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Oestrogen ameliorates blood-brain barrier damage after experimental subarachnoid haemorrhage via the SHH pathway in male rats

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ABSTRACT

Background Sex differences affect the occurrence, progression and regression of subarachnoid haemorrhage (SAH). Oestrogen plays a protective role in alleviating the vasospasm and neuronal apoptosis induced by SAH. However, whether oestrogen affects blood–brain barrier (BBB) integrity has not been fully studied. Oestrogen has been found to regulate the sonic hedgehog (SHH) signalling pathway through the oestrogen receptor in gastric cancer and adrenal glands, and the SHH signalling pathway has an important role in maintaining the BBB by upregulating the expression of tight junction proteins. In this study, we investigated the relationship between oestrogen and the SHH signalling pathway using clinical data and established an experimental SAH model to explore whether oestrogen could ameliorate BBB damage after SAH through the SHH pathway.

Methods Correlations between oestrogen and the SHH pathway were analysed by patients' cerebrospinal fluid (CSF) samples and the Genotype-Tissue Expression database (GTEx). Then, an experimental rat SAH model was established using the endovascular perforation method and treated with oestrogen, oestrogen inhibitors and SHH signalling pathway inhibitors. Then, the effects of oestrogen on BBB damage were analysed by western blot, immunofluorescence and neurobehavioural experiments.

Results ESLIA detection and correlation analysis showed that oestrogen levels in patients' CSF were positively correlated with the SHH pathway, which was further verified by GTEx gene-correlation analysis. SHH was found to be mainly expressed in neurons and astrocytes in rats under physiological conditions and was upregulated by oestrogen pretreatment. In the SAH model, oestrogen pretreatment was found to reverse SAH-induced decreases in the SHH pathway, which were counteracted by oestrogen receptor inhibitors. Furthermore, oestrogen pretreatment reduced SAH-induced BBB damage, brain oedema and neurological dysfunction, which were eliminated by SHH pathway inhibitors.

Conclusion In conclusion, we demonstrate here that oestrogen pretreatment ameliorates brain injury after SAH, at least in part through SHH pathway-mediated BBB protection.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Sex differences exist in the occurrence and prognosis of patients with aneurysmal subarachnoid haemorrhage (SAH), in which oestrogen plays an important role.

WHAT THIS STUDY ADDS

⇒ This study found that oestrogen attenuates blood–brain barrier damage after experimental subarachnoid haemorrhage via the sonic hedgehog pathway.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study may help explain the clinical sex differences associated with perihaematomal brain oedema and the prognosis of neurological function after SAH and provide potential targets for future treatment.

INTRODUCTION

Subarachnoid haemorrhage (SAH), caused mainly by ruptured aneurysms, has remarkably high mortality and disability rates, although it constitutes only 5%–7% of strokes.[1] In previous investigations, sex differences have been found to be associated with the incidence and outcome of SAH. There was a significant difference in the incidence of SAH in men versus women premenopause and postmenopause, with a higher prevalence in males than in premenopausal females and a lower prevalence in males than in postmenopausal females.[2 3] Oestrogen replacement therapy is considered to have protective effects against the occurrence of SAH.[4] Although the overall mortality after SAH is higher in women than in men, whether gender differences have an impact on functional outcome is controversial in previous studies. Whether oestrogen, an important factor influencing the incidence of SAH, also has a significant prognostic impact has not been adequately studied in clinical trials.

Blood–brain barrier (BBB) dysfunction, vasospasm, neuronal necrosis and apoptosis are important pathological processes of brain injury after SAH. Previous studies on the effect of oestrogen on the prognosis of

SAH have mainly focused on its antivasospasm, reduction of necrosis and apoptosis effects. Oestrogen downregulates TNF-α expression via oestrogen receptors and reduces apoptosis in neurons after SAH.[5 6] By reducing iNOS expression and maintaining eNOS expression in the basilar artery, oestrogen alleviates vasospasm after SAH.[7]

Oestrogen and its receptors are widely expressed in the brain and have regulatory functions on neurons, astrocytes and endothelial cells.[8] It has been shown that oestrogen can upregulate the sonic hedgehog (SHH) pathway through its receptor in adrenal cortical cells[9] and gastric cancer cells.[10] Importantly, the SHH signalling pathway plays a key role in maintaining BBB integrity.[11] In addition, studies have shown that upregulation of the SHH signalling pathway can improve the prognosis of SAH.[12] However, it is not clear whether oestrogen can ameliorate BBB damage after SAH through the SHH pathway.

In our study, we determined the correlation between oestrogen and the SHH signalling pathway and clarified the protective roles of oestrogen on BBB function after SAH. Furthermore, we explored the mechanisms by which oestrogen alleviates brain injury after SAH by using a variety of experimental methods.

MATERIALS AND METHODS

Patients

Cerebrospinal fluid (CSF) samples from 20 patients were obtained during ruptured aneurysm clamping surgery or during CSF examination. Each patient was informed of the purpose and procedure of the study, and written informed consent was obtained from each patient. Immediately after collection of all CSF samples, all samples were centrifuged at 1000 ×g for 20 min. The supernatant collected after centrifugation was used for the ELISA. A more detailed description of the materials and methods used is provided in online supplemental material and methods.

Genotype-Tissue Expression gene-correlation analysis

The Genotype-Tissue Expression database (GTEx) is an online database that provides expression data of different genes in different tissues of normal humans. All samples of the brain, excluding the cerebellum and spinal cord, were included. GEPIA (http://gepia2.cancer-pku.cn/) is a user-friendly analysis site that allows the use of data from GTEx to analyse correlations between genes.[13] By calculating Pearson correlation coefficients between gene expression of oestrogen receptors, SHH signalling pathway and some tight junction proteins (TJs), we plotted heatmaps using Python. Correlation coefficients between the expression of oestrogen receptors (ESR1, ESR2) and SHH, SHH signalling pathways (SHH, PTCH1, GLI1), and TJs in brain tissues were calculated by using GEPIA as well.

Animals

Male Sprague-Dawley (SD) rats weighing between 280 and 330 g were purchased from the Animal Center of Chinese Academy of Sciences (Shanghai, China). All SD rats were raised in temperature-controlled and humidity-controlled animal quarters with a 12-hour light/dark cycle and unlimited access to food and water. The animal protocols used in this study were approved by the Animal Ethics Committee, and all experiments complied with the Animal Research Reporting of In Vivo Experiments guidelines.

Sacrifice of experimental animals

Experimental rats were anaesthetised by isoflurane gas at the experimentally set time points.[14] After the disappearance of corneal and pain reflexes, the animals were sacrificed by transcardial perfusion with saline containing 0.1% EDTA after opening the thoracoabdominal cavity.

SAH model

The experimental SAH model was generated by an endovascular perforation model in rats. The rats were intubated under isoflurane anaesthesia and mechanically ventilated until the corneal and pain reflexes disappeared.[14] The external carotid artery was ligated and isolated, and a 4-0 nylon suture with the anterior end reshaped to a 60° angle was introduced through the dissected end of the external carotid artery to the left internal carotid artery. In the SAH group, after the nylon suture was inserted and resistance was felt, a further 3 mm was punctured, the bifurcation of the anterior and middle cerebral arteries was punctured, and the suture was immediately withdrawn. In the sham group, the wire embolus was introduced only into the internal carotid artery without perforation. At the time of sacrifice, the success of SAH was judged by assessing the SAH grade (≥8) as described previously. SAH models with insufficient scores were excluded from this study.[15]

Experimental groupings

The experimental design of the first experiment was as follows. All rats were randomly divided into three groups, including a normal group, a vehicle group and an oestrogen group. Each group included six rats that were euthanised after oestrogen intervention for 3 days, and brain tissue was harvested for immunofluorescence assays.

The second experimental design is as follows. All rats were randomly divided into seven groups, including one sham group and six SAH groups. Each group contained six rats. According to the experimental design at 6 hours, 12 hours, 24 hours, 48 hours, 72 hours and 168 hours after SAH, the rats were euthanised, and brain tissue was harvested for western blot assays.

The third experiment is designed as follows. All rats were randomly divided into six groups, including the sham group, SAH group, SAH+vehicle group, SAH+oestrogen (33 μg/kg) group, SAH+oestrogen+fulvestrant (ICI 182780, 2 mg/kg) group and SAH+oestrogen

+ cyclopamine (1 mg/kg) group.[5 16 17] Each group contained six rats, and vehicle, oestrogen, fulvestrant and cyclopamine were administered for 3 days before and after SAH. Brain tissue was harvested for western blotting and ELISAs after all rats were euthanised 3 days after SAH. A more detailed description of the materials and methods used is provided in online supplemental materials and methods.

Cell culture

Human brain microvascular endothelial cells (hBMVEC) (hCMEC/D3, Shanghai Zhong Qiao Xin Zhou Biotechnology) were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM) high-glucose complete medium with 10% fetal bovine serum and 1% penicillin/streptomycin to avoid the oestrogen-mimetic effects of phenol red.[18] The intervention groups were given oxyhaemoglobin (20 µM), oxyhaemoglobin+human recombinant SHH (HrSHH, 0.1 µg/mL), and oxyhaemoglobin+HrSHH+cyclopamine (10 µM) interventions for 24 hours and were harvested along with the control group.[11 16 19] These interventions were all dissolved in DMEM. All items required for in vitro experiments are shown in online supplemental table 1. A more detailed description of the materials and methods used is provided in online supplemental materials and methods.

Statistical analysis

Values are expressed as the mean and/or SD. Data were analysed using GraphPad Prism V.9.0.0 (GraphPad Software, La Jolla, California, USA), Python V.3.8. Data correlation was assessed using Pearson's correlation coefficient. Statistical comparisons between two groups were performed using t tests, statistical comparisons between more than two groups were performed using one-way analysis of variance, and comparisons between more than two groups were performed using the Turkish post hoc test to determine the significance of pairwise differences. A probability of $p<0.05$ was considered statistically significant.

