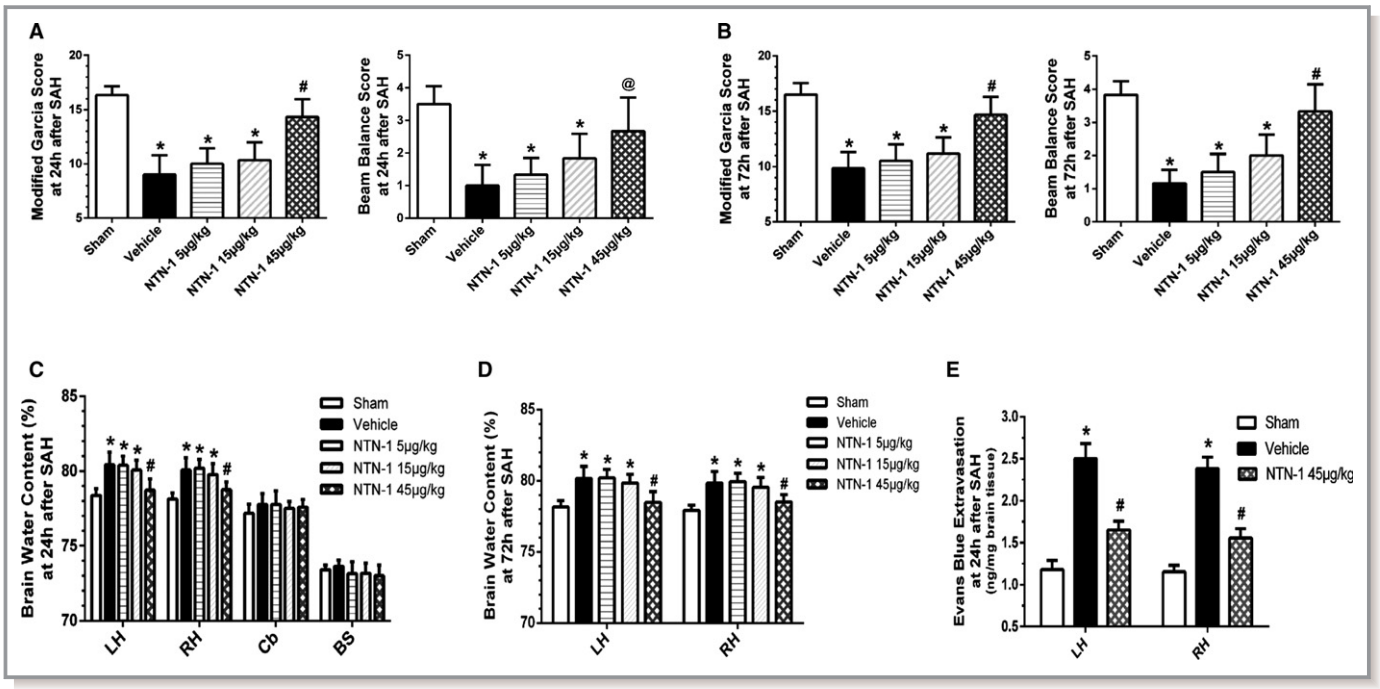

Figure 2. The neuroprotective effects of exogenous Netrin-1 (NTN-1) on neurological scores, brain edema, and blood-brain barrier integrity after subarachnoid hemorrhage (SAH). SAH significantly decreased neurological scores (A), and increased brain water content (C) and Evans blue (EB) extravasation (E) in both hemispheres. However, administration of exogenous NTN-1 at a dose of 45 μg/kg markedly ameliorated neurological deficits (A and B), and reduced brain water (C and D) at 24 and 72 hours as well as decreased EB extravasation at 24 hours after SAH (E). n=6 for each group. *P<0.05 vs sham; $^{\#}P$<0.05 vs vehicle, NTN-1 (5 μg/kg), and NTN-1 (15 μg/kg); $^{@}P$<0.05 vs Vehicle and NTN-1 (5 μg/kg). BS indicates brain stem; Cb, cerebellum; LH, left hemisphere; RH, right hemisphere.

