


17 β -Estradiol Attenuates Intracerebral Hemorrhage-Induced Blood–Brain Barrier Injury and Oxidative Stress Through SRC3-Mediated PI3K/Akt Signaling Pathway in a Mouse Model

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Abstract

Estrogen is neuroprotective in brain injury models, and steroid receptor cofactor 3 (SRC3) mediates estrogen signaling. We aimed to investigate whether and how SRC3 is involved in the neuroprotective effects of 17 β -estradiol (E2) in a mouse model of intracerebral hemorrhage (ICH). Ovariectomized female mice were treated with E2 after autologous blood injection-induced ICH. Brain damage was assessed by neurological deficit score, brain water content, and oxidative stress levels. Blood–brain barrier (BBB) integrity was evaluated by Evan's blue extravasation and claudin-5, ZO-1, and occludin levels. SRC3 expression and PI3K/Akt signaling pathway were examined in ICH mice treated with E2. The effect of SRC3 on E2-mediated neuroprotection was determined by examining neurological outcomes in SRC3-deficient mice undergone ICH and E2 treatment. We found that E2 alleviated ICH-induced brain edema and neurological deficits, protected BBB integrity, and suppressed oxidative stress. E2 enhanced SRC3 expression and PI3K-/Akt signaling pathway. SRC3 deficiency abolished the protective effects of E2 on ICH-induced neurological deficits, brain edema, and BBB integrity. Our results suggest that E2 suppresses ICH-induced brain injury and SRC3 plays a critical role in E2-mediated neuroprotection.

Keywords

17 β -estradiol, intracerebral hemorrhage, sSRC3, blood–brain barrier, oxidative stress

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Introduction

Intracerebral hemorrhage (ICH) is bleeding occurred in the brain parenchyma with or without external traumatic stimulation. A common condition in elderly individuals, ICH, has a high mortality and morbidity rate with a one-year survival rate of approximately 40% (Kazim et al., 2021). Research has revealed that ICH incidence in men is higher than that in women (Jolink et al., 2015). Studies of ICH patients also show that men have a younger age than women at ICH onset (Galati et al., 2015; Hsieh et al., 2016). ICH is associated with poor prognosis, and a high ratio of individuals suffered from ICH are accompanied by long-term neurological dysfunctions such as cognitive impairment, causing tremendous social and financial burden to the affected individuals and the society (Pinho et al., 2019). While current therapeutic

means are focused on treating the primary lesion, secondary injury following the initial ICH insult has been shown to greatly impact the long-term outcome (Lok et al., 2011). Attempts have been made to understand the mechanism underlying the secondary injury after ICH in order to explore potential therapeutic strategies.

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Among the molecular cascades involved in ICH-induced secondary injury, oxidative stress is a significant component (Duan et al., 2016). Suppression of oxidative stress correlates with reduced neurological deficits in the rodent model of ICH (Fu et al., 2020; Xie et al., 2020). We have previously shown that 17 β -estradiol (E2), an estrogen occurring naturally, played a neuroprotective effect in rats undergone ischemic brain injury through suppressing oxidative stress (Xiao et al., 2018). Additionally, E2 also attenuates brain injury in ICH animal models (Auriat et al., 2005; Nakamura et al., 2005). Research has also shown that E2 suppresses microglia activation and their pro-inflammatory polarization in a rodent model of ischemic brain injury (Thakkar et al., 2018). These previous findings suggest that E2 may suppress ICH-induced secondary brain injury through suppression of oxidative stress and microglia activation. To provide a better understanding of E2-mediated neuroprotection, this study further attempts to investigate the molecular mechanism underlying this protective effect.

A member of the p160 steroid receptor coactivator (SRC) family, SRC3, is a mediator of estrogens that modifies chromatin structure and induces target gene transcription by interacting with other receptors of the nucleus including estrogen receptor (Yan et al., 2006). SRC3, also known as amplified in breast cancer 1 (AIB1), is upregulated in various hormone-dependent cancers such as breast cancer, mediates estrogen signaling, and promotes tumorigenesis when overexpressed (Ma et al., 2011). SRC3 is important for the development of the mammary gland, reproductive function in females, as well as normal growth, and is extensively expressed in the brain (Xu et al., 2000). When regulating cell growth, SRC3 activates PI3K/Akt signaling (Zhou et al., 2003). It has been shown that the PI3K/Akt signaling pathway is activated and mediates the neuroprotective effect of Nle4-D-Phe7- α -melanocyte-stimulating hormone in ICH mice (Fu et al., 2020). A previous study also suggested activation of this pathway by E2 in cancer cells (Guo et al., 2006). Additionally, research has revealed a role of the PI3K/Akt signaling in suppression of oxidative stress induced by different stimulations (Hannan et al., 2020). We thus hypothesize that SRC3 and the PI3K/Akt signaling pathway are involved in mediating the neuroprotective effect of E2 in ICH mice.

Basal ganglion is a region highly susceptible to hypertension-induced ICH. As a result, autologous blood injection in the basal ganglia area has become a well-established method to generate ICH models in rodents (Sansing et al., 2011). In this study, we took advantage of this ICH mouse model and investigated how E2 played a neuroprotective effect.

Materials and Methods

ICH Induction and Drug Treatment in Mice

Cryopreserved SRC3-deficient sperms from Cyagen (strain symbol: KOCMP-17979-Ncoa3-B6J; strain name:

C57BL/6J-Ncoa3^{em1cyagen}) were used to generate SRC3 knock out (SRC3^{-/-}) mice on the C57BL/6J background. A total of 96 SRC3^{-/-} and 152 wild-type (WT) control C57BL/6J female mice were used in this study. We used ovariectomized mice in this study which is common in most publications that studied the effects of Estradiol on animal models. Two weeks prior to ICH, bilateral ovariectomy was performed in the mice under isoflurane-induced anesthesia. ICH was induced in 20–25 g 2-month-old SRC3^{-/-} and WT control C57BL/6J ovariectomized mice by injection of autologous blood according to previously established protocols (Fu et al., 2020). Following anesthesia by 10% chloral hydrate at a dose of 0.045 ml/kg, the mouse was placed in a prone position onto a stereotaxic device. Arterial blood was collected in a heparin-free capillary and was injected into the same mouse using a Hamilton syringe. The initial injection site was basal ganglion of the right hemisphere, and 5 μ l of autologous whole blood was injected at following stereotaxic coordinates: bregma dorsal–ventral 3.0 mm, medial–lateral 1.8 mm, and anterior–posterior 0.2 mm. The second injection included 25 μ l blood at the position of dorsal–ventral 3.0 mm at 5 min following the initial injection. No fluid was injected in the sham group with the other procedures being the same as ICH surgery.