RESULT

The SHH signalling pathway is correlated with oestrogen and its receptors

CSF samples were used to detect SHH concentrations and oestrogen concentrations by ESLIA kits. Correlation analysis suggested that SHH and oestrogen content were moderately positively correlated ($R=0.6$, $p<0.05$) (figure 1A, online supplemental table 2). To investigate the relationship between the transcriptional expression of oestrogen receptors, the SHH signalling pathway and TJs, we analysed the correlation between the expression of each of these genes in GTEx. The expression of each gene in the oestrogen receptors and SHH signalling pathway showed a positive correlation ($R>0.2$, $p<0.05$), except for ESR2, which was not correlated with SHH but had a p value>0.05 (figure 1B, online supplemental table 3). Then, we analysed the correlations between oestrogen receptor and SHH genes, between oestrogen receptor and SHH signalling pathway genes, between oestrogen receptor and TJs genes and between SHH signalling pathway and TJs genes based on the same data in GEPIA figure 1C-F. All showed a good positive correlation ($R>0.2$, $p<0.05$).

Tissue distribution of SHH and alterations after oestrogen intervention

Immunofluorescence was used to assess the expression of SHH in the brain. For immunofluorescence, we used GFAP as a marker for astrocytes and NeuN as a marker for neurons, and SHH was found to colocalise with cells positive for both markers. The expression of SHH was increased in temporal basal cortex brain tissue in the group treated with oestrogen for 3 days (figure 2A–B). Additionally, we used isolectin B4 as an endothelial cell marker and found that SHH failed to colocalise with endothelial cells (figure 2C). We then enumerated the proportion of cells costained with SHH as well as the total count of cells. Oestrogen intervention did not change the number of GFAP+astrocytes and NeuN+neurons, and the proportion of cells costained with SHH was not significantly altered (figure 2D–G). Additionally, we semiquantified SHH in temporal basal cortex brain tissue using western blotting, and SHH was significantly increased after 3 days of oestrogen intervention (figure 2H). These results suggest that oestrogen significantly increases the synthesis of SHH by neurons and astrocytes.

Protein expression of SHH is reduced and oestrogen reverses alterations in the SHH signalling pathway after experimental SAH via oestrogen receptors

Western blotting was used to assess the protein expression of SHH in temporal basal cortex brain tissue. Results showed that SHH was significantly reduced at 3 days and slightly recovered at 7 days compared with the sham group (figure 3A–B).

By performing oestrogen intervention in rats for 3 days each before and after SAH, we applied western blotting to determine the altered protein expression in the SHH signalling pathway. Expressions of SHH, PTCH1, SMO and GLI1 in the SHH signalling pathway were significantly reduced at 72 hours of SAH compared with the sham group. These decreased expression levels could be reversed by 6 days of oestrogen intervention. In contrast to the oestrogen alone administration group, no reversal was observed in the group given both oestrogen and the oestrogen receptor inhibitor fulvestrant (figure 3C–G).

After that, we observed the alteration of SHH expression in astrocytes and neurons after SAH by immunofluorescence. We found that the number of astrocytes increased after SAH but not neurons. SHH expression was reduced in both astrocytes and neurons after SAH, and some astrocytes were missing SHH colocalisation. The decrease in SHH expression was partially reversed by oestrogen (figure 3H–M). Additionally, we did not find

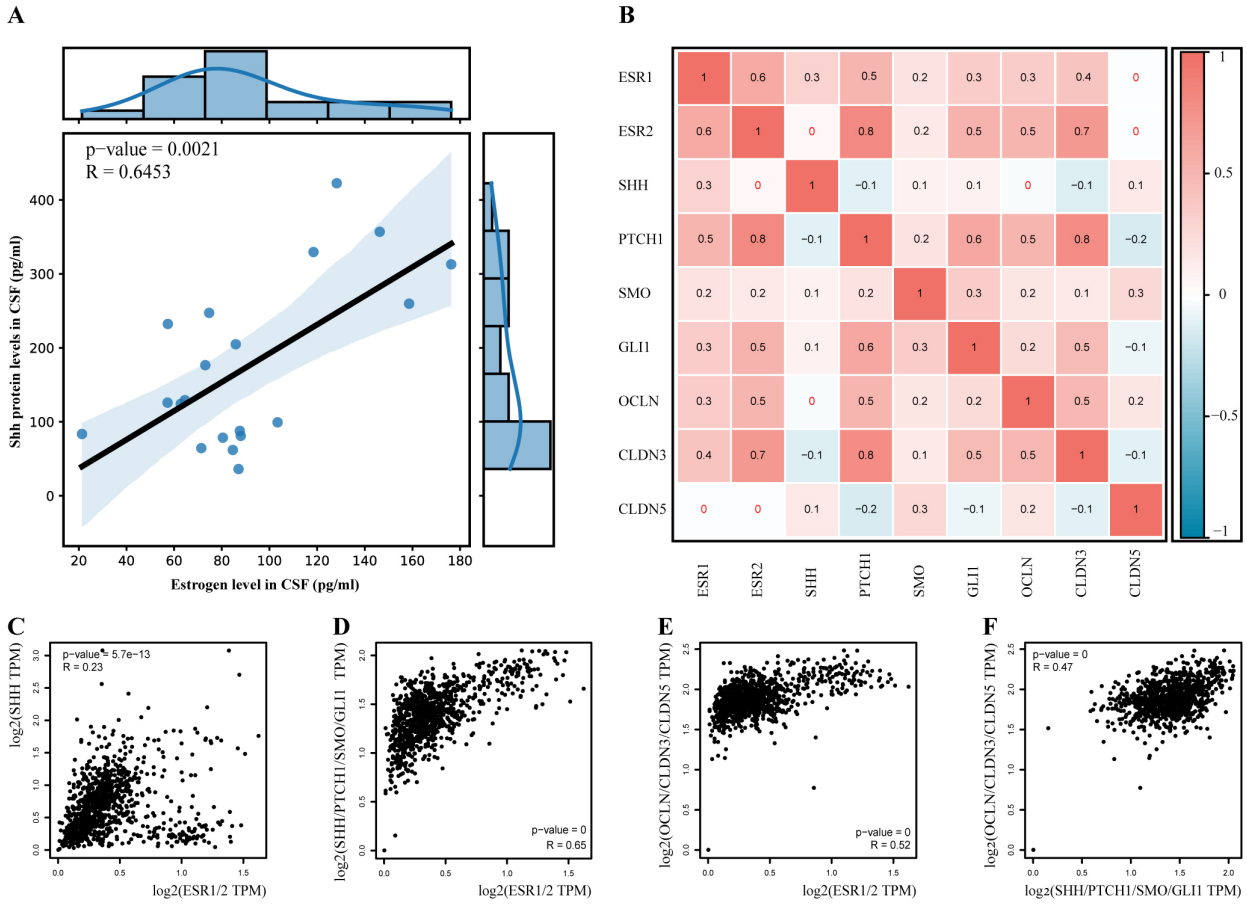

Figure 1 Positive correlation exists between oestrogen and the sonic hedgehog (SHH) signalling pathway in human samples. (A) Pearson correlation between SHH and oestrogen in cerebrospinal fluid samples from 20 patients with subarachnoid haemorrhage. (B) Heatmap of the correlation between oestrogen, the SHH signalling pathway and genes encoding tight junction proteins in normal human telencephalic tissue in the Genotype-Tissue Expression database (GTEx) database. p<0.05 is indicated in black font. p≥0.5 is indicated in red font. (C) Pearson correlation between SHH and ESR1/2 in normal human telencephalic tissue in the GTEx database. (D) Pearson correlation between the SHH signalling pathway and ESR1/2 in normal human telencephalic tissue in the GTEx database. (E) Pearson correlation between tight junction protein genes and ESR1/2 in normal human telencephalic tissue in the GTEx database. (F) Pearson correlation between tight junction protein genes and the SHH signalling pathway in normal human telencephalic tissue in the GTEx database.

SHH expression on the endothelium after SAH (online supplemental fig S1).

The protein expression of TJs is downregulated after SAH and reversed by ostrogen through the SHH signalling pathway

In vivo, western blotting was used to measure the protein expression of TJs. TJs were decreased at 72 hours after SAH compared with the sham group. Oestrogen intervention upregulated the expression of TJs at 72 hours after SAH. This intervention outcome could be reversed by cyclopamine, an inhibitor of the SHH signalling pathway (figure 4A–C). This alteration was subsequently verified by immunofluorescence costaining of TJs and endothelial marker isolectin B4 (figure 4D, online supplemental fig S2).

In vitro, we used western blotting to determine the expression of TJs in hBMVEC . The expression of TJs was decreased in the group with oxyhaemoglobin-mimicking SAH compared with the control group. Endothelial cells administered HrSHH protein intervention for 12 hours had significantly elevated TJs compared with the oxyhaemoglobin group. Cyclopamine inhibited the upregulation of TJs by HrSHH protein (figure 4E–G).

Oestrogen reverses BBB permeability and brain water content after experimental SAH

We used ELISA to determine the albumin concentration and Evans blue absorbance measurements to indirectly estimate changes in BBB permeability. BBB permeability was significantly higher at 72 hours after SAH than in the sham group. In contrast, in the oestrogen intervention group, there was no significant difference in BBB permeability at 72 hours after SAH, indicating that oestrogen significantly reversed the increase in BBB permeability after SAH (figure 5A–C).

Similarly, brain water content measurements revealed a significant increase in brain water content 72 hours after SAH, which was reversed by oestrogen. When the SHH signalling pathway was blocked by cyclopamine, this reversal by oestrogen could not be detected (figure 5D).

