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Critical role of EphA4 in early brain injury after subarachnoid hemorrhage in rat

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Abstract

Early brain injury (EBI) is reported as a primary cause of mortality in subarachnoid hemorrhage (SAH) patients. Eph receptor A4 (EphA4) has been associated with blood-brain barrier integrity and pro-apoptosis. We aimed to investigate a role of EphA4 in EBI after SAH. One hundred and seventy-nine male adult Sprague-Dawley rats were randomly divided into sham versus endovascular perforation model of SAH groups. SAH grade, neurological score, Evans blue dye extravasation, brain water content, mortality, Fluoro-Jade staining, immunofluorescence staining, and western blot experiments were performed after SAH. Small interfering RNA (siRNA) for EphA4, recombinant Ephexin-1 (rEphx-1), and Fasudil, a potent ROCK2 inhibitor, were used for intervention to study a role of EphA4 on EBI after SAH. The expression of EphA4, Ephexin-1, RhoA, and ROCK2 significantly increased after SAH. Knockdown of EphA4 using EphA4 siRNA injection intracerebroventricularly (i.c.v) reduced Evans blue extravasation, decreased brain water content, and alleviated neurobehavioral dysfunction after SAH. Additionally, the expression of Ephexin-1, RhoA, ROCK2 and cleaved caspase-3 were decreased. Tight junction proteins increased, and apoptotic neuron death decreased. The effects of EphA4 siRNA were abolished by

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Author contribution statement

The idea and design were made by Ruiming Fan, John H Zhang, Jiping Tang and Yangmei Chen. Drafting the article was done by Ruiming Fan, Budbazar Enkhjargal, and Richard Camara. Ruiming Fan, Budbazar Enkhjargal, and Feng Yan performed most experiments. Critically revising the article was made by all authors. Reviewed submitted version of manuscript was made by all authors. John H Zhang and Yangmei Chen approved the final version of the manuscript on behalf of all the authors.

Disclosures/conflict of interest

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rEphx-1. In contrast, Fasudil abolished the effects of rEphx-1. These results suggest that EphA4, a novel and promising target for treatment, exacerbates EBI through an Ephexin-1/ROCK2 pathway after SAH.

Keywords

Subarachnoid hemorrhage; EBI; EphA4; Blood-brain barrier; Neuronal apoptosis

1. Introduction

Subarachnoid hemorrhage, especially caused by aneurysm rupture, represents one of the most deadly cerebrovascular diseases with both high morbidity and mortality (Etminan, 2015). Although treatment of SAH includes drug therapy, neurosurgery and endovascular surgery technology, mortality is still high, and disability is severe (Grunwald et al., 2014). As cerebral vasospasm after SAH is strongly associated with clinical outcome, clinical trials in the last few decades focused on preventing it. Despite all efforts, no new pharmacological agents have shown to improve patient outcome (Etminan et al., 2011; Macdonald et al., 2008). Early brain injury (EBI) is a significant cause of poor prognosis in patients with SAH (Cahill and Zhang, 2009). The molecular mechanisms of EBI are complex with the major pathological alterations being blood brain barrier (BBB) disruption and apoptotic neuron death (Caner et al., 2012). Each of these are closely related to recovery of neurological function after SAH.

Eph receptors, the largest family of receptor tyrosine kinases in mammals, have been shown to promote cell death in adult germinal zones (Conover et al., 2000; Depaepe et al., 2005). Specifically, Eph receptor A4 (EphA4) exacerbates apoptosis after cerebral ischemia-reperfusion (Furne et al., 2009; Lemmens et al., 2013; Van Hoecke et al., 2012). The EphA4 receptor is not only upregulated but also a cause of poor outcomes after CNS injury, such as ischemia-reperfusion, degeneration disease, and spinal cord injury (Benson et al., 2005; Fu et al., 2014; Li et al., 2012; Spanevello et al., 2013). Once EphA receptors are activated, they induce phosphorylation of Ephexin-1, which preferentially activates RhoA (Sahin et al., 2005). Activation of RhoA/ROCK induces neuronal apoptosis and aggravates neurological function after ischemic stroke (Lemmens et al., 2013; YY et al., 2015). Moreover, activation of RhoA induces the BBB breakdown after intracerebral hemorrhage (Huang et al., 2012) and neuronal apoptosis (Lai, 2003). However, the role of EphA4 in EBI after SAH remains unknown.