Indicated mice were treated with 17 β -estradiol (E2) purchased from Sigma-Aldrich by intraperitoneal injection at 1, 12, 24, 48, and 72 h after ICH procedure at the dosages of 100, 200, and 300 μ g/kg, respectively, according to a previous study (Zheng et al., 2015). The serum E2 levels were determined before E2 injection and 2 h after each injection. All mouse protocols were approved by the Ethical Committee of Xiangya Second Hospital (#a453j).

Assessment of Neurological Deficit

Modified neurological severity score (mNSS) was used to determine the extent of neurological deficits, with 13–18 points reflecting severe abnormality, 7–12 points reflecting moderate abnormality, 1–6 points reflecting mild abnormality, and 0 points reflecting normal function (Kuramoto et al., 2019; Xu et al., 2017; Zhang et al., 2020). The score system included six subtests: spontaneous movement (0–3), limb symmetry (0–3), forward extension (0–3), climbing (0–3), body proprioception (0–3), and response to tentacles (0–3), with 0 for no abnormality, 1 for mild abnormality, 2 for moderate abnormality, and 3 for severe abnormality. Indicated mice were assessed one day before induction of ICH and 1, 7, 14, and 21 days after ICH.

Brain Water Content

Water content in different brain regions was measured to determine the severity of brain edema (Fu et al., 2020). Animals were deeply anesthetized with isoflurane and perfused with phosphate-buffered saline (PBS). Brains were

then removed and sliced into 4-mm thick sections around the injection site. The sections were divided into four regions including ipsilateral basal ganglia, ipsilateral cerebral cortex, contralateral basal ganglia, and contralateral cerebral cortex. The cerebellum was dissected out separately and used as an internal control. Each region was weighed, and the total weight of each region was referred to as wet weight (WW). The brain regions were weighed again after drying at 100 °C for 1 day (DW). The water content of each region was determined by $(WW - DW) / WW \times 100\%$.

Evan's Blue Extravasation

Blood–brain barrier integrity was assessed by extravasation of Evan's blue dye in the brain (Hu et al., 2009; Zheng et al., 2015). Briefly, a subset of mice were administered with Evan's blue dye by intravenous injection. Mice were then anesthetized and perfused transcardially with PBS one hour after dye injection. The levels of extravasated dye in the ipsilateral and contralateral hemispheres were measured by spectrofluorometry at the wavelength of 610 nm.

Western Blot Analysis

Western blot was carried out according to a previous protocol (Xie et al., 2020). Briefly, a subset of mice were anesthetized and sacrificed. The mice used for Western blot were different from mice used for Evan's blue extravasation. The ipsilateral hemisphere was extracted and homogenized in a lysis buffer. An equivalent amount of protein from each sample was separated through SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane. After blocking in 5% bovine serum albumin, the membrane was incubated in primary antibodies at 4 °C for overnight, followed by incubation in respective secondary antibodies. Proteins were detected by a chemiluminescence system. The following antibodies were used in this study: anti-claudin-5 (1:1000), anti-ZO-1 (1:1500), anti-occludin (1:1200), anti-GAPDH (1:3000), anti-SRC3 (1:2000), anti-phospho (p)-PI3K (1:800), anti-PI3K (1:1000), anti-p-Akt (1:1000), and anti-Akt (1:1000). All antibodies were purchased from Abcam or Sigma. For each analysis, tissue from eight mice was mixed and the experiments were repeated in triplicates.

Oxidative Stress

Three days after ICH induction, ipsilateral hemispheres from relevant mice were collected for the measurement of oxidative stress levels suggested by previous studies (Fu et al., 2020; Qu et al., 2019). Markers for oxidative stress including nitric oxide (NO), catalase (CAT), glutathione (GSH), superoxide dismutase (SOD), and malondialdehyde (MDA) were measured by kits purchased from Nanjing Jiancheng Bio-engineering Institute following respective instructions.

qRT-PCR

To measure the mRNA expression of SRC3, total RNA was collected from the ipsilateral injury hemispheres of relevant mice and quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed according to previous studies (Xie et al., 2020; Qu et al., 2019). The following primers (5'–3') were used: SRC3-Forward-AGTGGACTAGGCGAAAGCTCT and SRC3-Reverse-GTTGTTCGATGTCGCTGAGATT, and GAPDH-Forward-AATGGATTTGGACGCATTGGT and GAPDH-Reverse-TTTCACCTGGTACGTGTGAT. For each analysis, tissue from eight mice was mixed and the experiments were repeated in triplicates.

Immunohistochemistry

Following anesthesia, the brain was extracted and coronal sections were obtained at a thickness of 3 mm (Leica VT1200). The sections were fixed with 4% paraformaldehyde overnight at 4 °C and stained with an antibody of a microglia marker: ionized calcium-binding adapter molecule 1 (Iba-1). After washing with PBS, the sections were stained with goat anti-rabbit IgG conjugated to a peroxidase-labeled dextran polymer. Last, after rinsing with PBS, the immunoreactivity was visualized by diaminobenzidine staining. The immunopositive area was counted in a blinded manner, and the result was expressed as the percentage of positively stained area; the counting of positively stained area was completed using the manual counting program included in the Image J software.

Determination of Serum E2 Level

Serum E2 level was determined using 17 beta Estradiol ELISA assay (#ab108667, Abcam) following manufacturer's instruction.

Statistical Analysis

Differences among different treatment groups with a time course were evaluated by two-way analysis of variance (ANOVA) followed Tukey's multiple comparisons test. Differences among different treatment groups were evaluated by one-way ANOVA (Kruskal–Wallis test) followed Dunn's multiple comparisons test. Data were presented as Box–Whiskers plots to indicate the median and interquartile range. Differences with $p < .05$ were statistically significant. ns means no significance.

Results

E2 Alleviates ICH-Induced Brain Edema and Neurological Deficits in Wild-Type Mice

Two weeks prior to the ICH procedure, the experimental mice underwent ovariectomy to remove endogenous estradiol.