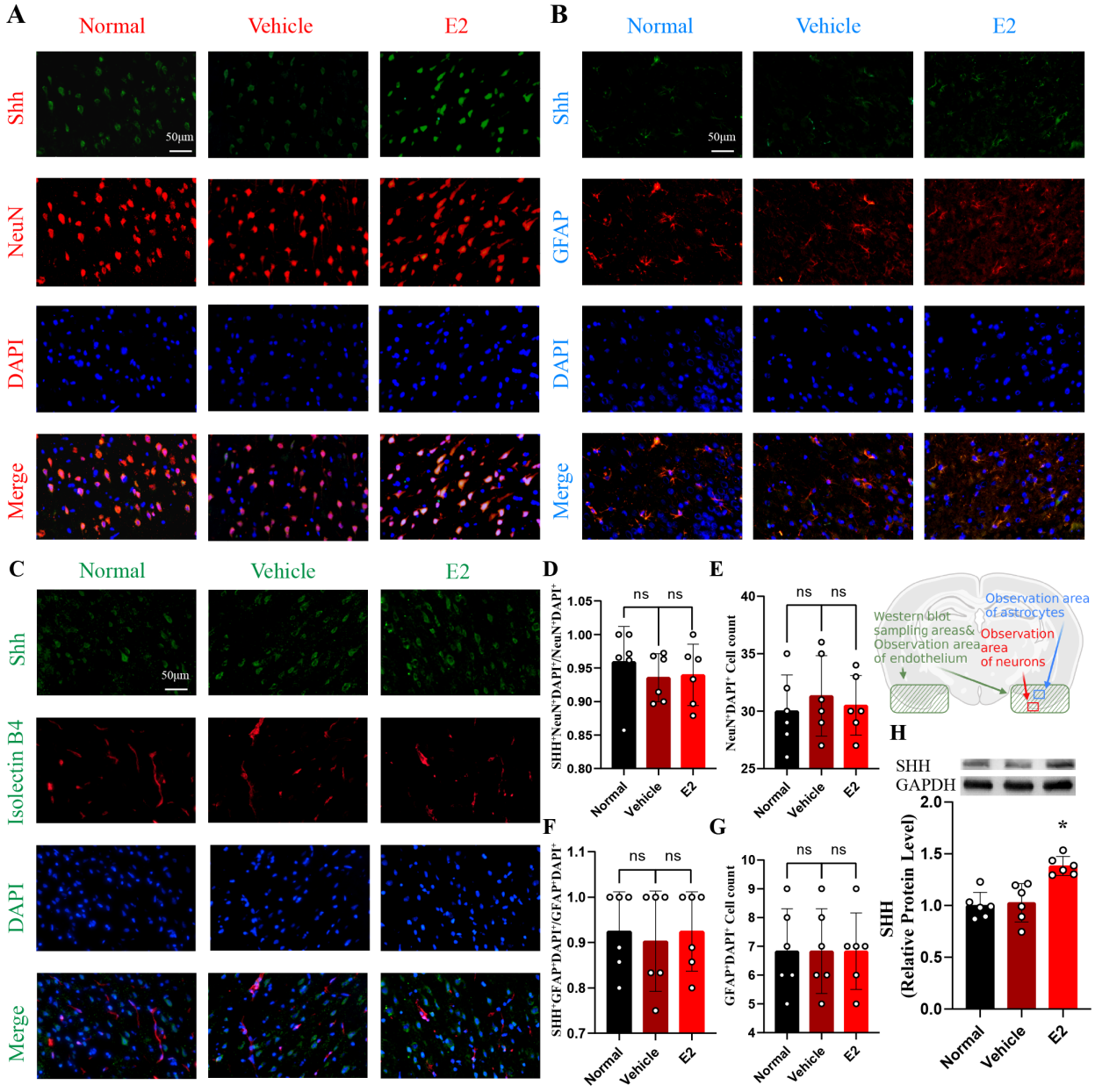

Figure 2 Both neurons and astrocytes in brain tissue at the base of the temporal lobe of rats have a distribution of sonic hedgehog (SHH, and oestrogen can increase Shh protein levels. (A) Immunofluorescence staining was performed with antibodies against SHH (green) and NeuN (red), while DAPI (blue) was used to label the nucleus. Scale bar=50 μm. (B) Immunofluorescence staining was performed with antibodies against SHH (green) and GFAP (red), while DAPI (blue) was used to label the nucleus. Scale bar=50 μm. (C) Immunofluorescence staining was performed with antibodies against SHH (green) and Isolectin B4 (red), while DAPI (blue) was used to label the nucleus. Scale bar=50 μm. (D) The ratio of the number of $SHH^+NeuN^+DAPI^+$ cells to the number of $NeuN^+DAPI^+$ cells in immunofluorescence staining. *p<0.05; n=6/group. One-way ANOVA and Tukey's multiple comparisons test. (E) $NeuN^+DAPI^+$ cell count in immunofluorescence. *p<0.05; n=6/group. One-way ANOVA and Tukey's multiple comparisons test. (F) The ratio of the number of $SHH^+GFAP^+DAPI^+$ cells to the number of $GFAP^+DAPI^+$ cells in immunofluorescence staining. *p<0.05; n=6/group. One-way ANOVA and Tukey's multiple comparisons test. (G) GFAP $^+DAPI^+$ cell count in immunofluorescence. *p<0.05; n=6/group. One-way ANOVA and Tukey's multiple comparisons test. (H) Western blotting was used to detect the protein levels of SHH in brain tissue at the base of the temporal lobe on day 3 after oestrogen intervention in rats. All images of original western blotting gels are pressented in online supplemental file 7. *p<0.05 compared with the normal group; n=6/group. One-way ANOVA and Tukey's multiple comparisons test. (E2): oestrogen. All data represent the means±SDs. ANOVA, analysis of variance.

Oestrogen alleviates neurological dysfunction after experimental SAH

We finally performed behavioural tests after the administration of oestrogen or oestrogen+cyclopamine in rats with experimental SAH. The modified Garcia score and the rotarod test showed similar results, with the oestrogen intervention group having significantly better scores in the modified Garcia score experiment again and longer