In the present study, we aimed to identify a role of EphA4 in EBI after SAH in rats and to find a translational approach for treatment of human SAH.

2. Materials and methods

2.1. Animals

One hundred and seventy-nine male adult Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 280 to 330 g were used in this study as shown in Table 1. All rats resided in a light and temperature-controlled room with sufficient food and water. All experimental

procedures were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

2.2. Experimental design

The experiment was designed as follows,

Part I—To determine expression of the key protein related to EphA4 dependent mechanism after SAH, 42 rats were randomly assigned into 6 groups in time course experiments: Sham ($n = 6$), 3 h after SAH ($n = 6$), 6 h after SAH ($n = 6$), 12 h after SAH ($n = 6$), 24 h after SAH ($n = 6$), and 72 h after SAH ($n = 6$). Western blots were used to detect the protein expression of EphA4, Ephexin-1, GTP-RhoA, and ROCK2 in the left hemisphere of the brain. Cellular localization of EphA4 on neurons and astrocytes was detected with IHC (Sham, $n = 3$; 24 h after SAH, $n = 3$) (Table 1).

Part II—To evaluate a role of EphA4 in outcomes after SAH, 84 rats were randomly divided into 4 groups: Sham ($n = 21$), SAH + Vehicle (10 μ L of sterile 0.9% NaCl; $n = 21$), SAH + 0.5 ng/ μ L scramble siRNA (5 ng in 10 μ L of sterile 0.9% NaCl; $n = 21$), SAH + 0.5 ng/ μ L EphA4 siRNA (5 ng in 10 μ L of sterile 0.9% NaCl; $n = 21$). Scrambled siRNA or EphA4 siRNA was injected intracerebroventricularly (i.c.v) 48 h before SAH. Neurological scores, brain water content ($n = 6$, each group), Evans blue extravasation ($n = 6$, each group), western blot ($n = 6$, each group), immunohistochemistry and Fluoro-Jade experiments ($n = 3$, each group) were assessed at 24 h after SAH (Table 1).

Part III—To determine molecular mechanism of EphA4 downstream, 18 rats were randomly assigned into 2 additional groups: SAH + EphA4 siRNA+ rEphx-1 (50 ng in 10 μ L of sterile 0.9% NaCl i.c.v and 0.5 mL of sterile 0.9% NaCl i.p; $n = 9$), SAH + EphA4 siRNA +rEphx-1+Fasudil (10 mg/kg in 0.5 mL of sterile 0.9% NaCl i.p; $n = 9$). Fasudil, a ROCK2 inhibitor, and sterile 0.9% NaCl were administered i.p 1 h after SAH induction. Western blots ($n = 6$, each group) and Fluoro-Jade staining ($n = 3$, each group) were performed 24 h after SAH in all groups (Table 1).

2.3. SAH model and experimental protocol

The endovascular perforation model of SAH was produced in rats as described previously (Suzuki et al., 2010). Briefly, with 3% isoflurane, a sharpened 4–0 monofilament nylon suture was inserted gently into the internal carotid artery through the external carotid artery stump and common carotid artery bifurcation. The 3 cm suture was advanced until resistance was felt and perforated the bifurcation of the anterior and middle cerebral arteries. Sham operated rats underwent the same procedures, except the suture was withdrawn without puncture.

2.4. Severity of SAH

The severity of SAH was blindly assessed at the time of euthanasia as previously described (Sugawara et al., 2008a, 2008b). Briefly, animals were euthanized and the brains were removed. The basal cistern was divided into 6 segments. Each segment was allotted a grade from 0 to 3 depending on the amount of subarachnoid blood in the segment. The animals

received a total score ranging from 0 to 18 after adding the scores from all 6 segments. The rats that received a score of <9 were excluded from the study.

2.5. Neurological outcome assessment

Neurological impairments were blindly evaluated using an 18-point score test of modified Garcia and 4-point score test of beam balance as previously described (Altay et al., 2014).