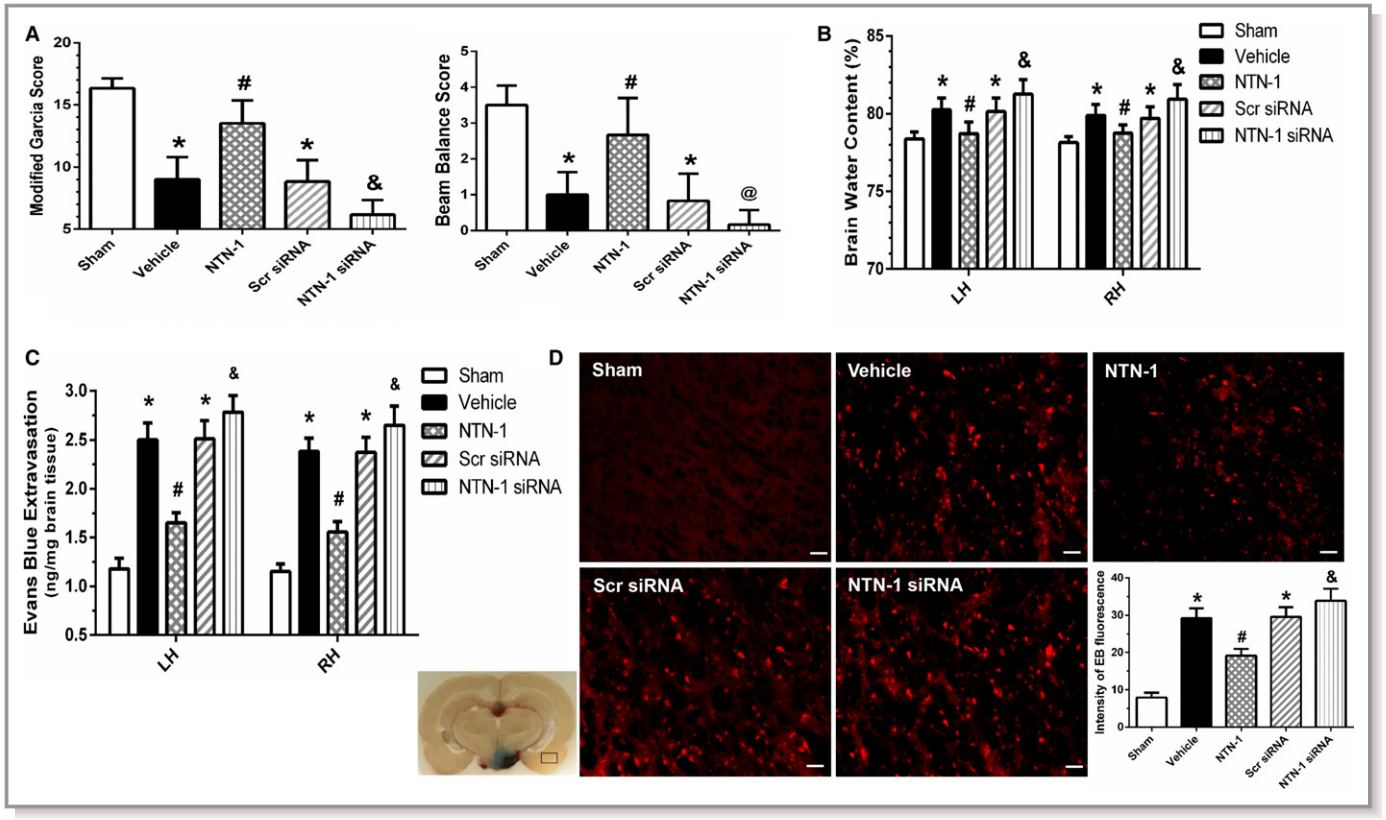

Figure 3. The effects of silencing of endogenous Netrin-1 (NTN-1) by NTN-1 small interfering RNA (siRNA) on blood-brain barrier integrity at 24 hours after subarachnoid hemorrhage (SAH). NTN-1 siRNA aggravated neurological deficits (A) and increased brain water content (B) and Evans blue (EB) extravasation (C) in both hemispheres. n=6 for each group. D, Representative fluorescent micrograph of EB extravasation and quantitative analyses of the intensity of EB fluorescence in the ipsilateral cortex. n=2 for each group. *P<0.05 vs sham, $^{\#}P$<0.05 vs Vehicle, $^{\&}P$<0.05 vs NTN-1 and Scr siRNA; $^{@}P$<0.05 vs NTN-1. LH indicates left hemisphere; RH, right hemisphere; Scr siRNA, scramble siRNA. Scale bar=25 μm.

Silencing of Endogenous NTN-1 Aggravated Neurological Deficits and Increased Brain Edema and BBB Permeability After SAH

To assess the role of NTN-1 in BBB integrity, NTN-1 siRNA was administered by ICV injection in order to silence endogenous NTN-1. The silencing efficacy of NTN-1 siRNA was validated by Western blot. The result showed that NTN-1 expression was inhibited by NTN-1 siRNA at 72 hours after siRNA injection (Figure S3A). Silencing of endogenous NTN-1 significantly aggravated neurological impairments (Figure 3A), brain edema (Figure 3B), and EB extravasation (Figure 3C and 3D) in both hemispheres at 24 hours after SAH, compared with NTN-1 treatment and scramble siRNA groups.

Effect of Exogenous NTN-1 and Depletion of Endogenous NTN-1 on Expression of Downstream Signaling Pathway of FAK/RhoA After SAH

As shown in Figure 4, the expression of phosphorylated FAK (p-FAK) and RhoA activity were increased, whereas endothelial junction proteins (ZO-1 and Occludin) were remarkably decreased at 24 hours after SAH, when compared with the sham group. However, administration of exogenous NTN-1 further augmented p-FAK expression (Figure 4A), decreased RhoA activity (Figure 4B), and thereby increased expressions of endothelial junction proteins (Figure 4C and 4D) compared with vehicle group. Conversely, NTN-1 siRNA pretreatment resulted in opposite changes of downstream signaling molecules (Figure 4) in contrast to that found in the NTN-1 treatment group.