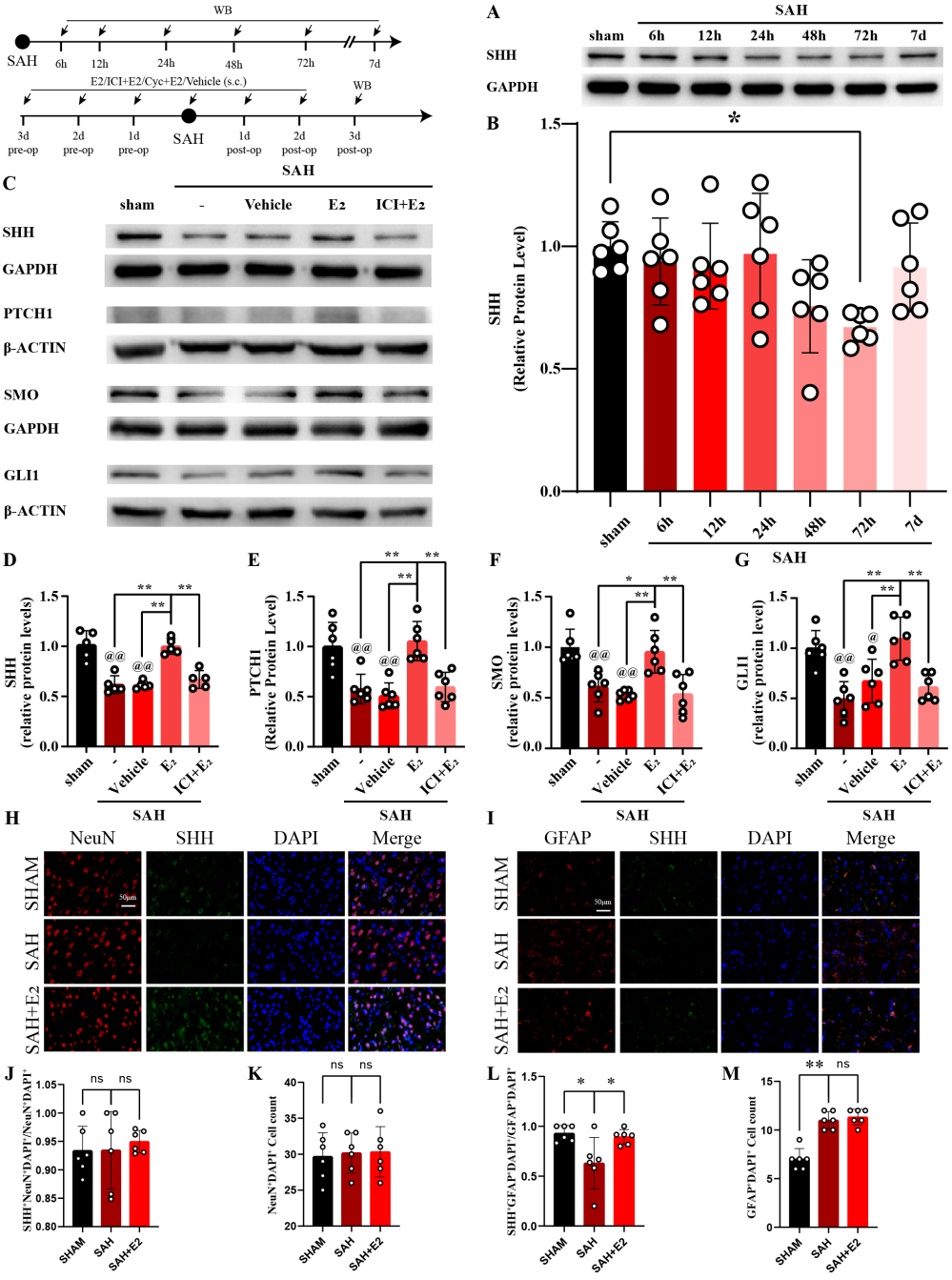

Figure 3 Sonic hedgehog (SHH) decreases after subarachnoid haemorrhage (SAH) in brain tissue at the base of the temporal lobe in rats. Oestrogen has an upregulatory effect on the post-SAH SHH signalling pathway via oestrogen receptors. (A, B) Time course of SHH expression in rat brain tissue at the base of the temporal lobe after SAH using Western blotting. *p<0.05 compared with the sham group; n=6/group. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. (C) Western blotting was used to detect SHH (D), PTCH1 (E), SMO (F), and Gli1 (G) protein levels in brain tissue at the base of the temporal lobe on day 3 after SAH under oestrogen and oestrogen+fulvestrant intervention. @p<0.05 compared with the sham group, *p<0.05 compared with the oestrogen group; n=6/group. One-way ANOVA and Tukey's multiple comparisons test. (H) Immunofluorescence staining was performed with antibodies against SHH (green) and NeuN (red), while DAPI (blue) was used to label the nucleus. Scale bar=50 µm. (I) Immunofluorescence staining was performed with antibodies against SHH (green) and GFAP (red), while DAPI (blue) was used to label the nucleus. Scale bar=50 µm. (J) The ratio of the number of $SHH^+NeuN^+DAPI^+$ cells to the number of $NeuN^+DAPI^+$ cells in immunofluorescence staining. *p<0.05; n=6/group. One-way ANOVA and Tukey's multiple comparisons test. (K) $NeuN^+DAPI^+$ cell count in immunofluorescence. *p<0.05; n=6/group. One-way ANOVA and Tukey's multiple comparisons test. (L) The ratio of the number of $SHH^+GFAP^+DAPI^+$ cells to the number of $GFAP^+DAPI^+$ cells in immunofluorescence staining. *p<0.05; n=6/group. One-way ANOVA and Tukey's multiple comparisons test. (M) GFAP $^+DAPI^+$ cell count in immunofluorescence. *p<0.05; **p<0.01; n=6/group. One-way ANOVA and Tukey's multiple comparisons test. (–): SAH only; (Vehicle): Vehicle+SAH; (E2): oestrogen+SAH; (ICI+E2): oestrogen+fulvestrant + SAH. ns: No significant. All data represent the means±SDs.

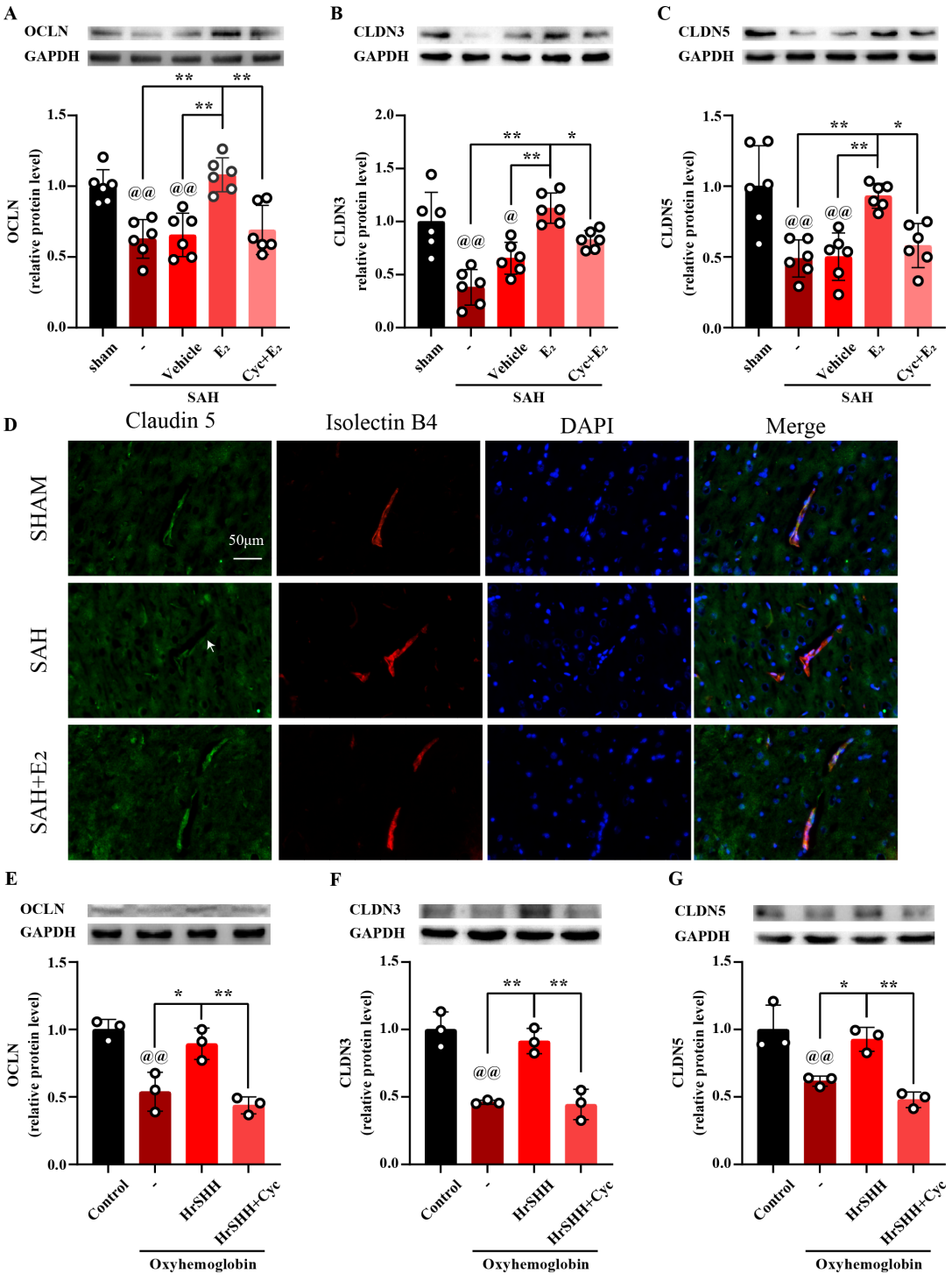

Figure 4 Upregulation of tight junction proteins in rat temporal lobe basal brain tissue by oestrogen via the sonic hedgehog (SHH) signalling pathway in an in vivo subarachnoid haemorrhage (SAH) model and in vitro in experimental oxyhaemoglobin-mimicking SAH endothelial cells. Western blotting was used to detect occludin (A), claudin-3 (B), and claudin-5 (C) protein levels in brain tissue at the base of the temporal lobe on day 3 after SAH under oestrogen and oestrogen+cyclopamine intervention in vivo. @ p<0.05 compared with the sham group, *p<0.05, **p<0.01 compared with the oestrogen group; n=3/group. One-way ANOVA and Tukey's multiple comparisons test. (–): SAH only; (Vehicle): Vehicle+SAH; (E2): oestrogen+SAH; (Cyc+E2): oestrogen+cyclopamine + SAH. (D) Immunofluorescence staining was performed with antibodies against Claudin 5 (green) and Isolectin B4 (red), while DAPI (blue) was used to label the nucleus. Scale bar=50 μm. Western blotting was used to detect occludin (E), claudin-3 (F), and claudin-5 (G) protein levels in experimental oxyhaemoglobin-mimicking SAH endothelial cells 12 hours after SAH under HrSHH and HrSHH+cyclopamine intervention in vitro. @ p<0.05 compared with the control group, *p<0.05, **p<0.01 compared with the HrSHH group; n=3/group. One-way ANOVA and Tukey's multiple comparisons test. (–): oxyhaemoglobin only; (HrSHH): HrSHH+oxyhaemoglobin; (E2+HrSHH): HrSHH+cyclopamine + oxyhaemoglobin. All data represent the means±SDs. ANOVA, analysis of variance.