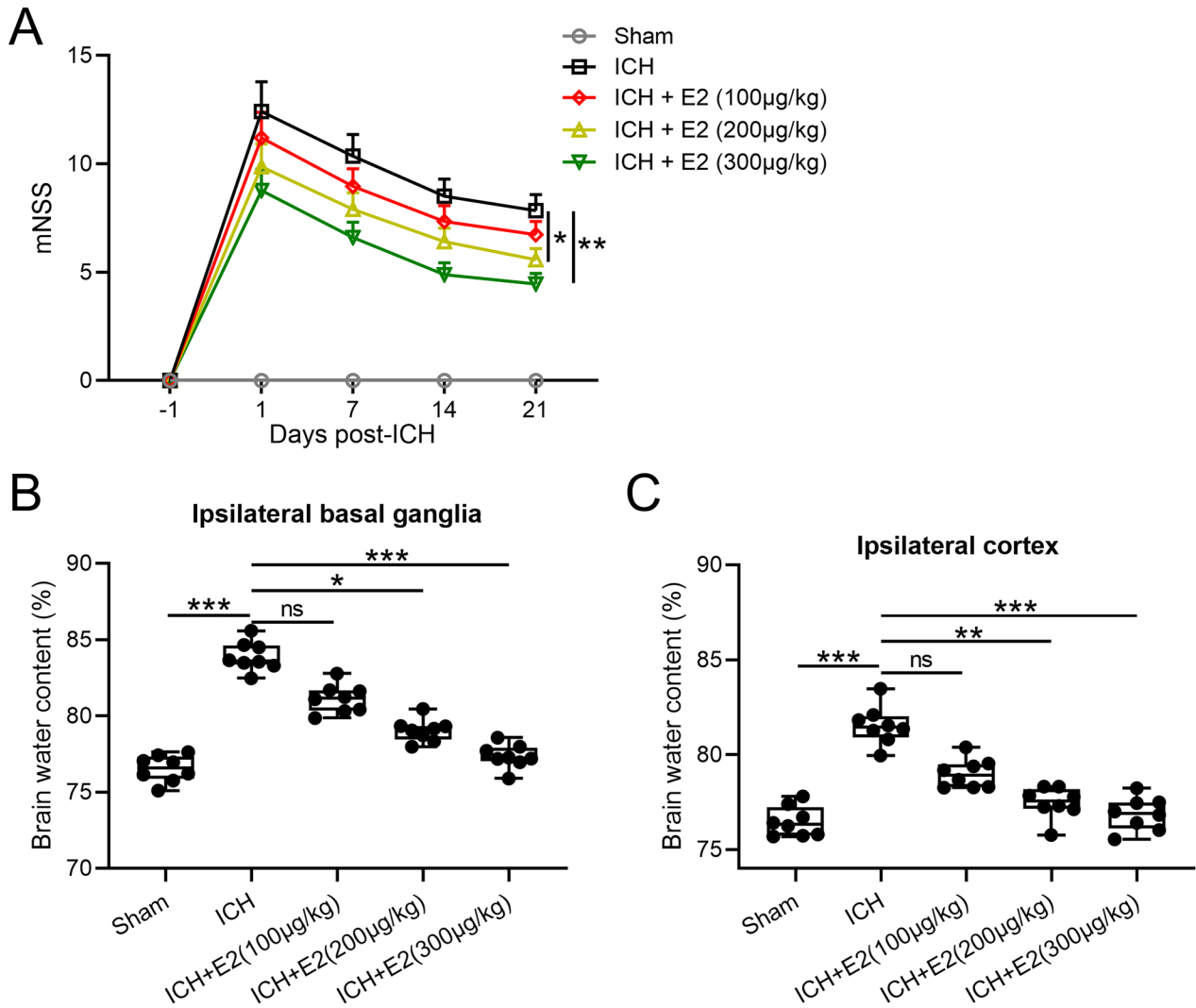


Figure 1. E2 attenuates ICH-induced brain edema and neurological deficit in wild-type mice. Neurological deficit scores were measured pre, 1, 7, 14, and 21 days postintracerebral hemorrhage (A). One-way ANOVA followed Dunn's multiple comparisons test. Brain water content was compared 3 days postintracerebral hemorrhage (B and C). $N = 8$ for each group. One-way ANOVA followed Dunn's multiple comparisons test in (B and C). * $p < .05$, ** $p < .01$, *** $p < .001$, ns indicates no significance. Note. ICH = intracerebral hemorrhage; ANOVA = analysis of variance.

Following ICH, the ovariectomized mice were then treated with E2 and serum E2 levels were assessed before E2 administrations and 2 h after each injection. We found E2 administration at different doses all resulted in supraphysiological level of E2 in the serum (Figure S1). Then, we explored the neuroprotective effects of E2 in WT mice undergone the ICH procedure. We used the extensively used mNSS scale to evaluate the neurological function in mice with a higher score corresponding to more severe neurological deficits (Tian et al., 2019; Zhou et al., 2016). mNSS assessment showed that ICH significantly increased mNSS score and E2 dose-dependently reduced mNSS score (Figure 1A). Compared to ICH mice, mice treated with 300 µg/kg E2 showed significantly alleviated neurological deficits after 3 weeks of ICH.

We then examined the severity of edema in different brain regions. We showed that water content in the basal ganglia (Figure 1B) and cortex (Figure 1C) of the ipsilateral hemisphere of mice undergone ICH was significantly elevated. E2 dose-dependently reduced the water contents in both brain regions. On the other hand, we did not detect any significant differences in the water content of the cerebellum among the mice from all the groups (Figure S2).

Our results showed that 100 µg/kg E2 had no significant neuroprotective effects against ICH-induced brain injury. We did not detect any statistically significant differences between mice treated with 200 µg/kg E2 and mice treated with 300 µg/kg E2. However, there is a trend showing that the effect was most pronounced when E2 was treated at 300 µg/kg, and a previous study also used this dose (Zheng