DCC In Vivo Knockdown Abolished the Protective Effects of Exogenous NTN-1 on BBB Integrity After SAH

The knockdown efficacy of DCC siRNA was confirmed by Western blot. DCC siRNA significantly inhibited DCC receptor expression in the ipsilateral hemisphere at 72 hours after siRNA injection (Figure S3B). DCC siRNA pretreatment sufficiently abolished the protective effect of exogenous NTN-1 on neurological deficits (Figure 5A), brain edema (Figure 5B), and BBB disruption (Figure 5C). Moreover, DCC

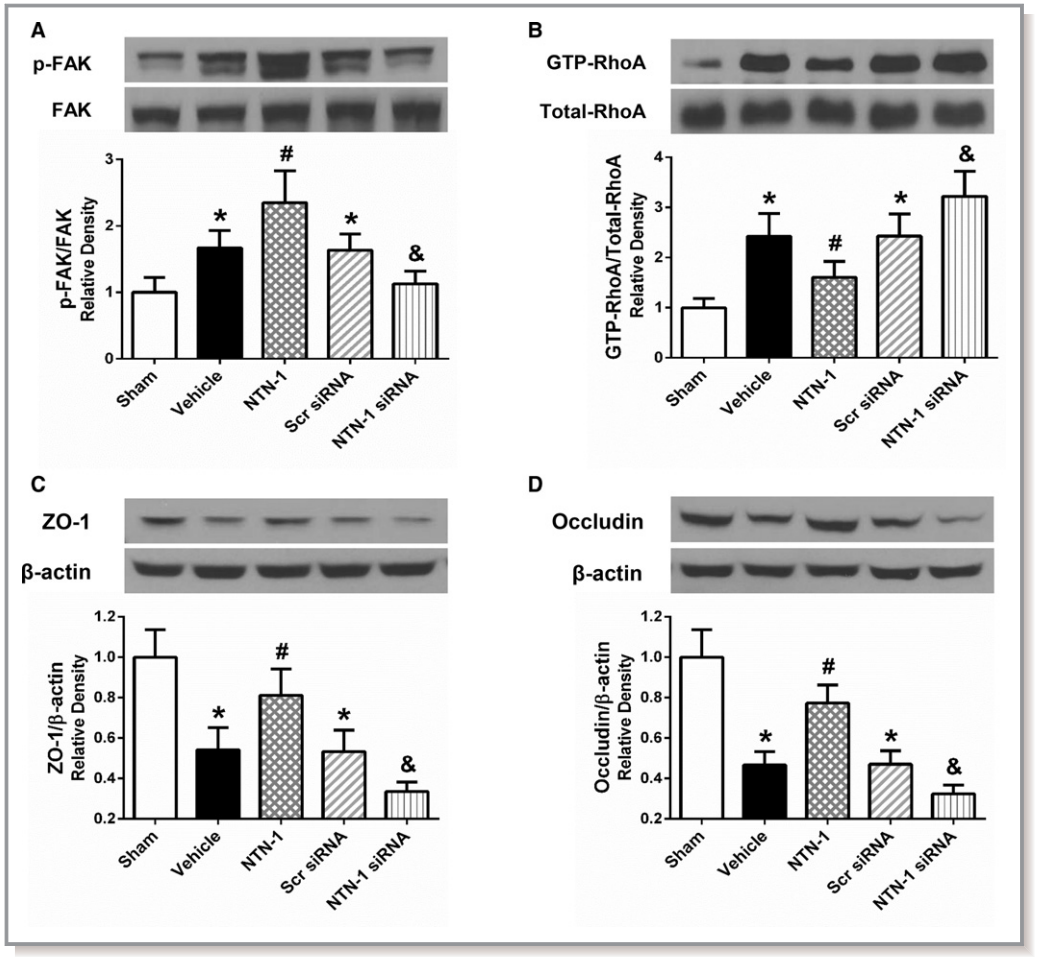

Figure 4. Changes in expressions of downstream signaling pathway of Focal Adhesion Kinase (FAK)/RhoA after exogenous Netrin-1 (NTN-1) treatment and NTN-1 siRNA pretreatment at 24 hours post–subarachnoid hemorrhage (SAH). Exogenous NTN-1 significantly further enhanced the FAK phosphorylation (A), which resulted in the suppression of RhoA activity (B). While the endogenous NTN-1 was depleted by use of NTN-1 siRNA, the p-FAK level decreased (A), which augmented the RhoA activation (B). The RhoA activity was negatively related to the levels of endothelial tight junction proteins including ZO-1 (C) and Occludin (D) in the ipsilateral hemisphere. Relative densities of each protein have been normalized against the sham group. n=6 for each group. $^{*}P<0.05$ vs sham, $^{\#}P<0.05$ vs vehicle, and $^{\&}P<0.05$ vs NTN-1 and Scr siRNA. Scr siRNA indicates scramble siRNA; siRNA, small interfering RNA.

in vivo knockdown significantly decreased expressions of p-FAK, ZO-1, and Occludin but increased RhoA activity (Figure 5D) when compared with NTN-1 treatment alone or with scramble siRNA at 24 hours after SAH.

Inhibition of FAK Reversed the Protective Effects of Exogenous NTN-1 on BBB Integrity After SAH

To further confirm whether the protective effect of NTN-1 on BBB integrity is mediated by interacting with FAK, Fib-14 was administered by intraperitoneal injection at 1 hour before SAH induction and then followed by NTN-1 treatment in SAH rats. As shown in Figure 6A and 6B, inhibition of FAK by Fib-14 significantly reversed the protective effects of exogenous NTN-1 treatment on neurological deficits and brain edema at 24 hours after SAH. Consistently, Fib-14 pretreatment abolished the protection of NTN-1 treatment on BBB, as significant increases in albumin leakage (Figure 6C) were observed at 24 hours after SAH. Furthermore, pretreatment with Fib-14 significantly increased RhoA activity (Figure 6D) and MMP-9 (Figure 6C) expression with a reduction in ZO-1 and Occludin expressions (Figure 6D), when compared with NTN-1 treatment, and NTN-1+PBS groups.