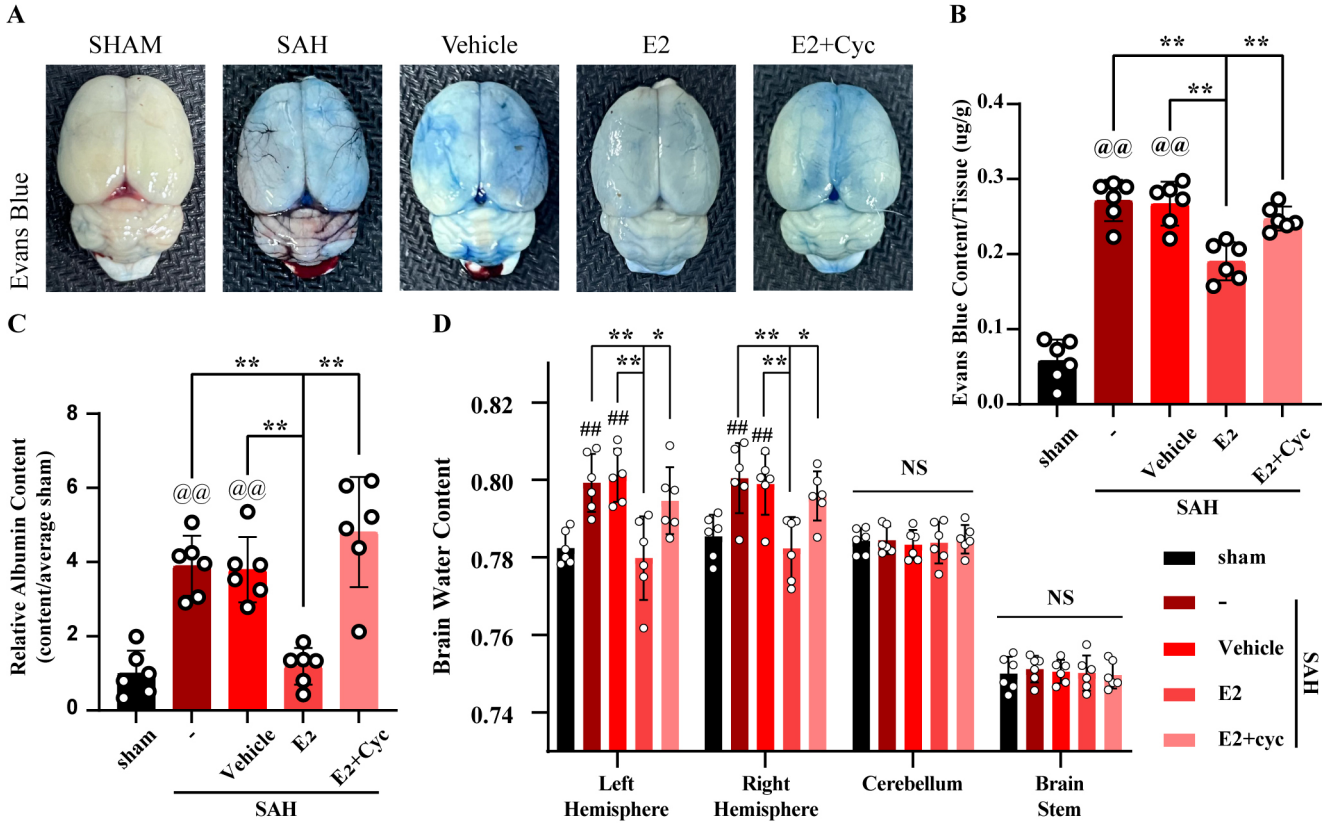

Figure 5 Oestrogen ameliorates blood–brain barrier (BBB) impairment and reduces brain water content in rats after subarachnoid haemorrhage (SAH) via the sonic hedgehog (SHH) signalling pathway. (A) Wide field observation of the Evans blue difference in brain tissue on day 3 after SAH under oestrogen and oestrogen+cyclopamine intervention. (B) Evans blue was quantified by absorbance in brain tissue on day 3 after SAH under oestrogen and oestrogen+cyclopamine interventions. n=6/group. One-way ANOVA and Tukey's multiple comparisons test. (C) Relative albumin content (content/average sham) determined by ELISA in brain tissue on day 3 after SAH under oestrogen and oestrogen+cyclopamine interventions. n=6/group. One-way ANOVA and Tukey's multiple comparisons test. (D) Water content of brain parts was measured on day 3 after SAH under oestrogen and oestrogen+cyclopamine interventions. n=6/group. One-way ANOVA and Tukey's multiple comparisons test. @@ p<0.01 compared with the sham group, *p<0.05, **p<0.01 compared with the oestrogen group. NS: No significant. (–): SAH only; (Vehicle): Vehicle+SAH; (E2): oestrogen+SAH; (Cyc+E2): oestrogen+cyclopamine + SAH. All data represent the means±SDs. ANOVA, analysis of variance.

dwell time in the rotarod experiment compared with the SAH group compared with the control group, suggesting that oestrogen can improve neurological dysfunction after experimental SAH (figure 6A–C). In contrast, this improvement was not observed in the group given cyclopamine concomitantly. In addition, in the Morris water maze, we chose escape latency and lane length as evaluation indexes. Rats in the oestrogen intervention group showed the shortest latency and swim path length compared with the other groups, which also implies better locomotor ability (figure 6D–G). The results suggest that oestrogen attenuates neurological dysfunction through the SHH signalling pathway.

DISCUSSION

As a common and important factor, gender difference was shown to have an important impact on the onset and progression of a variety of brain disorders. Oestrogen, a sex cortisol hormone that has an important effect on the development and maintenance of brain structures, plays an important role in this difference.[20 21] Experimentally and clinically, oestrogen has been shown to have a role in reducing the development of aneurysmal SAH and an effect that explains the difference in the incidence of SAH in women before and after menopause.[22] However, due to the lack of oestrogen testing in current clinical studies or subgroup analysis before and after menopause, the effect of oestrogen on brain injury after SAH has not been uniformly concluded. On the other hand, laboratory evidence has all agreed that oestrogen alleviates vasospasm, inflammation and necrotic effects via NF-κB, NOS and other signalling pathways after SAH.[5–7] Previous studies have found that oestrogen improves BBB permeability.[23] In our study, we investigated whether oestrogen improved BBB permeability or neurological impairment after SAH and the underlying mechanism.

Under physiological conditions, oestrogen and classical oestrogen receptors are extensively distributed in

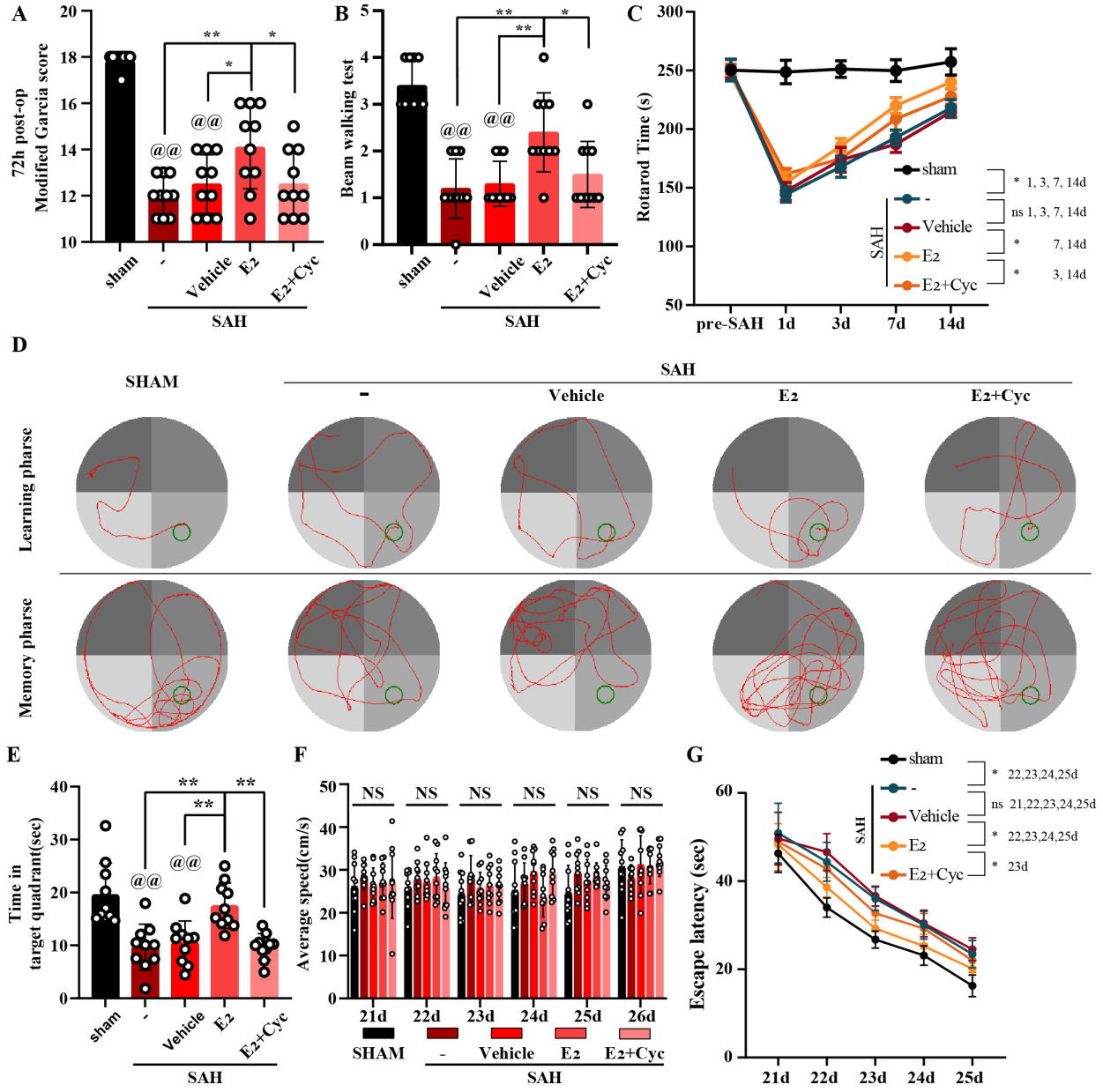

Figure 6 Oestrogen improves neurobehavioural function in rats after subarachnoid haemorrhage (SAH) via the sonic hedgehog (SHH) signalling pathway. (A) The modified Garcia score was detected on day 3 of SAH in rats under oestrogen and oestrogen+cyclopamine intervention. n=10/group. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. (B) The beam walking test was performed on day 3 of SAH in rats under oestrogen and oestrogen+cyclopamine intervention. n=10/group. One-way ANOVA and Tukey's multiple comparisons test. (C) Rotarod time was detected 1 day pre-SAH and 1, 3, 7 and 14 d post-SAH in rats under oestrogen and oestrogen+cyclopamine intervention. n=10/group. Two-way RM ANOVA and Tukey's multiple comparisons test (D) Morris water maze experiment in rats, swimming trajectory map of rats in learning phase and memory phase. (E) The dwell time in the target quadrant was examined on day 26 after SAH in rats under oestrogen and oestrogen+cyclopamine intervention. n=10/group. One-way ANOVA and Tukey's multiple comparisons test. (F) Swimming speed of each group in the Morris water maze experiment at days 21–26 after SAH. n=10/group. One-way ANOVA and Tukey's multiple comparisons test. (G) The escape latency of rats in each group was examined 21–25 days after SAH. n=10/group. Two-way RM ANOVA and Tukey's multiple comparisons test @ p<0.05, @@ p<0.01 compared with the sham group, *p<0.05, **p<0.01 compared with the oestrogen group; NS: No significant. (–): SAH only; (Vehicle): Vehicle+SAH; (E2): oestrogen+SAH; (Cyc+E2): oestrogen+cyclopamine + SAH. All data represent the means±SDs.

the brain. In previous research, oestrogen in blood could interfere with neuronal and astrocyte regulation through the BBB. We found a positive correlation between SHH protein levels and oestrogen levels in the CSF of SAH patients by collecting real-world human CSF samples. Oestrogen has been shown to upregulate the expression of the SHH signalling pathway through oestrogen receptors in adrenal cells and gastric cancer models. In our study, this correlation between oestrogen receptors and the SHH signalling pathway was analysed through GEPIA