2.6. Intracerebroventricular drug administration

Intracerebroventricular (i.c.v) drug administration was performed as reported previously (Chen et al., 2015; Tang et al., 2015). Briefly, rats were laid in a stereotaxic apparatus under 2.5% isoflurane anesthesia. A burr hole was drilled into the skull according to the following coordinates relative to bregma: 0.92 mm posterior and 1.5 mm lateral. The needle of a 10 μ L Hamilton syringe (Microliter 701; Hamilton Company, Reno, NV) was inserted into the left lateral ventricle through the burr hole 3.3 mm below the horizontal plane of bregma. Sterile 0.9% NaCl or 10 μ L rEphx-1 (5 ng/ μ L; MyBioSource, CA) were administered 1 h after SAH induction by a pump at a rate of 1 μ L/min. EphA4 siRNA or scrambled siRNA (5 ng/10 μ L, i065462, ABM Inc., Canada) was infused at the same rate 48 h before SAH induction.

2.7. Brain water content

At 24 h after SAH, the left hemisphere, right hemisphere, cerebellum, and brain stem were separated, and each part was immediately weighed (wet weight) after removal. After dehydration in 105 °C for 72 h, brain part was weighed again (dry weight). The percentage of water content was calculated as (wet weight – dry weight)/wet weight (Yin et al., 2016).

2.8. Blood–brain barrier disruption

The permeability of BBB was evaluated on the basis of Evans blue extravasation, as described previously (Guo et al., 2016). The brain level of Evans blue was determined at 615 nm for spectrophotometric quantification.

2.9. Western blot analysis

The left hemisphere brain tissues were collected at 24 h after SAH. Western blot tests were performed as described previously (Enkhjargal et al., 2016). Briefly, Animals were euthanized at 24 h following SAH. Intracardiac perfusion with cold phosphate-buffered saline (PBS, pH 7.4) was performed, followed by removal of the brain and separation into left and right hemispheres. The brain parts were stored appropriately at –80 °C immediately until analysis. Protein extraction from the left hemisphere was obtained by gently homogenizing them in RIPA lysis buffer (Santa Cruz Biotechnology Inc., sc-24948) with further centrifugation at 14,000 rounds per minute at 4 °C for 30 min. The supernatant was used as whole cell protein extract and the protein concentration was determined using a spectrophotometer (Thermo GENESYS 10S UV-VIS). Equal amounts of sample protein (50 μ g) were loaded onto an SDS-PAGE gel. After electrophoresis and transfer of the samples to a nitrocellulose membrane, the membrane was blocked and incubated with the primary antibody overnight at 4 °C. Primary antibodies used in western blot were anti-EphA4 (1:1000, ab5396, abcam, MA,USA); anti-Ephexin-1(1:1000, ab157594, abcam, MA,USA);

RhoA Activation Assay Kit (ab211164, abcam, MA,USA); anti-ROCK2(1:1000, ab71598, abcam, MA,USA); anti-zonula occludens-1 (ZO-1) (1:1000, sc10804, Santa Cruz Biotechnology, CA); anti-Claudin-5(1:1000, sc374221, Santa Cruz Biotechnology, CA); cleaved caspase-3 (CC-3) (1:1000, ab90437, abcam, MA,USA), β -actin (1:5000, I-19, Santa Cruz Biotechnology, CA). Subsequently, membranes were incubated at room temperature for 2 h with the appropriate secondary antibodies (1:5000, Santa Cruz Biotechnology Inc.). Immunoblots were then probed with an ECL Amersham Western blotting detection reagents (Amersham Biosciences UK Ltd., PA, USA). Blot bands were quantified by densitometry using image J software (Image J 1.46r, NIH, USA). B-Actin was used as loading control.