Discussion

In the present study we first elucidated the NTN-1–mediated signaling pathway in BBB protection following SAH in rats. Our

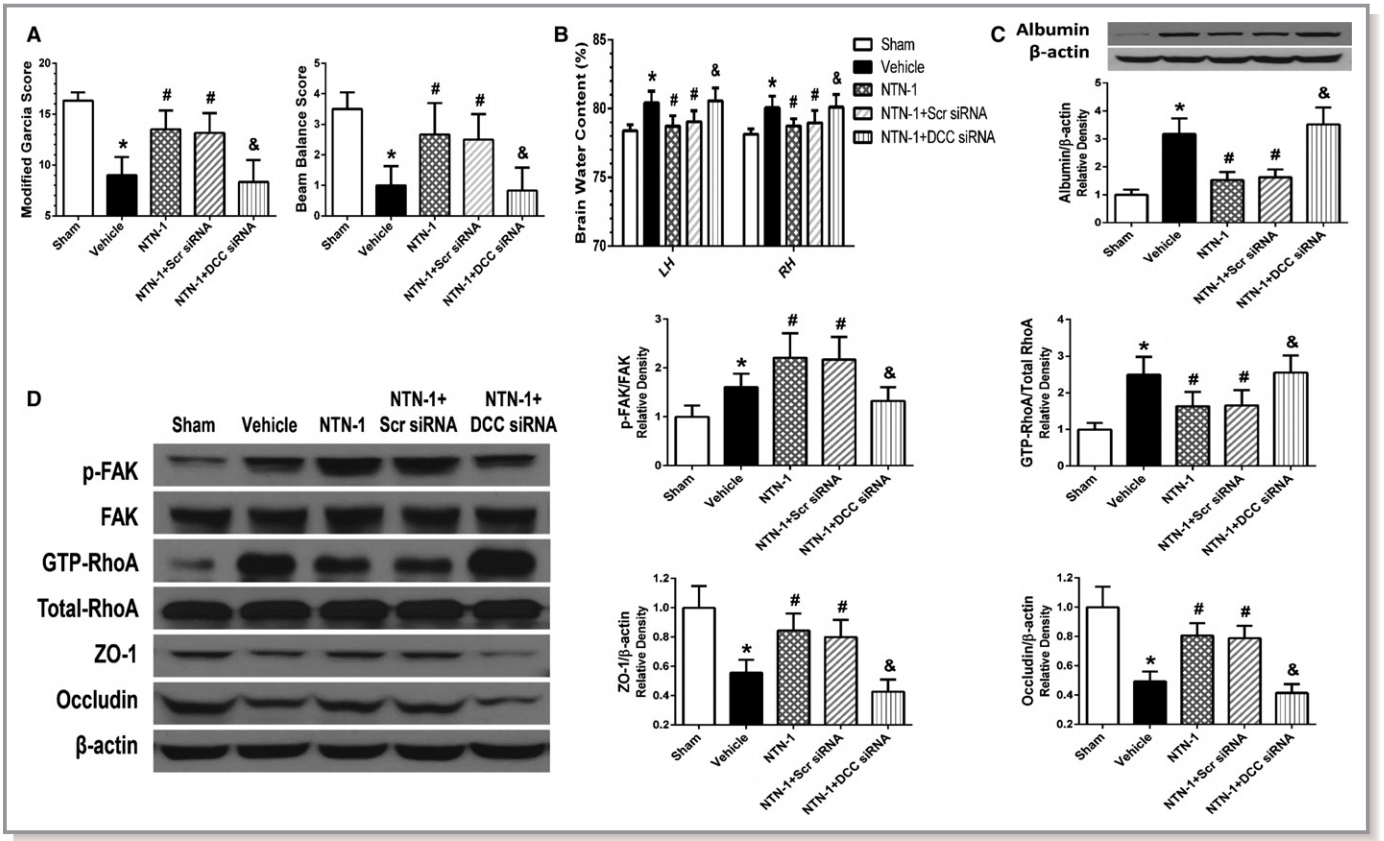

Figure 5. Knockdown Deleted in Colorectal Cancer (DCC) using DCC siRNA abolished the protective effects of exogenous Netrin-1 (NTN-1) on blood-brain barrier integrity at 24 hours after subarachnoid hemorrhage (SAH). DCC siRNA aggravated neurological impairments (A) and increased brain water content (B) and albumin leakage (C). Consistently, DCC siRNA abolished the effects of exogenous NTN-1 on the enhancement of Focal Adhesion Kinase (FAK) phosphorylation, inhibition of RhoA activation, and promotion of tight junction protein expression (D) in the ipsilateral hemisphere. Relative densities of each protein have been normalized against the sham group. n=6 for each group. $^{*}P<0.05$ vs sham, $^{\#}P<0.05$ vs vehicle, and $^{\&}P<0.05$ vs NTN-1 and NTN-1+Scr siRNA. LH indicates left hemisphere; RH, right hemisphere; Scr siRNA, scramble siRNA.