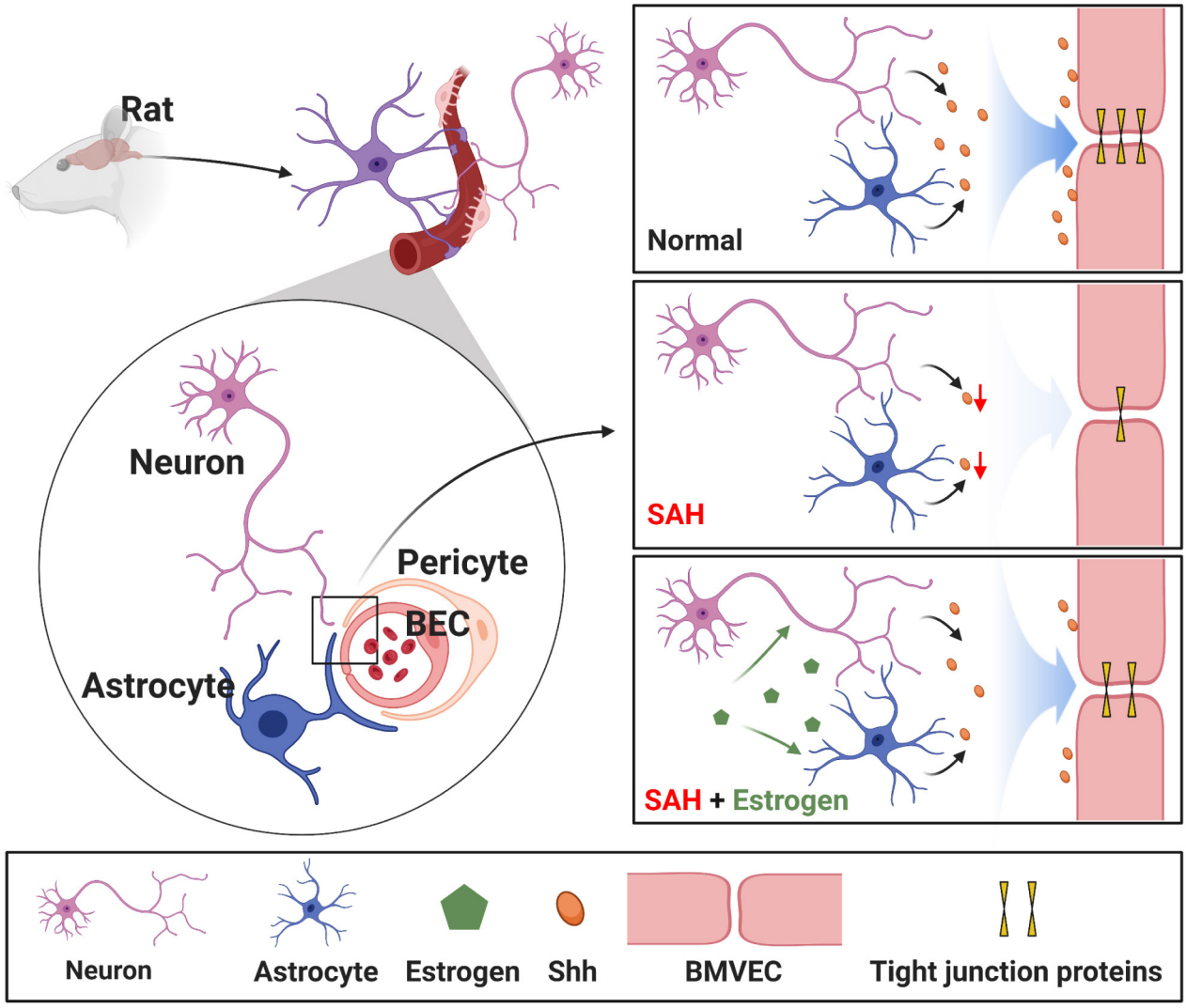

Figure 7 Schematic representations of the potential mechanisms of oestrogen in blood–brain barrier (BBB) protection in rats exposed to subarachnoid haemorrhage (SAH). Under physiological conditions, sonic hedgehog (SHH) released by neurons and astrocytes plays a key role in maintaining BBB integrity. The decline in the SHH pathway caused by SAH is one of the causes of BBB damage. Exogenous oestrogen intervention can effectively reverse SAH-induced SHH pathway decline and BBB damage. BMVEC, brain microvascular endothelial cells.

in transcriptional data from GTEx. The transcriptional expression of two oestrogen receptor classical receptors, ESR1/2, positively correlated with the transcript levels of SHH signalling pathway components.

In previous studies, hBMVEs were regulated by extracellular sources of SHH, which in turn expressed more TJs and maintained the stability of the BBB.[11] However, a few related studies have shown that neurons are the main source of SHH in the brain rather than astrocytes.[24] The BBB is regulated not only primarily by astrocyte-endothelial communication but also by neuron–endothelial communication. The distribution of SHH and which types of cells had elevated SHH expression after oestrogen stimulation remain worthy of investigation. In our experiments, oestrogen was expressed in both astrocytes and neurons, and an increase in SHH content was found in both types of cells without any change in cell count after oestrogen intervention, again confirming the relationship between SHH and oestrogen from the previous section.

Alternations in SHH after SAH have not been uniformly identified in previous studies. Hu *et al* found that SHH expression was downregulated 48 hours after SAH, and Zuo *et al* also found that expression was downregulated 24 hours after SAH.[12 25] In contrast, Tao *et al* found that SHH was elevated 48 hours after SAH.[26] Meanwhile, Hu *et al* and Li *et al* had different results for the changes in the SHH signalling pathway component GLI1 after SAH. In our study, we added the post-SAH time point setting compared with previous works and obtained similar results to Hu *et al* 72 hours after SAH, SHH protein in the temporal cortex was significantly decreased. Interestingly, this result was consistent with the decrease in TJs and brain oedema to a trough at 72 hours after SAH. For changes in downstream components after SAH, GLI1 and SMO were also both downregulated by changes in

SHH after SAH. This process was reversed through the oestrogen receptor signalling pathway when oestrogen was given.

Tight junctions between hBMVEs are important proteins that maintain BBB stability.[27] There are two peaks of TJs reduction after SAH, 3 hours and 72 hours.[28] The mechanism of reduction may be related to the activation of apoptotic pathways in endothelial cells after SAH. SHH from extraendothelial cells increases the release of GLI1 into the nucleus by binding to PTCH1 on the endothelial cell membrane. The entry of GLI1 into the nucleus promotes the transcription of SOX18 and increases the expression of proteins such as claudin-3, claudin-5 and occludin.[11] Seventy-two hours after SAH, our experiments revealed that the expression level of TJs was downregulated. In vivo, we found that TJs on endothelial cells were upregulated after oestrogen intervention through the SHH pathway. In vitro, by mimicking SAH for 24 hours, we found a decrease in the amount of TJs in endothelial cells, and SHH alleviated this decrease.

TJs are key proteins that maintain BBB stability. However, it is not known whether TJs can properly assemble into cell junctions after oestrogen stimulation to play a role in maintaining the stability of the BBB.[29] There is no suitable method to detect this assembly process. In our experiments, we assessed BBB stability by performing a western blot assay of albumin and measurements of Evans blue absorbance and brain water content. Increased TJs after oestrogen stimulation reduced the increased BBB permeability due to SAH, indicating that these TJs are correctly assembled to the cell junctions.

Neurological injury after SAH is affected not only by oedema due to increased BBB permeability but also by decreased cerebral blood flow due to vasospasm and increased intracranial pressure. Previous studies have also found that oestrogen can reduce vasospasm after SAH.[7] Whether oestrogen can improve neurological function after SAH by reducing acute brain injury after SAH through an improvement effect on BBB function remains unclear. In previous studies, there were no relevant behavioural tests on the role of SHH and oestrogen post-SAH.[12 25] In our experiments, oestrogen improved neurological function in rats after SAH, and the reversal occurred when the SHH signalling pathway was blocked by cyclopamine. However, whether this conclusion can be generalised to clinical patients still needs to be confirmed by more clinical trials in the future.

Our experiments suggest that oestrogen is a protective factor for neurological function after SAH, which is consistent with the results of previous experiments. However, this conclusion was verified in male rats. We used only male rats to avoid endogenous oestrogen fluctuations in female rats and pathological alterations of sex hormones such as progesterone brought about by ovariectomy in female rats. Whether female rats differ from male rats after experimental SAH remains to be analysed in future studies. Meanwhile, we still note that clinical data suggest a higher mortality rate in women than in men after SAH, which is inconsistent with the results of many previous experiments. This may be related to differences in androgen, progesterone, and some nongender-related factors, such as smoking.[30] The effect of a single-factor intervention of oestrogen in experiments may be masked by the effect of other factors in clinical patients.

Overall, our experiments explain the role of oestrogen in improving BBB stability and ameliorating neurological impairment after SAH through upregulation of the SHH signalling pathway by oestrogen receptors (figure 7). This study may help to explain the clinical sex differences in relation to perihaematomal brain oedema and the prognosis of neurological function after SAH. However, how to specifically agonise oestrogen receptors in the brain and thus reduce brain damage caused by SAH while avoiding the systemic side effects of oestrogen application remains to be studied in the future.

Contributors GC is responsible for the overall content as the guarantor. JZ and HL contributed equally to this work. GC and WY were the cocorresponding authors involved in the conception, design and execution of the study. All authors made significant contributions to the manuscript.JZ and HL contributed to the manuscript writing, ELISA, Western blot assay, immunofluorescence assay and data analysis. JZ contributed to the GTEx data analysis. ZX and JL were involved in the neurobehavioural experiments. CC and HS were involved in animal experiments, rat brain tissue collection and human CSF collection. XL performed the data analysis. GC and WY completed the study design and manuscript review and revision.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University (2022-294). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request.

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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.

