Supplemental materials and methods

Immunofluorescent Microscopy

Normal rats and rats treated with vehicle or estrogen (subcutaneous injection) for three days were euthanized, and brain tissues were harvested. Harvested brain tissues were fixed in 4% paraformaldehyde. After dehydration in 10% sucrose solution, the tissues were embedded in OCT and then sectioned at a thickness of 10 μm. Sections were blocked with BSA at 37°C for 60 min and then incubated with primary antibody at 4°C overnight. After washing three times with PBST, sections were incubated with secondary antibodies for 60 min at 37°C. Sections were visualized using a fluorescence microscope (Olympus BX50/BX-FLA/DP70, Olympus Corporation, Japan).

Western Blotting

Bilateral temporal floor brain tissues were stored in liquid nitrogen. After removal from the liquid nitrogen, 1 ml of IP lysis buffer (Beyotime, Shanghai, China) was added to grind thoroughly on ice to lyse the brain tissue for 30 min. The lysate was centrifuged at 12,000 × g for 5 min at 4°C, and the supernatant was collected. The centrifugation step was repeated twice. We then used the standard BCA method (Beyotime, Shanghai, China) to determine the protein concentration in each sample. Subsequently, protein samples (20 μg/lane) were separated by 10% SDS polyacrylamide gels and electrophoretically transferred to NC membranes. The membranes were blocked with quickblock block solution (Beyotime, Shanghai, China) for 20 min and then incubated with the primary antibody overnight at 4°C. Then, the membrane was incubated with the secondary antibody for 60 min at 37°C. Chemiluminescence bands were visualized

using the Enhanced Chemiluminescence Detection Kit (3100 Mini, Clinx Scientific Instruments Ltd.) and grayscale values were quantified using ImageJ software. The grayscale value of each group was divided by the grayscale value of the internal reference, including GAPDH and β-actin, in each group to obtain the relative protein level of the target protein to the internal reference. Afterwards, the data from the 6 biological replicates were normalized to the mean relative protein level of the 6 replicates of the sham group. All antibody details are shown in Supplemental Table 1.

ELISA

Cerebrospinal fluid samples from patients and brain tissue grinds from the base of the temporal lobe of rats were removed from -20 degrees freezing. The samples were diluted using sample diluents according to the method in the kit and added to wells coated with primary antibodies. The wells were incubated, washed, and then again incubated and washed after the secondary antibody was given. The final luminescent solution was added, and the absorbance of each well was measured afterwards. The concentration of each sample was determined according to the absorbance of the standards. The concentrations of SHH and estrogen in patients' cerebrospinal fluid samples were used to calculate the Pearson correlation coefficient. Albumin concentrations in rat temporal lobe base brain tissue grinding fluid samples were used to calculate the ratio of each sample group to the mean concentration of the sham group. All ELISA kit details are shown in Supplemental Table 1.

Brain water content

The brain water contents of SAH model rats and SAH model rats with estrogen

intervention for 3 days before and after piercing were measured 3 days after modeling. After anesthesia, the rats were euthanized, and brain tissue was harvested. Each brain was divided into three parts, including the right hemisphere, left hemisphere, and cerebellum. The weight of each part was immediately weighed as the wet weight. After drying in an oven at 100°C for 72 hours, the dry weight was determined. The percentage of brain water content was calculated according to the following formula. [(wet weight - dry weight)/wet weight] × 100%.

Evans blue assay

Rats were sacrificed 3 days after SAH, and BBB permeability was measured indirectly by the Evans blue assay. Three hours before sacrifice, the rats were given 2% Evans blue solution by intraperitoneal injection to bring it into the blood circulation. Afterwards, the rats were anesthetized and perfused with PBS through the left ventricle for 5 minutes to cleanse the blood vessels of residual Evans Blue. The brain tissue was then removed and frozen in liquid nitrogen. The right hemisphere samples were homogenized in 1,100 μL of PBS and centrifuged at 15,000 rpm for 5 min at 4°C, and then the supernatant was collected. Next, 500 μL of 50% trichloroacetic acid (TCA) was added to each 500 μL of supernatant and incubated overnight at 4°C. Finally, these samples were centrifuged at 15,000 rpm for 30 min at 4°C. The absorbance of Evans blue was measured at 610 nm using a spectrophotometer. Meanwhile, different concentrations (0.025 to 1000 μg/ml) of Evans blue dye were added to 50% TCA to prepare the standard solution. Standard solution diluted 4 times in 50% TCA was then added to each well in a 96-well plate, and the absorbance at 610 nm was measured. The

standard curve was determined by fitting the concentration and absorbance of the standard solution. The amount of Evans blue in tissue was then calculated from a standard curve and normalized to the tissue weight (μg of Evans blue stain/g of brain tissue).

Neurobehavioral experiments

For short-term behavioral studies, we incorporated the modified Garcia score and the beam balance score in our study. Seventy-two hours after SAH, neurological function was evaluated using a modified Garcia score for each group of rats. The scoring system included six aspects: spontaneous activity, limb motor, forepaw abduction, beard tactility, trunk tactility and climbing ability. Each item is scored from 0 to 3, with higher scores representing better neurobehavioral function. In the beam balance test, we evaluated the distance walked and balance maintained on the beam for 1 minute at 72 hours after SAH. A total of five scores from 0 to 4 were assigned, with higher scores representing better neurobehavioral function.

The rotarod test and the Morris water maze experiment were used for long-term behavioral studies. The rotarod test experiment was performed to analyze the neurobehavioral function of rats by recording the time spent on the rotating wheel in each group 1 day before SAH and 1 day, 3 days, 7 days, and 14 days after SAH. The longer the time, the better the neurobehavioral function. The water tank was 50 cm deep and 180 cm in diameter, filled with water at a height of 30 cm, and the water temperature was controlled at 20-22°C. The platform was placed 2 cm below the water surface. The rats were trained on the Morris water maze from 3 days postoperatively, 4 times a day

for 4 days. The starting point was changed daily. Each test was continued until the rat found the platform or until 60 seconds had elapsed.