results showed that endogenous NTN-1 and DCC receptor were upregulated in the early stage after SAH. Exogenous NTN-1 treatment reduced brain edema and BBB permeability and thereby alleviated neurological deficits after SAH, which were accompanied by an increase in FAK phosphorylation and a decrease in RhoA activity as well as endothelial junction protein upregulation. In contrast, silencing of endogenous NTN-1 by special siRNA exacerbated brain edema, BBB disruption, and neurological deficits. Furthermore, knockdown DCC using DCC siRNA or inhibition of FAK by Fib-14 abolished the neuroprotective effects of exogenous NTN-1 on BBB integrity and brain edema formation, which were associated with the increased RhoA activity and MMP-9 and the degraded endothelial junction proteins and basal lamina at 24 hours after SAH. Taken together, these findings support our hypothesis that exogenous NTN-1 could preserve BBB integrity after SAH at least in part via a DCC/FAK/RhoA signaling pathway.

NTN-1 binding to its canonical receptors initiates multiple signaling pathways and mediates diverse biological functions in the brain.[12,26-30] Recently, the potential role of NTN-1 in preserving BBB integrity has been demonstrated in rodent models of traumatic brain injury and experimental autoimmune encephalomyelitis.[13,14] There is compelling evidence that exogenous NTN-1 significantly diminished the diffusion of albumin and dextran across human brain-derived endothelial cells in vitro, whereas NTN-1 knockout mice (NTN-$1^{-/-}$) were associated with significantly increased extravasation of plasma proteins into the brain parenchyma, indicating that exogenous NTN-1 maintained and stabilized optimal BBB function after experimental autoimmune encephalomyelitis .[13] In the present study we observed that endogenous NTN-1 expression was increased at 24 hours after SAH, and NTN-1 and DCC receptor were mainly expressed in endothelial cells. These findings were consistent with the observation in a cerebral ischemia model.[12,28-30] Similar to the protection by NTN-1 of BBB integrity shown in a preclinical study of experimental autoimmune encephalomyelitis and traumatic brain injury, the administration of exogenous NTN-1 significantly diminished brain edema and BBB disruption and