Supplemental Table 1: Item list

Items	Company	Item No.
Primary Antibodies		
Anti-Shh Antibody	Santa Cruz	sc-365112
Anti-PTCH Polyclonal Antibody	Thermofisher	PA1-46222
Anti-Smoothened antibody	Abcam	ab236465
Anti-Gli1	Abcam	ab273018
Anti-Occludin Antibody	Abcam	ab216327
Anti-Claudin3 Antibody	Thermofisher	PA5-16867
Anti-Claudin5 Antibody	Thermofisher	35-2500
Anti-NeuN antibody	Abcam	ab177487
Anti-GFAP Antibody	Abcam	ab7260
Anti-β-Actin Antibody	Santa Cruz	sc-47778
Anti-GAPDH Antibody	Affinity	AF7021
GSL Isolectin-B4	Thermofisher	L32473
Secondary Antibodies		
Anti-rabbit IgG, HRP-linked Antibody #7074	CST	7074s
Anti-mouse IgG, HRP-linked Antibody #7076	CST	7076s
Donkey Anti-Mouse IgG H&L (Alexa Fluor® 488)	Abcam	ab150105
Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 568)	Abcam	ab175470
ELISA kits		
Human Sonic Hedgehog/Shh N-Terminus Quantikine ELISA Kit	Rnd systems	DSHH00
Human Estrogen ELISA Kit	Finetest	EH4184
Rat Albumin ELISA kit	Abcam	ab108789
Intervention drugs		
Cyclopamine	Shanghai YUANYE	B20547

Fulvestrant	Shanghai YUANYE	S41859
Estrogen	Shanghai YUANYE	S30633
Corn oil	Shanghai YUANYE	S50856
Cell culture		
DMEM high glucose (no phenol red, sodium pyruvate)	Procell	PM150225
Penicillin/streptomycin solution	Shanghai Zhong Qiao Xin Zhou Biotechnology Co.,Ltd	503
Fetal bovine serum	Shanghai Zhong Qiao Xin Zhou Biotechnology Co.,Ltd	25
Trypsin/EDTA digestion solution	Gibco	25200056
Hemoglobin, freeze dried	MP Biomedical	02151234-CF
HrSHH	Abcam	ab268966

Supplemental Table 2: Shh protein and estrogen levels in CSF

Sample Number	Age	Sex	shh protein level (pg/ml)	estrogen level (pg/ml)
1	63	Female	78.552	80.4562
2	48	Male	329.54118	118.4834
3	51	Female	422.62326	128.2442
4	38	Female	312.91938	176.2774
5	57	Female	124.26195	62.7896
6	69	Male	247.26327	74.6908
7	46	Male	81.04527	87.9594
8	45	Male	87.69399	87.5602
9	41	Female	204.87768	85.8528
10	62	Female	83.53854	21.3406
11	65	Male	129.24849	64.5362
12	63	Female	64.42347	71.4128
13	58	Female	99.32925	103.4108
14	61	Male	36.16641	87.031
15	79	Male	232.30365	57.3756
16	63	Male	176.62062	73.0868
17	47	Female	356.96715	146.2598
18	43	Female	259.72962	158.6058
19	58	Male	61.9302	84.628
20	49	Male	125.92413	57.2488

Supplemental Table 3: Pearson correlation coefficient of genes calculated in GTEx

	ESR1	ESR2	SHH	PTCH1	SMO	GLI1	OCLN	CLDN3	CLDN5
ESR1	R=1 P=0	R=0.59 P=0	R=0.3 P=0	R=0.5 P=0	R=0.19 P=3.6e-09	R=0.34 P=0	R=0.34 P=0	R=0.4 P=0	R=-0.00061 P=0.98
ESR2	R=0.59 P=0	R=1 P=0	R=0.049 P=0.13	R=0.81 P=0	R=0.16 P=1.1e-06	R=0.53 P=0	R=0.5 P=0	R=0.71 P=0	R=-0.013 P=0.68
SHH	R=0.3 P=0	R=0.049 P=0.13	R=1 P=0	R=-0.12 P=0.00031	R=0.08 P=0.012	R=0.088 P=0.0061	R=-0.039 P=0.22	R=-0.14 P=1.2e-05	R=0.14 P=1.1e-05
PTCH1	R=0.5 P=0	R=0.81 P=0	R=-0.12 P=0.00031	R=1 P=0	R=0.19 P=9.6e-10	R=0.61 P=0	R=0.51 P=0	R=0.78 P=0	R=-0.16 P=3.9e-07
SMO	R=0.19 P=3.6e-09	R=0.16 P=1.1e-06	R=0.08 P=0.012	R=0.19 P=9.6e-10	R=1 P=0	R=0.34 P=0	R=0.17 P=8.1e-08	R=0.14 P=9.9e-06	R=0.26 P=0
GLI1	R=0.34 P=0	R=0.53 P=0	R=0.088 P=0.0061	R=0.61 P=0	R=0.34 P=0	R=1 P=0	R=0.24 P=7.5e-14	R=0.46 P=0	R=-0.079 P=0.014
OCLN	R=0.34 P=0	R=0.5 P=0	R=-0.039 P=0.22	R=0.51 P=0	R=0.17 P=8.1e-08	R=0.24 P=7.5e-14	R=1 P=0	R=0.48 P=0	R=0.17 P=4e-08
CLDN3	R=0.4 P=0	R=0.71 P=0	R=-0.14 P=1.2e-05	R=0.78 P=0	R=0.14 P=9.9e-06	R=0.46 P=0	R=0.48 P=0	R=1 P=0	R=-0.11 P=0.00053
CLDN5	R=-0.00061 P=0.98	R=-0.013 P=0.68	R=0.14 P=1.1e-05	R=-0.16 P=3.9e-07	R=0.26 P=0	R=-0.079 P=0.014	R=0.17 P=4e-08	R=-0.11 P=0.00053	R=1 P=0

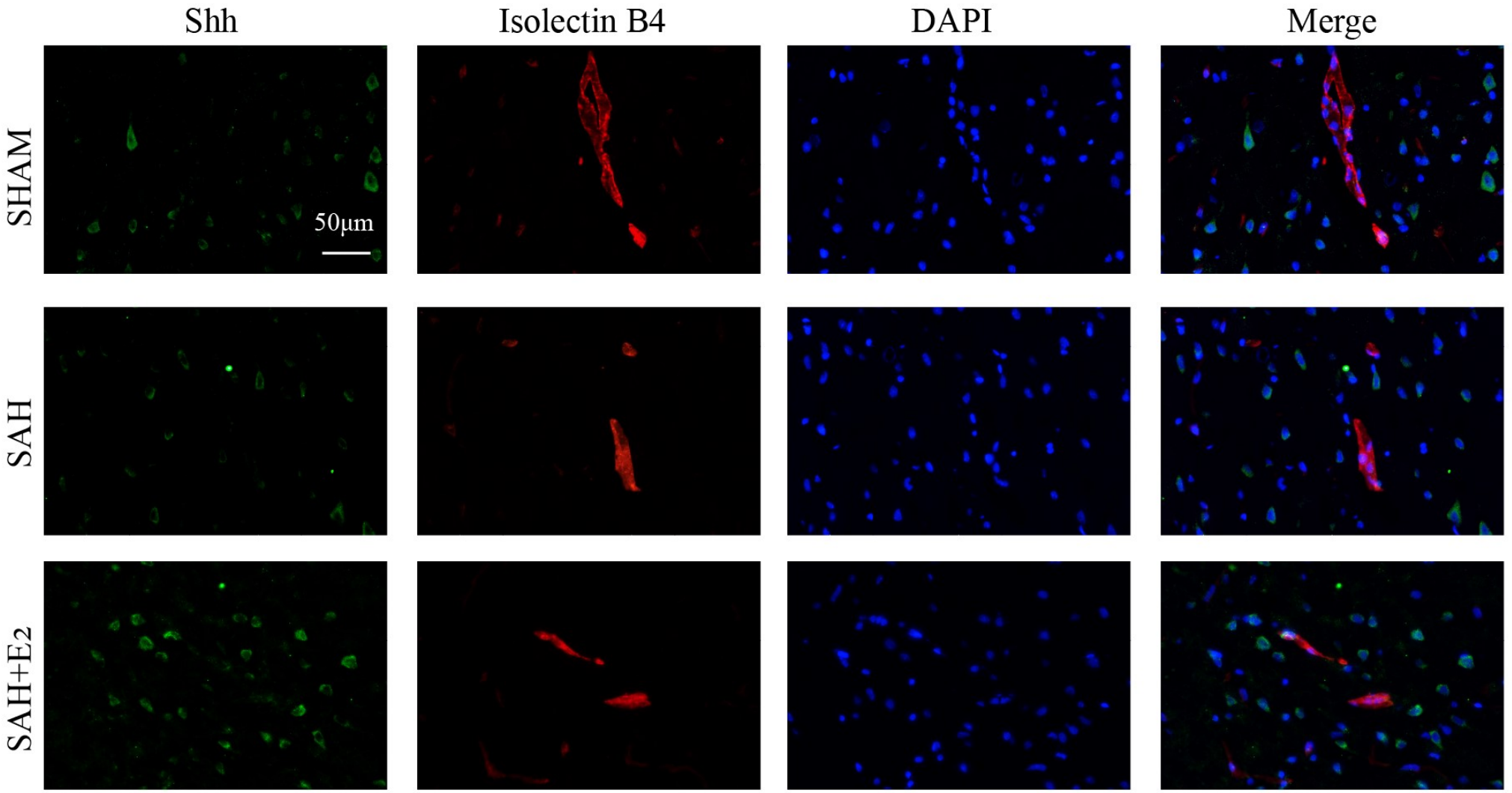


Fig S1 | No SHH distribution was found in the endothelial cells after subarachnoid hemorrhage. Immunofluorescence staining was performed with antibodies against Shh (green) and Isolectin B4 (red), while DAPI (blue) was used to label the nucleus. Scale bar = 50 μm. [E2]: estrogen.