2.10. Immunofluorescence staining

The brain slices for double-fluorescence staining was performed at 24 h after SAH as described previously (Sugawara et al., 2008a, 2008b). Under deep anesthesia, the rats were transcardially perfused with ice-cold 0.1 mol/L PBS (pH 7.4) for 15 min, followed by 15 min of ice-cold 10% paraformaldehyde for fixation. Whole brains were quickly removed and fixed in 10% paraformaldehyde for 1 day, followed by 30% sucrose for an additional 3 days. Ten-micrometer-thick coronal sections cut by a cryostat (Leica Microsystems LM3050S) were mounted on poly-L-lysine-coated slides (Richard Allen, Kalamazoo, MI). Sections were incubated overnight at 4 °C with anti-EphA4 primary antibody (1:200, ab5396, MA, USA), anti-GFAP (1:500, ab110062), anti-NeuN (1:500, ab104224), and anti-Iba-1 (1:100, ab107159). Appropriate fluorescence dye-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were applied in the dark for 2 h at room temperature at a dilution of 1:500. For negative controls, the primary antibodies were omitted, and the same staining procedures were performed. Eight random microscope fields ($\times 400$) in the base of each brain coronal section were imaged by Leica DMi8 (Leica microsystems, Germany).

2.11. Fluoro-Jade C staining

Apoptotic neuron death was observed by Fluoro-Jade C (TR-100-FJ, Biosensis, USA) staining, which was performed at 24 h after SAH as the instruction of the kit. Eight random microscope fields ($\times 200$) in the base of each brain coronal section were imaged by Leica DMi8 (Leica microsystems, Germany). The number of positive cells was calculated as the mean of the numbers obtained from the 8 pictures.

2.12. Statistical analysis

Neurological score were expressed as median $\pm$ 25th to 75th percentiles and analyzed with Mann-Whitney tests, followed by Steel-Dwass multiple comparisons. Other values were expressed as mean $\pm$ SD. Statistical significance was verified with Analysis of Variance (ANOVA), followed by Tukey multiple comparison tests. The analysis of mortality was done with χ^2 test. $P < 0.05$ were considered statistically significant.

3. Results

3.1. SAH grade

Mean SAH severity scores were measured in survived rats, the mean SAH grading was 11.27 ± 1.86 . There was no significant difference in average SAH grading score among the

groups in each experiment. 5 rats that received a score of <9 were excluded from the study (Table 1).

3.2. Mortality

Of the 179 total animals, 30 rats were subjected to sham and 149 rats were subjected to SAH induction, with total 30 (17%) animals died within 24 h after surgery. The animals were divided randomly in each groups. The mortality within 24 h after surgery in this study was as follows in each group: Sham, 0% (0 died of 9 rats in time course study); SAH, 20% (9 died of 44 rats in time course study); Sham, 0% (0 died of 21); SAH + Vehicle, 24% (7 died of 29); SAH + Scramble siRNA, 21% (6 died of 28); SAH + EphA4 siRNA, 13% (3 died of 24); SAH + EphA4 + rEphx-1, 23% (3 died of 13); SAH + EphA4 siRNA + rEphx-1 + Fasudil, 18% (2 died of 11) (Table 1).

3.3. Expression profile of EphA4 and the downstream proteins after SAH

We investigated the effect of EBI after SAH on EphA4 and down-stream protein. Western blot analysis was performed to determine the protein expression of EphA4, Ephxin-1, RhoA, and ROCK2 in the left hemisphere (Fig. 1A at 3 h, 6 h, 12 h, 24 h, and 72 h after SAH. Results showed that EphA4, Ephxin-1, RhoA, and ROCK2 were significantly upregulated after SAH (Fig. 1B–E, $P < 0.05$ vs. Sham). Double immunostaining of EphA4 with neuronal nuclei (NeuN, marker for neuron), glial fibrillary acidic protein (GFAP, marker for astrocyte), and ionized calcium binding adaptor molecule-1 (Iba-1, marker for microglia) showed that EphA4 is intensely expressed in neurons, astrocytes and microglia at 24 h after SAH (Fig. 2A–C).

3.4. Knockdown EphA4 decreased brain edema and blood-brain barrier permeability, attenuates neurobehavioral deficits after SAH

EphA4 siRNA was administrated i.c.v. 48 h before SAH. Brain water content, Evans blue extravasation and neurological scores were measured at 24 h after SAH. The data showed that in rats receiving EphA4 knockdown, the modified Garcia (Fig. 3A) and beam balance score (Fig. 3B) were higher, the brain water content (Fig. 3C) and BBB permeability (Fig. 3D) were lower at 24 h after SAH.