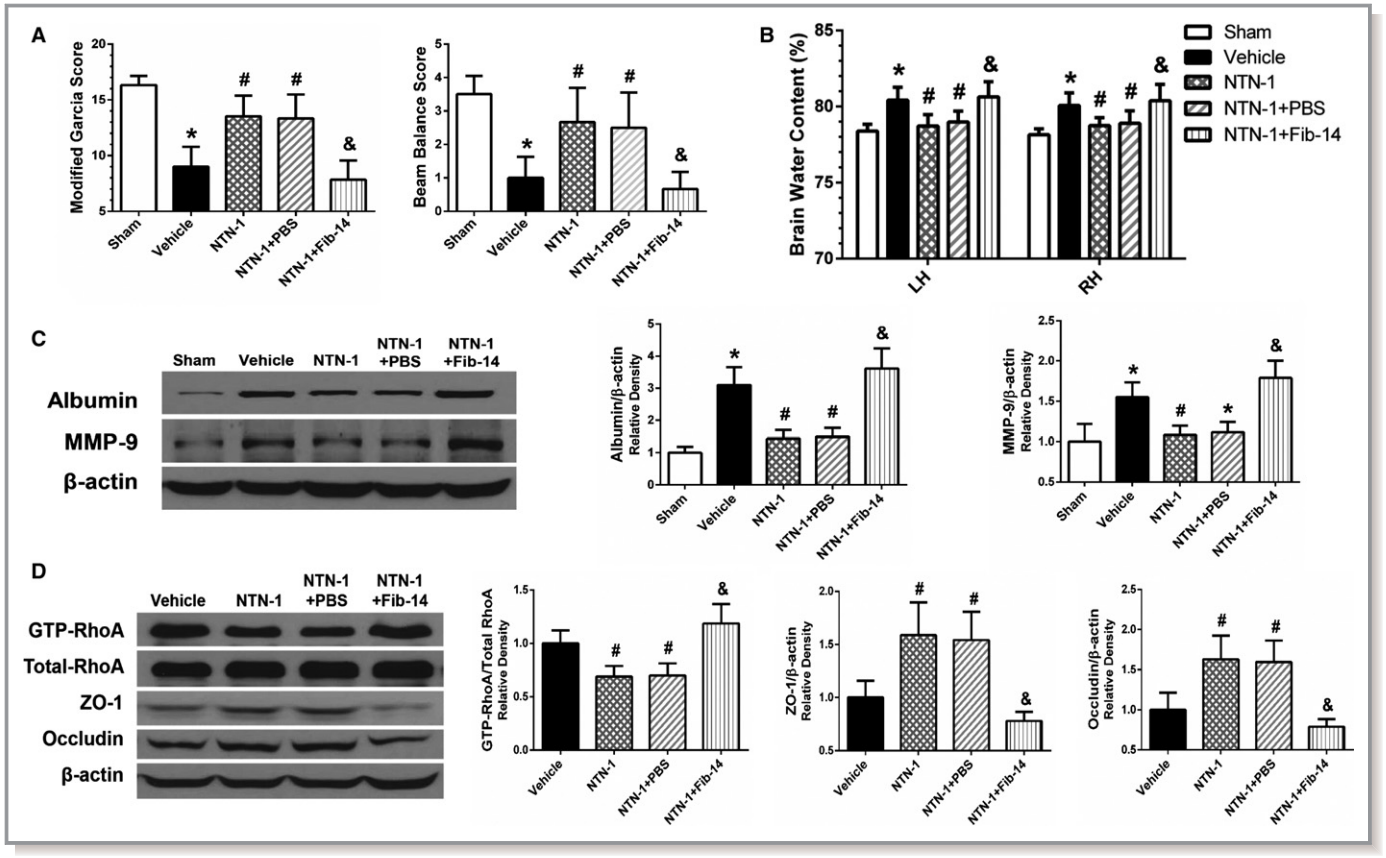

Figure 6. Inhibition of Focal Adhesion Kinase (FAK) by Fib-14 reversed the protective effects of exogenous Netrin-1 (NTN-1) on blood-brain barrier integrity at 24 hours after subarachnoid hemorrhage (SAH). Fib-14 significantly worsened neurological deficits (A) and increased brain water content (B). The effects of NTN-1 on albumin and MMP-9 protein reduction (C), RhoA activity suppression, and tight junction protein enhancement were reversed by Fib-14 (D). Relative densities of each protein have been normalized against the sham group. n=6 for each group. $^{*}P<0.05$ vs sham, $^{\#}P<0.05$ vs Vehicle, and $^{\&}P<0.05$ vs NTN-1 and NTN-1+PBS. LH indicates left hemisphere; RH, right hemisphere.

improved neurological functions after SAH, whereas silencing endogenous NTN-1 and DCC receptor by siRNA aggravated BBB breakdown and neurological deficits.[13,14]

Although the exact mechanisms of NTN-1–mediated BBB regulation are not well clarified, FAK may play a critical role in the NTN-1/DCC-mediated signaling pathway as an immediately downstream mediator after NTN-1 binding to the DCC receptor.[31] Ren et al[31] revealed that NTN-1 induction of FAK tyrosine phosphorylation was DCC specific, and inhibition of FAK tyrosine phosphorylation blocked NTN-1 function. Indeed, FAK is required in regulating endothelial cell function and barrier function.[32-34] Endothelial-specific deletion of FAK in mouse was embryonically lethal due to impaired vascular development.[32,33] In another study Zhao et al[34] demonstrated that endothelial cells lacking FAK exhibited a significant disruption in barrier integrity associated with an abnormal distribution of vascular endothelial cadherin. In the present study we found that FAK phosphorylation level was increased after SAH, which was consistent with the previous observation in a SAH model.[35] Furthermore, several lines of evidence have confirmed that FAK is a key regulator of RhoA activity.[16,36] Deletion of FAK in mouse endothelial cells resulted in a significant increase in RhoA activity correlating with increased endothelial barrier permeability through disrupting the balance between the activities of RhoA and Rac1 GTPases, which is a critical determinant of a stable endothelial barrier.[37,38] Previous observations implied that RhoA activation contributed to Rho kinase phosphorylation, upregulated MMP-9 expression, and degraded endothelial junction proteins and basal lamina to impair BBB integrity.[39-43] If FAK phosphorylation negatively related RhoA activation, there seems to be a paradoxical result that both p-FAK and RhoA expression were increased in the ipsilateral hemisphere in vehicle-treated SAH rats. Given that RhoA is a common downstream protein, it could be affected by multiple upstream signaling pathways. Some signaling proteins have been shown to activate RhoA directly, whereas others may inhibit RhoA activity.[39,40] Thus, we speculated that the elevated extent of endogenous p-FAK after SAH might not be sufficient to inhibit RhoA activity. It is the administration of exogenous NTN-1 that further significantly increased the p-FAK level, resulting in an effective inhibition of RhoA activity and MMP-9

expressions. Nevertheless, our data demonstrated that the FAK inhibitor Fib-14 specifically inhibited FAK phosphorylation at Y397 and significantly reversed the BBB protection of exogenous NTN-1, which resulted in the increased RhoA activity and MMP-9 expression and downregulated expressions of endothelial junction proteins as well as degraded basal lamina, thereby aggravating BBB disruption at 24 hours after SAH. Those findings supported the DCC/FAK/RhoA signaling pathway as part of underlying BBB protective mechanism of exogenous NTN-1 after SAH.

There are some limitations in the present study. NTN-1 exerts multiple protective properties via different signaling pathways in the nervous system.[26-30] In the present study we only focused on the neuroprotective effects of NTN-1 on BBB integrity after SAH. Hence, we cannot exclude the possibility that antiapoptosis, anti-inflammatory, preservation of axonal integrity, or other effects of NTN-1 also play neuroprotective roles in EBI after SAH. Future studies are needed to investigate the other protective functions as well as the long-term neurological benefits of NTN-1 after SAH. Additionally, we acknowledged that a statistically significant result may have occurred by chance due to the large number of analyses performed.

In conclusion, the administration of exogenous recombinant NTN-1 after SAH could improve neurological impairment, reduce brain edema, and preserve BBB integrity with upregulation of brain endothelial junction proteins in rats. Such neuroprotection by NTN-1 was most likely through a DCC/FAK/RhoA signaling pathway. Therefore, recombinant NTN-1 might be a promising treatment strategy for BBB protection in patients with SAH.