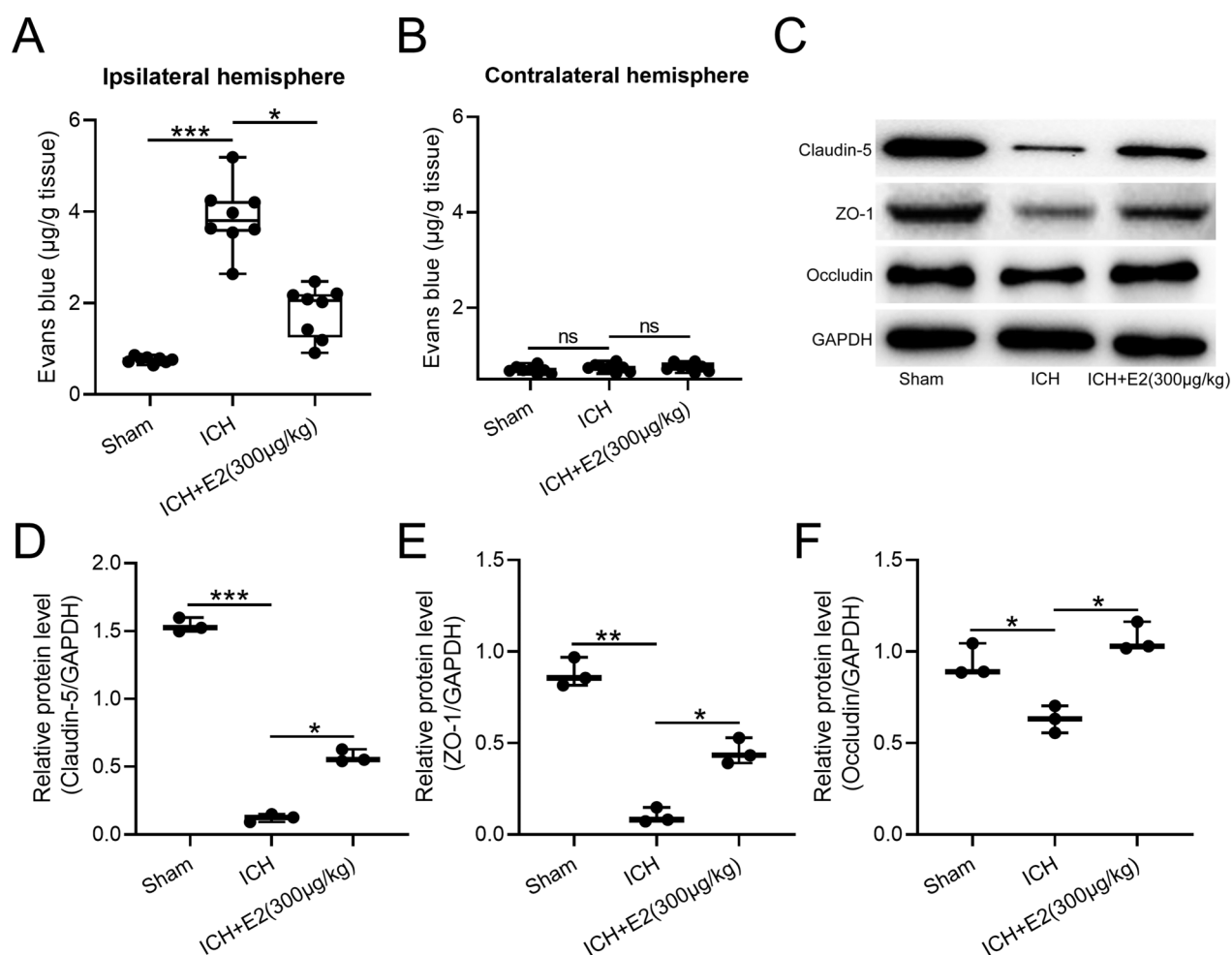


Figure 2. E2 attenuates ICH-induced BBB disruption in wild-type mice. BBB permeability was assessed by Evan's blue extravasation 3 days post-ICH in the ipsilateral injury hemisphere (A) and contralateral uninjury hemisphere (B). claudin-5, ZO-1, and occludin in the ipsilateral injury hemispheres 3 days postintracerebral hemorrhage were assessed by Western blot (C). Experiments were repeated in triplicate using tissues from eight mice. The relative expressions were normalized to GAPDH (D–F). One-way ANOVA followed Dunn's multiple comparisons test. * $p < .05$, ** $p < .01$, *** $p < .001$, ns indicates no significance.

Note. ICH = intracerebral hemorrhage; ANOVA = analysis of variance; BBB = blood–brain barrier.

et al., 2015). Therefore, we only focused on this dose for the subsequent assays to reduce the number of animals.

E2 Alleviates ICH-Induced Blood–Brain Barrier Disruption in Wild-Type Mice

To investigate how E2 mediates neuroprotection in mice undergone ICH, we examined the integrity of BBB following E2 treatment. Evan's blue extravasation showed that, in the ipsilateral hemisphere, ICH significantly increased extravasated Evan's blue dye compared to sham operation, which was significantly reduced by 300 µg/kg E2 treatment (Figure 2A). Neither ICH procedure nor E2 treatment significantly impacted Evan's blue extravasation in the contralateral hemisphere (Figure 2B). A clearer distribution of the data in the sham-operated ipsilateral hemisphere and the contralateral

hemisphere of all groups is shown in Figure S2 with a smaller range in Y-axis. Figure S3A shows each data point of the ipsilateral hemisphere of sham-operated mice, and Figure S3B shows each data point of the ipsilateral hemisphere of all groups of mice. The levels of extravasated Evans blue were low since these regions were not damaged. Western blot analysis (Figure 2C) showed that ICH significantly reduced claudin-5 (Figure 2D), ZO-1 (Figure 2E), and occludin (Figure 2F) protein levels, which were partially or completely restored by E2 treatment.

E2 Suppresses ICH-Induced Oxidative Stress in Wild-Type Mice

We further examined how E2 impacted ICH-induced oxidative stress in WT mice. We confirmed that ICH significantly