3.5. Knockdown of EphA4 exacerbated neuronal apoptosis and BBB disruption after SAH

After knockdown of EphA4 using specific siRNA, the expression of EphA4 was significantly lower, compared to negative control groups (Fig. 4A). To identify downstream pathways of EphA4, the expression of active Ephexin-1 and ROCK2 were determined. The upregulated expression of active Ephexin-1 and ROCK2 was significantly reversed in SAH + EphA4 siRNA group compared with the SAH + Vehicle group, whereas scrambled siRNA did not show these effects after SAH (Fig. 4B, C). Moreover, tight junction proteins such as ZO-1 (Fig. 4D), claudin-5 (Fig. 4E) were significantly increased in SAH + EphA4 siRNA group. While, apoptotic protein CC-3 was decreased in the SAH + EphA4 siRNA group (Fig. 4F) compared with SAH + Vehicle group at 24 h after SAH. To determine the role of Ephexin-1 and ROCK2 in the downstream pathway, rEphx-1 (3.33 μ g/kg) and Fasudil (10 mg/kg; ROCK2 inhibitor) were administered. The protective effect of knockdown EphA4 on

alleviating apoptotic neuron death and preserving BBB integrity in the brain was significantly abolished, if rEphx-1 was infused i.c.v. after SAH, however, Fasudil administration decreased the levels of CC-3 and increased ZO-1, claudin-5, compared with the SAH + EphA4 siRNA + rEphx-1 group (Fig. 4D–F).

Fluoro-Jade C staining was performed at 24 h after SAH to measure the apoptotic neuron death in the left hemisphere near the blood clot. The positive cells decreased in SAH + EphA4 siRNA and SAH + EphA4 siRNA + rEphx-1 + Fasudil, compared to SAH + Vehicle, SAH + Scramble siRNA, and SAH + EphA4 siRNA + rEphx-1 groups (Fig. 5A, B).

4. Discussion

The novel finding of the present study was that EphA4 has a critical role in EBI through exacerbation of neuronal apoptosis and BBB disruption. First, we observed that EphA4, expressed on neurons and associated with reactive astrocytes, is markedly upregulated early after SAH, and its downstream proteins, such as Ephexin-1, RhoA, and ROCK2, are also augmented. Second, EphA4, capable of triggering caspase-dependent neuronal death and promoting BBB permeability, exacerbates EBI through an Ephexin-1/ROCK2 pathway.

Accumulating scientific evidence supports that SAH induces BBB breakdown and neuronal apoptosis (Fujii et al., 2013). In contrast, EphA signaling has been reported to control cell survival and apoptosis of neural progenitors during CNS development (Depaepe et al., 2005; Van Hoecke et al., 2012). In particular, EphA4 is predominantly expressed in the central nervous system (Leighton et al., 2001). A recent in vitro study has associated EphA4 with triggering of caspase-dependent cell death (Furne et al., 2009). Furthermore, inhibition of EphA4 signaling after ischemia–reperfusion reduces neuronal apoptosis (Li et al., 2012). In the present study, we observed upregulation of EphA4 at 24 h after SAH. Meanwhile, its downstream proteins Ephexin-1, RhoA/ROCK2 were also augmented as predicted from previous studies (Rosas et al., 2011; Lemmens et al., 2013; Shamah et al., 2001).

As reported previously, astrocytes actively participate in BBB disruption secondary to overactivation, while inhibition of the ROCK pathway by Fasudil stabilizes astrocytes after stroke (Abeyasinghe et al., 2016). In connection, we found that upregulated EphA4 exists on astrocytes, microglia as well as neurons after SAH. EphA4 knockdown by siRNA attenuated elevations in brain water content, reduced Evans blue extravasation, and improved the neurological function after SAH, suggesting that increased levels of EphA4 is linked to reduction of BBB integrity, increased neuronal apoptosis, even involving in inflammation. Our EphA4 results were congruent with studies of EphA4 in ischemia, trauma, and spinal injury (Biervet and Fahrig, 2001; Figueroa et al., 2006; Li et al., 2012). EphA4 is predicted to bind with other Ephrin ligands simultaneously (Barquilla and Pasquale, 2015). This predicted interactive mechanism between Ephrin and EphA4 in the brain after stroke remains to be elucidated in further studies.