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Disclosures

None.

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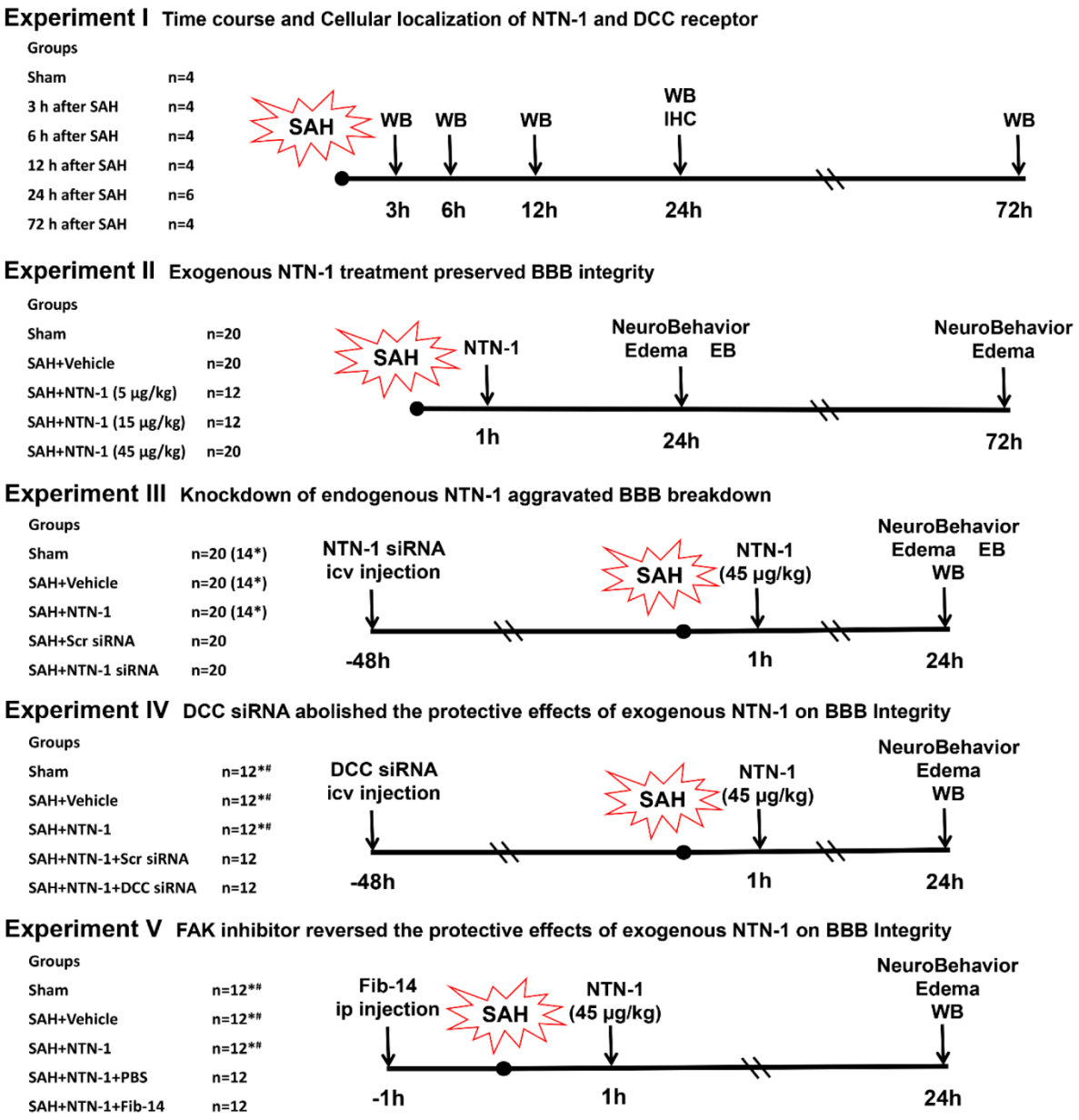
Supplemental Material

Table S1. Numbers of animals used in each group

Experimental Group	Neurobehavioral test Brain water content	BBB permeability	WB	IHC	Death	Exclusion	Subtotal
Sham	12	8	10		0	0	30
SAH (3h, 6h, 12h, 24h, 72h)			4×5	2	7	3	32
SAH+Vehicle	12	8	6		9	5	40
SAH+NTN-1 (5 μg/kg)	12				4	2	18
SAH+NTN-1 (15 μg/kg)	12				3	1	16
SAH+NTN-1 (45 μg/kg)	12	8	6		5	3	34
SAH+Scr siRNA	6	8	6		6	3	29
SAH+NTN-1 siRNA	6	8	6		8	2	30
SAH+NTN-1+Scr siRNA	6		6		3	1	16
SAH+NTN-1+DCC siRNA	6		6		5	2	19
SAH+NTN-1+PBS	6		6		2	1	15
SAH+NTN-1+Fib-14	6		6		4	1	17
Sham+Scr siRNA			3		0	0	3
Sham+NTN-1 siRNA			3		0	0	3
Sham+DCC siRNA			3		0	0	3
SAH+DCC siRNA			3		1	0	4
Total					57	24	309

Figure S1.