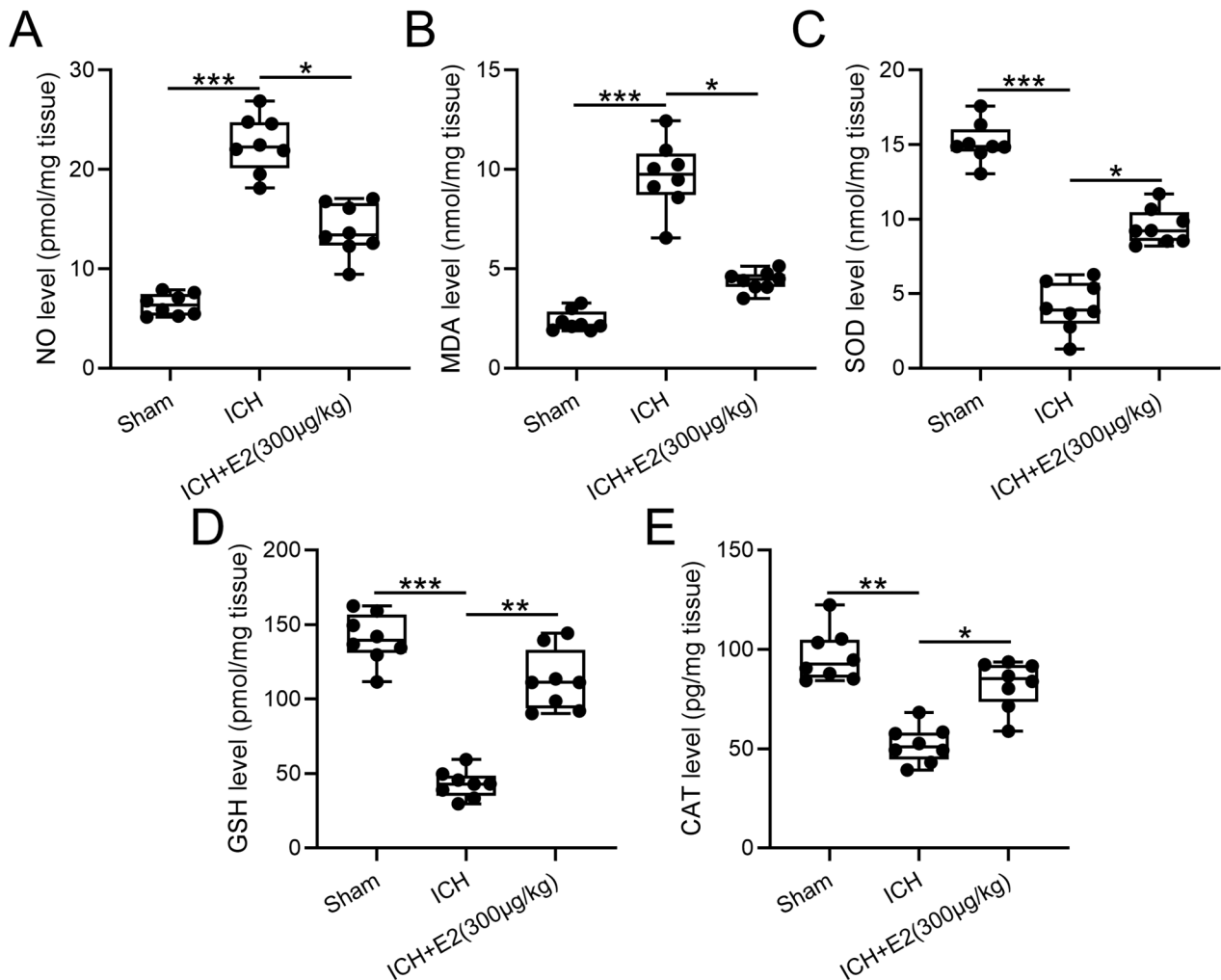


Figure 3. E2 attenuates ICH-induced oxidative stress in wild-type mice. The levels of oxidative stress indicators NO (A), MDA (B), SOD (C), GSH (D), and CAT (E) in the ipsilateral injury hemispheres 3 days post-intracerebral hemorrhage were examined. One-way ANOVA followed Dunn's multiple comparisons test. * $p < .05$, ** $p < .01$, *** $p < .001$.

Note. ICH = intracerebral hemorrhage; ANOVA = analysis of variance.

increased NO (Figure 3A) and MDA (Figure 3B) levels and decreased SOD (Figure 3C), GSH (Figure 3D), and CAT (Figure 3E) levels, consistent with an elevation of oxidative stress following ICH. Importantly, 300 $\mu\text{g}/\text{kg}$ E2 significantly suppressed ICH-induced changes in oxidative stress markers.

E2 Leads to Microglia Activation and Upregulates SRC3 and PI3K/Akt Pathway in Wild-Type Mice Following ICH

We then investigated the cellular and molecular mechanisms underlying the neuroprotective effects of E2 in mice undergone ICH. Immunohistochemistry of the cerebral cortex with microglia marker Iba-1 (Figure S4A) showed that the ICH procedure significantly increased the ratio of Iba-1-positive area in WT cortex, which was suppressed by E2 treatment (Figure S4B). qRT-PCR showed that SRC3

mRNA expression was significantly suppressed following ICH which was partially restored by 300 $\mu\text{g}/\text{kg}$ E2 (Figure 4A). Similarly, Western blot analysis (Figure 4B) showed that ICH significantly suppressed SRC3 protein expression which was also restored by E2 treatment (Figure 4C). We also examined the activity of PI3K-Akt pathway by Western blot (Figure 4D) and found that ICH significantly inhibited PI3K (Figure 4E) and Akt (Figure 4F) phosphorylation, which were both increased by E2 treatment.

SRC3 Deficiency Abolishes E2-Mediated Protection From Intracerebral Hemorrhage-Induced Brain Injury

To determine if SRC3 is required for E2-mediated neuroprotection in mice undergone ICH, we induced ICH in SRC deficient mice and assessed neurological outcomes after E2 treatment. We found that ICH significantly increased brain