The expression of Ephexin-1, a key member of the family of guanine nucleotide exchange factors (GEFs), was increased after SAH in this study which supports a role for Ephexin-1 in EBI after SAH. Once EphA receptors are activated, they induce increased levels of Ephexin-1, (Rosas et al., 2011; Sahin et al., 2005) which preferentially activates RhoA, as

well as Cdc42, and Rac1 (Shamah et al., 2001). Studies support that activation of the Rho/ROCK signaling pathway increases BBB permeability (Gibson et al., 2014; Takemoto, 2002). While, inhibition of this pathway maintains the BBB integrity and decreases apoptosis through inhibition of caspase-3 (Lai, 2003; YY et al., 2015). The end result is reduction of brain injury after stroke (Fujii et al., 2012; Rikitake et al., 2005). Interestingly, in this study, knockdown of EphA4 by specific siRNA decreased the expression of Ephexin-1, ROCK2, and CC-3. This knockdown also increase tight junction proteins ZO-1 and claudin-5. rEphx-1 administration reversed these results. While, administration of Fasudil, a ROCK2 inhibitor, reversed the effects of rEphx-1 and mirrored the effects of EphA4 knockdown by specific siRNA. These observations support that EphA4 aggravates BBB disruption and apoptotic neuron death through an Ephexin-1, RhoA, ROCK2 signaling pathway.

Apoptosis can be induced by multiple mechanisms, such as death receptor activation, radial or chemical exposure and viral infections, which includes caspase-dependent and caspase-independent type with the former consisting of the intrinsic and extrinsic pathways (Krantic et al., 2007). As an apoptotic marker protein, CC-3 can be activated by the intrinsic or extrinsic pathway. The observation of apoptotic neuron death and CC-3 elevation in this study was consistent with our published literature (Cahill et al., 2006) and other published results (Cheng et al., 2009; Endo et al., 2006; Gao et al., 2008). In regards to apoptosis, this study supports that EphA4 up-regulates CC-3, and since apoptosis is a major cause of neuron death after SAH, knockdown of EphA4 by siRNA and subsequent reduction of CC-3 levels is one explanation for our observations of better neurological outcomes after EphA4 knockdown.

Our previous study showed that SAH significantly decreased the levels of tight junction proteins, ZO-1 mediated by ROCK (Fujii et al., 2012). In this study, EphA4 knockdown reversed these effects. Moreover, rEphx-1 abolished the protective effects of EphA4 knockdown on tight junction proteins and CC-3. While, Fasudil administration after rEphx-1 administration rescued the effects of EphA4 knockdown. It is likely that EphA4/Ephexin-1/ROCK2 participate in the exacerbation of BBB permeability and apoptosis after SAH. Pragmatically, this study does not rule out the potential participation of caspase-independent pathways in EphA4-related neuronal death after SAH. The Ephrin ligands binds to the EphA4 which potentially has other effects after SAH; therefore, further studies are needed to investigate other remaining roles of Ephrins and associated receptors after SAH.

In conclusion, we demonstrate that increased levels of EphA4 worsens SAH outcomes and aggravates BBB disruption and neuronal death, both key components of EBI. Our observations supporting downstream signaling by EphA4 through an Ephexin-1/ROCK2 pathway may provide new clues for treatment of SAH in humans.

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Fig. 1.

Expression changes of EphA4, Ephexin-1, RhoA, and ROCK2 after SAH. A, representative western blot images. B, C, D, E, quantitative analyses of time-dependent expression of EphA4, Ephexin-1, RhoA, and ROCK2 in the left hemisphere at 24 h after SAH. Relative densities of each protein have been normalized against the Sham group. $n = 6$ per group. $*P < 0.05$ vs. Sham.

Fig. 2.