* Shared with Experiment II, # shared with Experiment III

Figure S2.

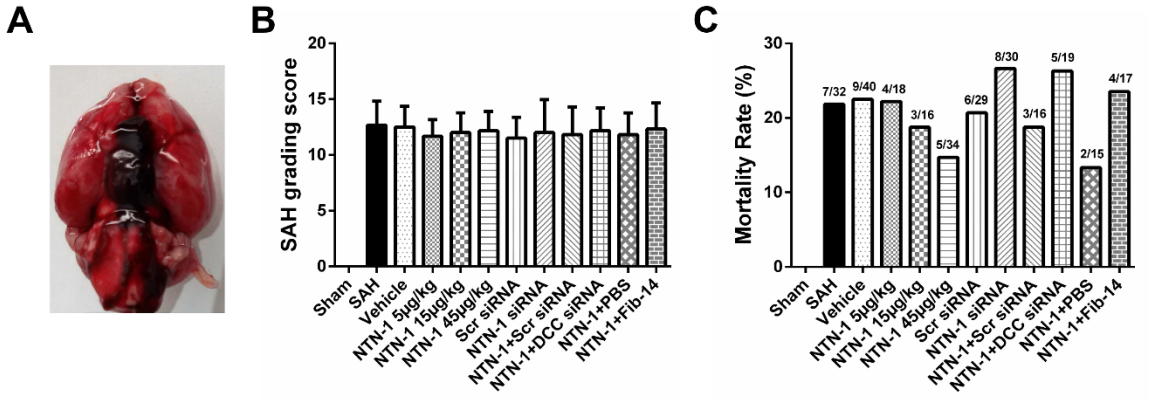
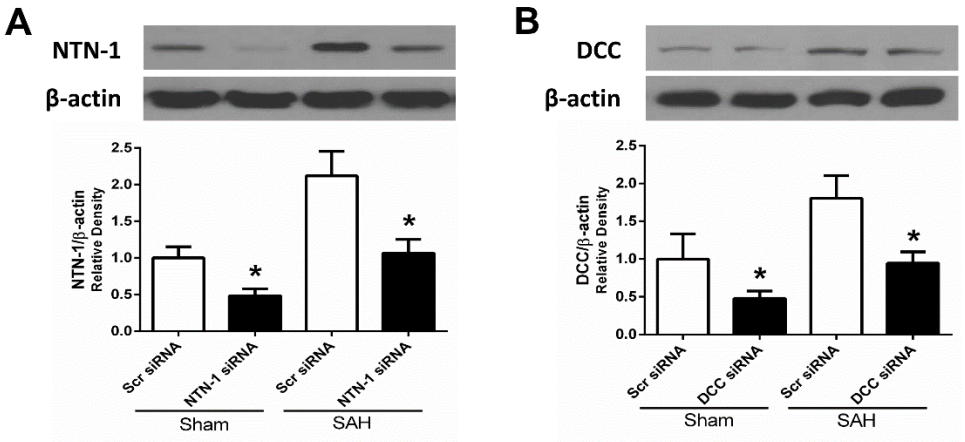


Figure S3.



Supplemental Figure Legends:

Figure S1. Experimental design and animal group classification. BBB, Blood brain barrier; DCC, deleted in colorectal cancer; EB, Evans blue extravasation and fluorescence; FAK, Focal adhesion kinase; Fib-14, FAK inhibitor 14; IHC, immunohistochemistry; icv, intracerebralventricular; ip, intraperitoneal; NTN-1, Netrin-1; SAH, Subarachnoid hemorrhage; Scr siRNA, Scramble siRNA; siRNA, small interfering RNA; WB, Western blot.

Figure S2. Representative image of subarachnoid hemorrhage (SAH) model, SAH grading score and mortality rates among each group. **A**, Representative image of SAH model in rat. SAH was induced in the basal cisterns. **B**, There was no significant difference of SAH grading score among all the experimental groups at 24 hours after SAH. **C**, Mortality rate for each group was listed as follow: SAH group 21.87% (7/32), SAH+vehicle group 22.5% (9/40), SAH+NTN-1 (5 μg/kg) group 22.22% (4/18), SAH+NTN-1 (15 μg/kg) group 18.75% (3/16), SAH+NTN-1 (45 μg/kg) group 14.71% (5/34), SAH+Scr siRNA group 20.69% (6/29), SAH+NTN-1 siRNA group 26.67% (8/30), SAH+NTN-1+Scr siRNA group 18.75% (3/16), SAH+NTN-1+DCC siRNA group 26.31% (5/19), SAH+NTN-1+PBS group 13.33% (2/15), SAH+NTN-1+Fib-14 group 23.53% (4/17). Mortality rates were not significantly different among these operated groups.

Figure S3. The knockdown efficiency of Netrin-1 (NTN-1) siRNA and DCC siRNA. Representative Western blot bands and quantitative analysis of NTN-1 (**A**) and DCC (**B**) expressions in ipsilateral hemisphere at 72 hours after siRNA injection. Relative densities of each protein have been normalized against the sham Scr siRNA group. Scr siRNA, scramble siRNA; NTN-1 siRNA, Netrin-1 siRNA mixtures; DCC siRNA, DCC siRNA mixtures, n=3 for each group. $^{*}P<0.05$ vs Scr siRNA.