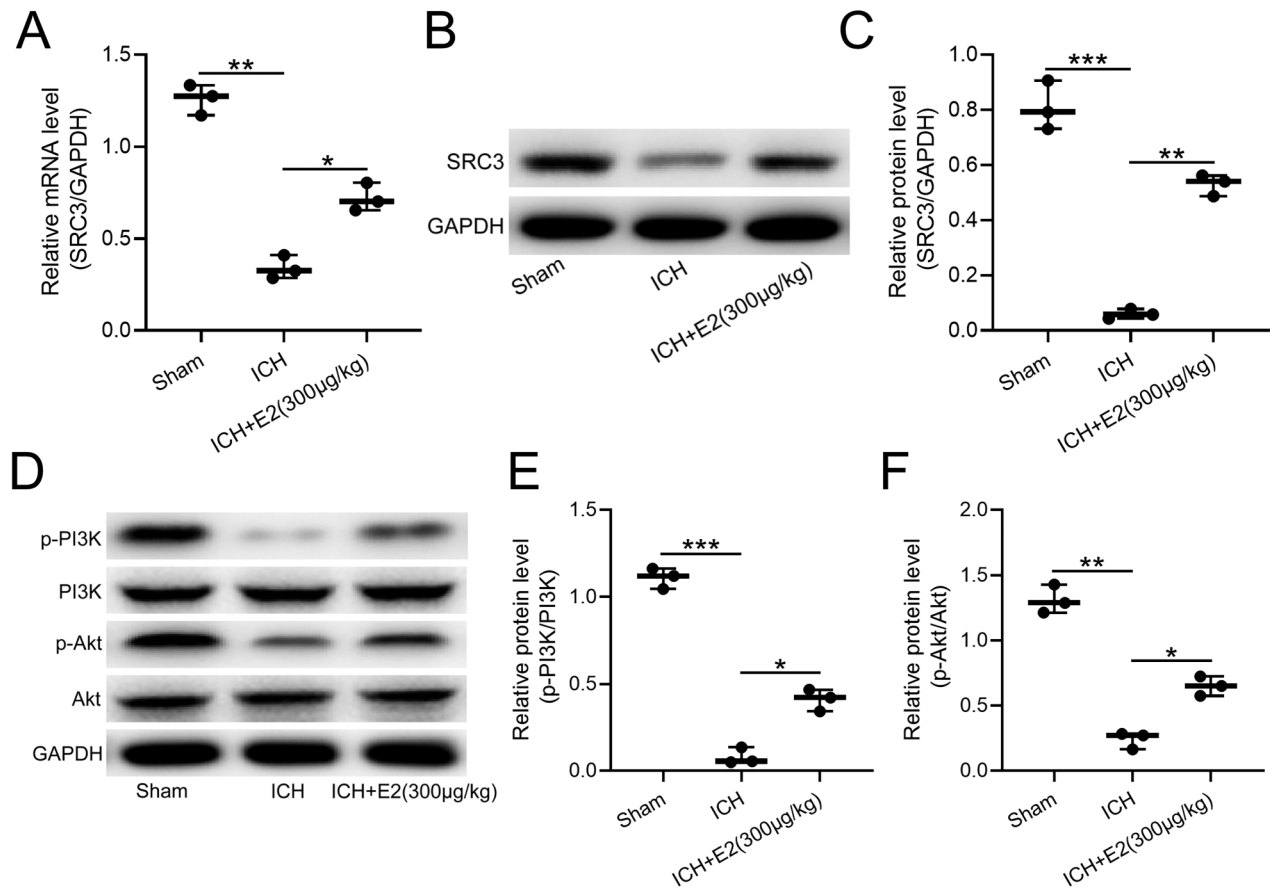


Figure 4. E2 activates SRC3 and upregulates PI3K/Akt pathway in wild-type mouse model of intracerebral hemorrhage. mRNA and proteins levels of SRC3 in the ipsilateral injury hemispheres 3 days post-intracerebral hemorrhage were assessed by qRT-PCR and Western blot (A–C). p-PI3K, PI3K, p-Akt, and Akt levels in the ipsilateral injury hemispheres 3 days postintracerebral hemorrhage were assessed by Western blot (D). The relative expressions were shown in (E and F). Experiments were repeated in triplicate using tissues from eight mice. One-way ANOVA followed Dunn's multiple comparisons test. * $p < .05$, ** $p < .01$, and *** $p < .001$.

water contents in both basal ganglia (Figure 5A) and ipsilateral (Figure 5B) of the ipsilateral hemisphere of SRC3^{-/-} mice, which were not reduced by E2 treatment. Similarly, mNSS assessment showed that ICH significantly increased neurological deficits in SRC3^{-/-} mice, which were not attenuated by E2 treatment (Figure 5C). These findings suggest that SRC3 is required for E2-mediated protection from ICH-induced brain injury.

SRC3 Deficiency Prevents E2-Mediated Protection of Blood–Brain Barrier Integrity in Mice Following ICH

Finally, we investigated the effects of SRC3 deficiency on the integrity of BBB in mice undergone ICH and E2 treatment. We found that ICH significantly increased extravasated Evan's blue in the ipsilateral hemisphere of SRC3^{-/-} mice, which was not suppressed by E2 treatment (Figure 6A). Similarly, Western blot analysis (Figure 6B) showed that E2 treatment had no impact on claudin-5 (Figure 6C), ZO-1

(Figure 6D), and occludin (Figure 6E) protein levels in the ipsilateral hemisphere of SRC3^{-/-} mice following ICH.

Discussion

This study investigated the molecular mechanism underlying the neuroprotective effects of E2 against ICH-induced injury in mice. We confirmed that mice treated with E2 following induction of ICH showed attenuated brain edema and neurological deficits and better preserved BBB integrity. E2 treatments results suppressed oxidative stress levels following ICH. In an attempt to dissect cellular and molecular networks activated by E2, we showed that ICH-induced microglia activation and suppressed PI3K/Akt signaling pathway, which were partially restored by E2 treatment. Similarly, SRC3 expression in the brain was also suppressed by the ICH procedure and partially recovered by E2. Importantly, our study revealed that SRC3 was required for E2-mediated neuroprotection in mice undergone ICH insult. SRC3 deficiency abolished E2-mediated alleviation of brain edema and