Cellular localization of EphA4 after SAH. Representative images of immunofluorescent staining of EphA4 (red) and neurons (NeuN, green) (A), EphA4 (green) and astrocytes (GFAP, red) (B), and EphA4 (red) and microglia (Iba-1, green) (C) in Sham and SAH rats after 24 h surgery. Nuclei are stained with DAPI (blue). Top panel indicates the location of staining (small black box). Arrows indicate colocalization of EphA4/NeuN, EphA4/GFAP, and EphA4/Iba-1. $n = 3$ per group. Bar = 30 μm .

Fig. 3.

The effect of Ephrin A4 (EphA4) knockdown on neurological deficits and BBB disruption after subarachnoid hemorrhage (SAH). In rats receiving intracerebraventricular (i.c.v.) administration of small interfering ribonucleic acid (siRNA) for EphA4, the modified Garcia (A) and beam balance score (B) were significantly higher, and brain water content (C) and Evans blue extravasation (D) were lower at 24 h after SAH. $n = 6$ per group. Vehicle, phosphate-buffered saline; * $P < 0.05$ vs. Sham; @ $P < 0.05$ vs. SAH + Vehicle; # $P < 0.05$ vs. SAH + Scramble siRNA.

Fig. 4.

Ephrin receptor A4 (EphA4) knockdown upregulates the expression of tight junction proteins, and inhibits cleaved caspase 3 (CC-3) from the left hemisphere at 24 h after SAH. Silencing EphA4 by using small interfering ribonucleic acid (siRNA) significantly decreases the expression of EphA4 (A). Representative western blot images and quantitative analysis of Ephexin-1 (B), ROCK2 (C), zonula occludens-1 (ZO-1) (D), Claudin-5 (E), and CC-3 (F) from the left hemisphere at 24 h after SAH. $n = 6$ per group. rEphx-1, recombinant Ephexin-1; Vehicle, phosphate-buffered saline; * $P < 0.05$ vs. Sham; @ $P < 0.05$ vs. Vehicle; # $P < 0.05$ vs. Scramble siRNA; & $P < 0.05$ vs. EphA4 siRNA; \$ $P < 0.05$ vs. EphA4 siRNA + rEphx-1.

Fig. 5. Effect of Ephrin receptor A4 (EphA4) knockdown on apoptotic neuron death after SAH. **(A)** Representative images of Fluoro-Jade staining at 24 h hours after SAH. **(B)** Quantitative analyses of mean apoptotic cell counts from 8 fields of left hemisphere at 24 h hours after SAH (NO/mm²). *n* = 3 per group. Vehicle, phosphate-buffered saline; siRNA, small interfering ribonucleic acid; rEphx-1, recombinant Ephexin-1. Top panel indicates the location of staining (small black box). **P* < 0.05 vs. Sham; @*P* < 0.05 vs. Vehicle; #*P* < 0.05 vs. Scramble siRNA; &*P* < 0.05 vs. EphA4 siRNA; \$*P* < 0.05 vs. EphA4 siRNA + rEphx-1. Bar = 50 μm.

Table 1

Experimental design and number of animals used per group.

Part	Groups	Neuro test BWC	Evans blue	Western blot	Fluoro-Jade IHC	Mortality	Exclusion	Sum
I	Time course study ($n = 6$; 9* per group):							
	Sham*	–	–	6	3	–	–	9
								44
	SAH (3, 6, 12, 24*, 72 h)	–	–	30	3	9 (20%)	2	9
II	Sham	6	6	6	3	–	–	21
	SAH + Vehicle	6	6	6	3	7 (24%)	1	29
	SAH + Scramble siRNA	6	6	6	3	6 (21%)	1	28
	SAH + EphA4 siRNA	6	6	6	3	3 (13%)	–	24
III	SAH + EphA4 siRNA + rEphx-1	–	–	6	3	3 (23%)	1	13
	SAH + EphA4 siRNA + rEphx-1 + Fasudil	–	–	6	3	2 (18%)	–	11
	Total	24	24	72	24	30 (17%)	5	179

Sprague-Dawley male rats with 280–330 g body weight were used. SAH, subarachnoid hemorrhage; Vehicle, sterile 0.9% NaCl; siRNA, small interfering ribonucleic acid; EphA4, Eph receptor A4; rEphx-1, Ephexin-1; BWC, brain water content; IHC, immunohistochemistry.