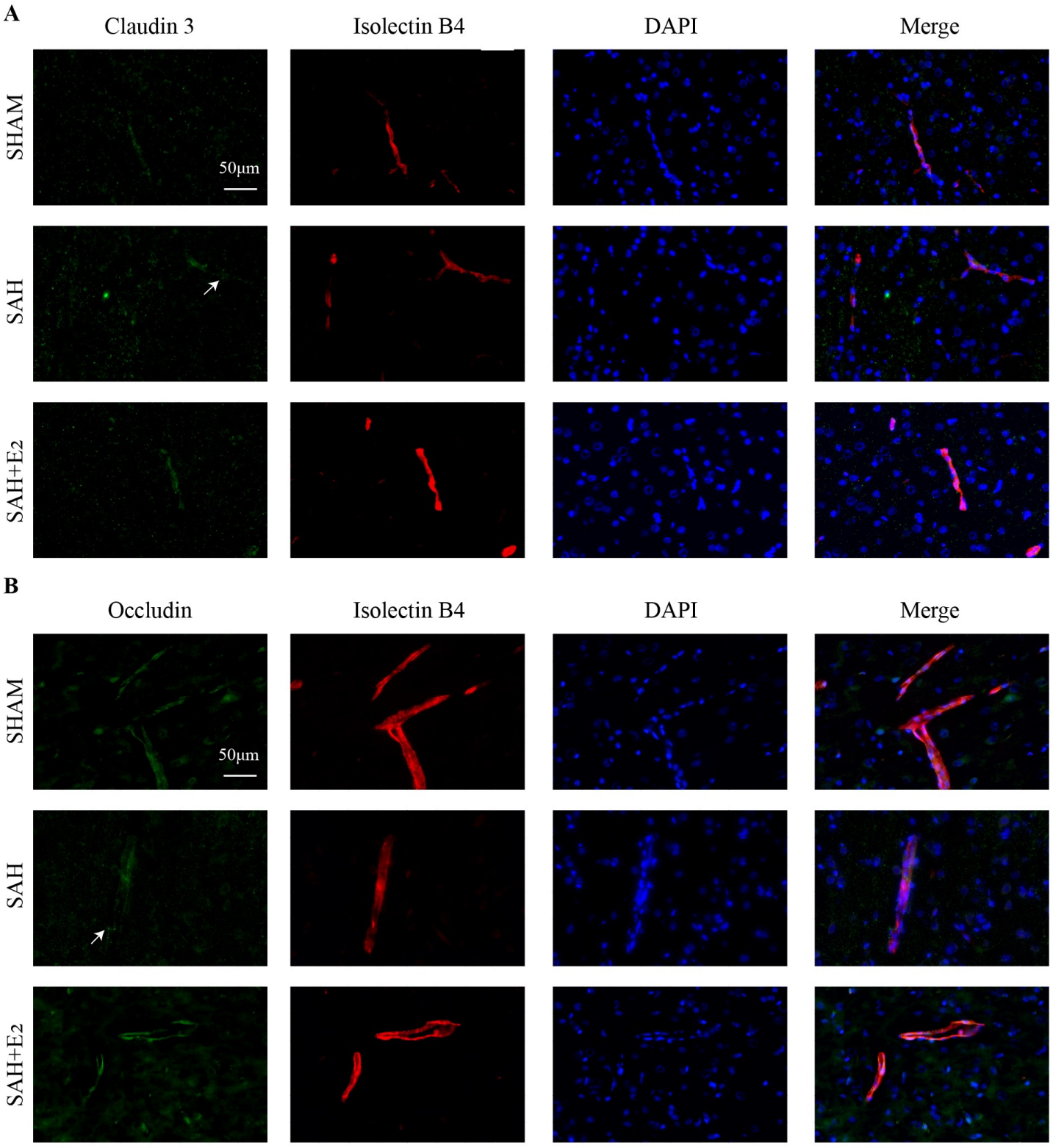
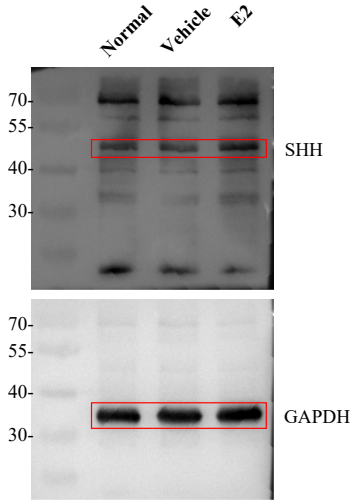
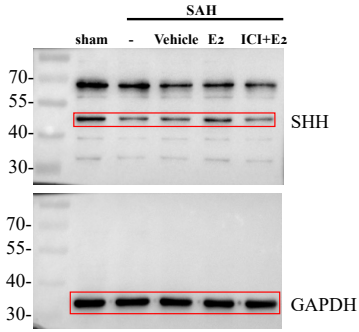


Fig S2 | Estrogen upregulates tight junction protein expression after subarachnoid hemorrhage. (A)Immunofluorescence staining was performed with antibodies against Claudin 3 (green) and Isolectin B4 (red), while DAPI (blue) was used to label the nucleus. (B)Immunofluorescence staining was performed with antibodies against Occludin (green) and Isolectin B4 (red), while DAPI (blue) was used to label the nucleus. Scale bar = 50 μm. [E2]: estrogen.

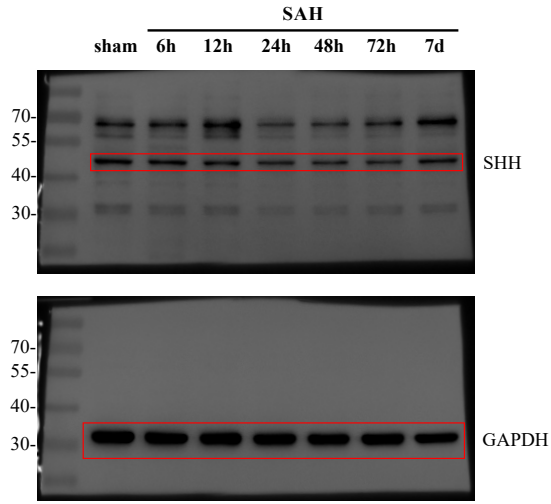
Western blots related to Figure 2H

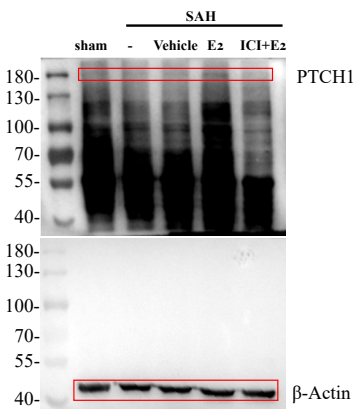


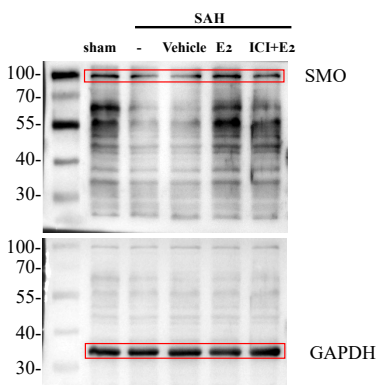
Western blots related to Figure 3C

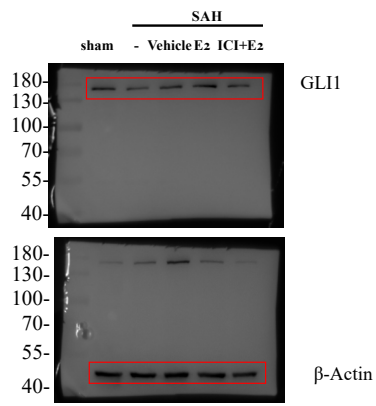


Western blots related to Figure 3A

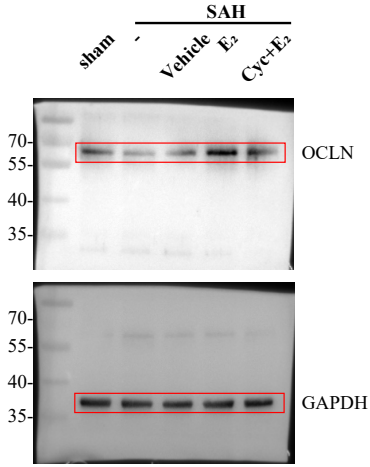




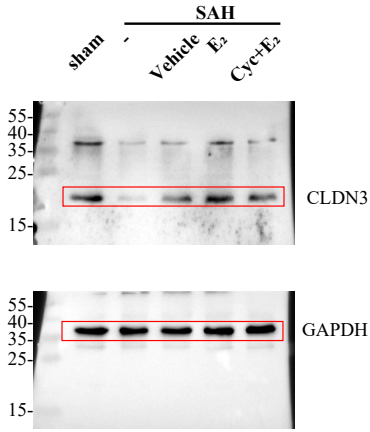




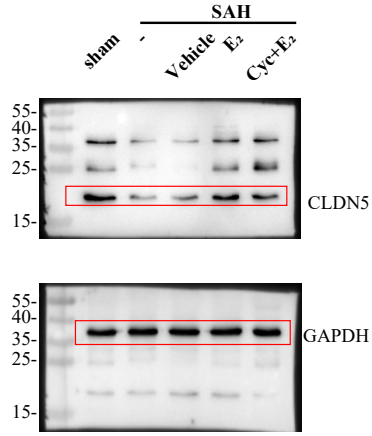
Western blots related to Figure 4A



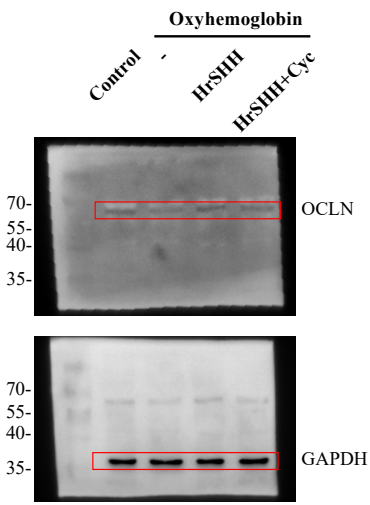
Western blots related to Figure 4B



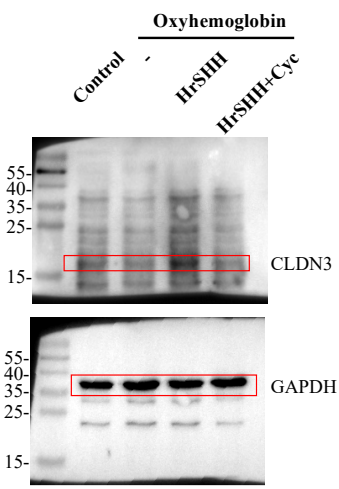
Western blots related to Figure 4C



Western blots related to Figure 4E



Western blots related to Figure 4F



Western blots related to Figure 4G