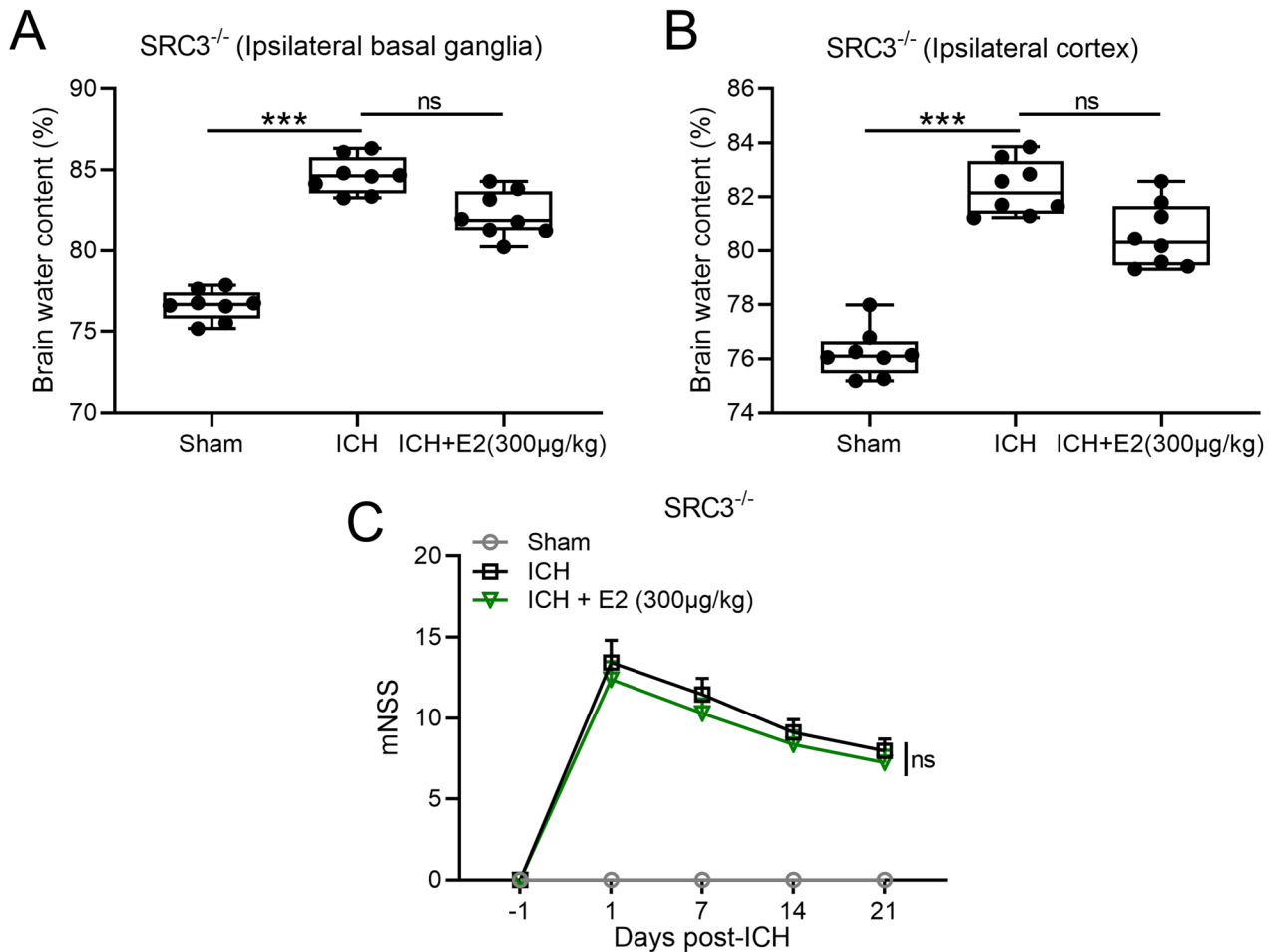


Figure 5. E2 has limited effects on ICH-induced brain edema and neurological deficit in SRC3^{-/-} mice. Brain water content was compared 3 days postintracerebral hemorrhage (A and B). One-way ANOVA followed Dunn's multiple comparisons test. Neurological deficit scores were measured pre, 1, 7, 14, and 21 days postintracerebral hemorrhage (C). $N = 8$ for each group. Student's t -test. *** $p < .001$, ns indicates no significance.

Note. ICH = intracerebral hemorrhage; ANOVA = analysis of variance.

neurological deficit and prevented E2-induced restoration of BBB integrity in ICH mice. Our study thus first identifies a critical role of SRC in mediating the neuroprotective function of E2 in ICH mice.

Several previous studies, including a study by our group, have suggested a neuroprotective function of E2 in various brain injury models. We have previously shown that E2 attenuated ischemic brain injury by protecting BBB integrity and suppressing oxidative stress (Xiao et al., 2018). Another study showed that E2 alleviated neurological deficits and BBB disruption and prevented hyperglycemia-induced hematoma expansion in ICH mice (Zheng et al., 2015). In this current study, prior to dissecting the molecular mechanism, we confirmed that E2 alleviated ICH-induced brain injury. Consistent with previous studies, we showed that treatment of ICH mice with E2 resulted in better preserved BBB integrity and reduced neurological deficits and brain edema, corresponding to alleviated brain injury following ICH insult.

Here, we also found that E2 suppressed ICH-induced oxidative stress. Since oxidative stress is a critical mediator of the secondary injury following ICH, we believe E2 may suppress ICH-induced secondary injury to the brain.

Various studies have indicated that the PI3K/Akt signaling pathway is neuroprotective in different brain injury models. Activation of this pathway is important for the suppressive effects of Nle4-D-Phe7- α -melanocyte-stimulating hormone on neuronal apoptosis and oxidative stress in ICH mice (Fu et al., 2020). Similarly, this pathway is also implicated in the antiapoptotic and anti-inflammatory effects of various pharmacological drugs including statins and methylene blue (Chen et al., 2019; Yang et al., 2012). Importantly, PI3K/Akt pathway has been implicated in mediating estrogen signaling. For example, PI3K/Akt pathway is activated by estrogen in luminal epithelial cells (Kazi et al., 2009), and E2 has also been shown to promote the activation of this pathway in various cancer cells (Guo et al., 2006; Lee et al., 2005).

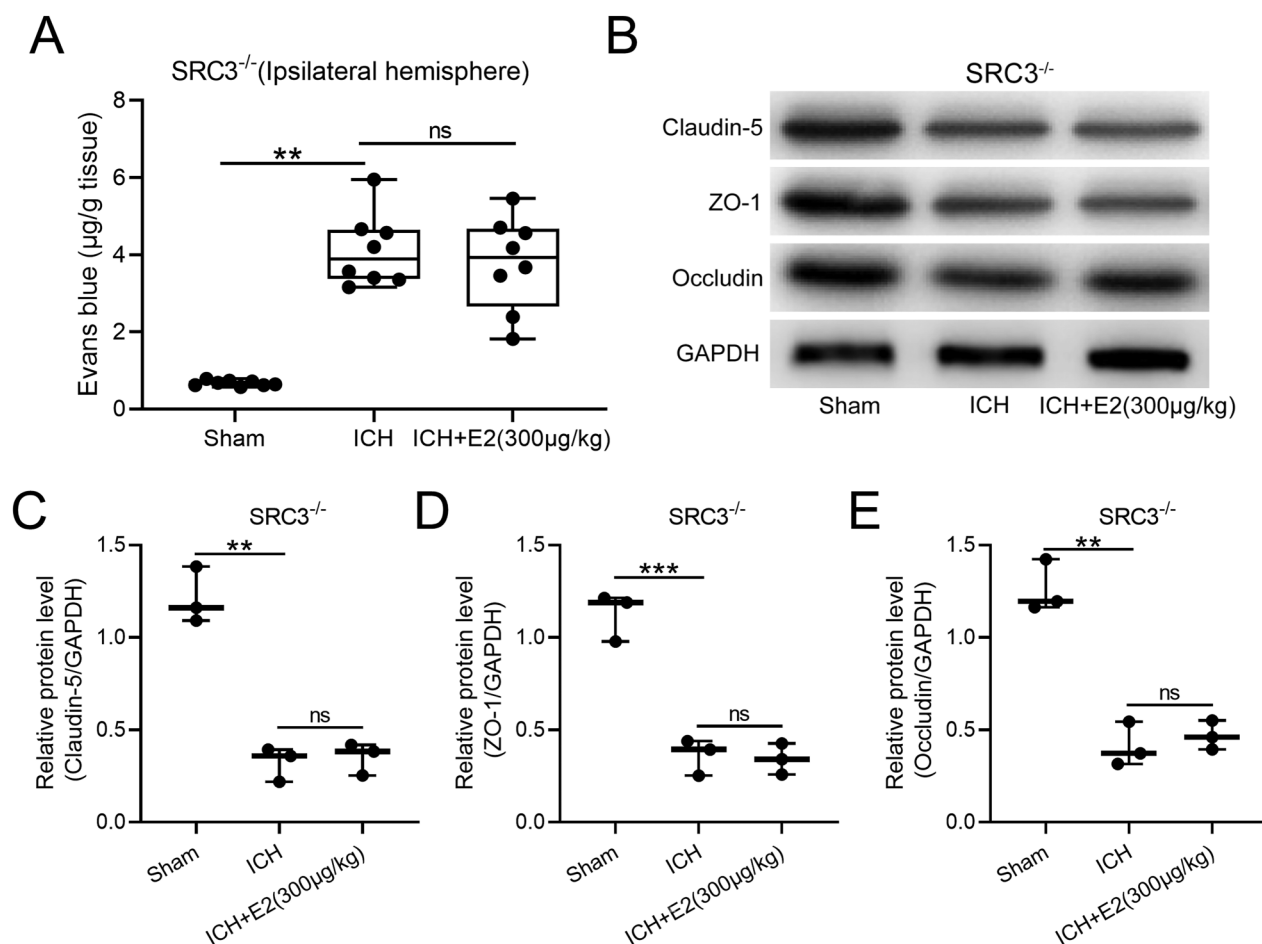


Figure 6. E2 has limited effects on ICH-induced BBB disruption in SRC3^{-/-} mice. BBB permeability was assessed by Evan's blue extravasation 3 days post-ICH in the ipsilateral injury hemisphere (A). Claudin-5, ZO-1, and occludin in the ipsilateral injury hemispheres 3 days postintracerebral hemorrhage were assessed by Western blot (B). Experiments were repeated in triplicate using tissues from eight mice. The relative expressions were normalized to GAPDH (C–E). One-way ANOVA followed Dunn's multiple comparisons test. ** $p < .01$, *** $p < .001$, ns indicates no significance.

Note. ICH = intracerebral hemorrhage; ANOVA = analysis of variance; BBB = blood–brain barrier.

Interestingly, a connection between the PI3K/Akt pathway and E2 has not been previously established in the neuroprotection against ICH-induced brain injury. In an effort to search for the intracellular signaling that mediates the neuroprotective effects of E2 in ICH mice, we speculated that E2 activated PI3K/Akt pathway in mouse brain with ICH. In fact, examination of this pathway by the phosphorylation status of PI3K and Akt showed that ICH mice treated with E2 had high levels of pPI3K and pAkt, indicative of an elevation of this pathway in the brain of these mice.

SRC3, an important coactivator of estrogen signaling, is upregulated in various cancers and is extensively expressed in the brain (Stashi et al., 2013). Loss of SRC3 results in slowed overall growth and gender-dependent behavioral abnormalities. While SRC3-deficient female mice showed various disruptions in behavior including reduced exploratory activity and increased anxiety, male mice lacking SRC3 behaved largely normal (Stashi et al., 2013). Importantly,

SRC3 has also been shown to regulate the PI3K/Akt pathway. SRC3 overexpression induced growth of cancer cells is suppressed by PI3K inhibitor (Xu et al., 2016). Downregulation of SRC3 correlates with Akt inhibition in preeclampsia pathogenesis (He et al., 2019). Interestingly, the role of SRC3 in E2-mediated neuroprotection has not been previously explored. We therefore investigated whether and how SRC3 is involved in E2-mediated neuroprotection in ICH mice. We hypothesized that SRC3 was required in mediating E2-induced neuroprotection. In our study, we first found that E2 enhanced both SRC3 expression and PI3K/Akt signaling in the brain of mice undergone ICH which were both suppressed by ICH in the absence of E2 treatment. To test our hypothesis, we explored the neuroprotective effects of E2 in mice deficient of SRC3. Importantly, our results showed that deletion of SRC3 completely blocked the effects of E2. While ICH procedure induced similar brain injury including neurological deficits, brain

edema, and disruption in BBB integrity, E2 treatment following ICH had no impact on these abnormalities in SRC3-deficient mice, indicating that SRC3 is important for mediating the neuroprotective effects of E2 against ICH-induced brain injury. BBB dysfunction is a hallmark of ICH-induced secondary brain injury and preservation of BBB integrity has been shown to suppress ICH-induced neurological deficits (Keep et al., 2008, 2014). Our findings that E2 restored BBB tight junction components and reduced BBB permeability indicate that E2-mediated BBB functional recovery may be involved in its role in improving neurological functions subsequent to ICH insult.

Although we did not provide any evidence on the involvement of estrogen receptors in E2-mediated neuroprotection, a previous study showed that administration of an estrogen receptor antagonist in female rats exacerbated ICH-induced brain injury, suggesting that estrogen receptor may be involved in E2-mediated neuroprotection in ovariectomized female mice (Nakamura et al., 2006). Additionally, we believe that neurotrophins such as BDNF may be involved in this regulation since previous studies have revealed a strong connection between estrogen, estrogen receptor, and neurotrophin expression in various neurological diseases (Kovesdi et al., 2020; Sohrabji & Lewis, 2006). Further study is needed to dissection the role of estrogen receptors and neurotrophin in E2-mediated neuroprotection.

In our study, we showed that ICH-induced microglia activation was suppressed by E2 treatment. This is in line with a previous study that E2 suppresses microglia activation and promotes anti-inflammatory M2 polarization of the microglia in the hippocampus of ovariectomized rats following ischemic brain injury (Thakkar et al., 2018). Although beyond the scope of our current study, in the future we will explore in more detail how E2 impacts different cell types in the brain to mediate its neuroprotective effects.

Conclusion

In summary, our study has revealed a neuroprotective role of E2 that alleviates brain edema and neurological deficits in mice undergone ICH. We show here that SRC3 may be involved in alleviating ICH-induced brain injury by E2. Our findings further suggest that administration of E2 subsequent to ICH insult may be a therapeutic strategy for patients suffering from ICH and worth further investigation for clinical application.

Author Contribution

HX, JL, JH, ZL, MD, and ZH contributed to the data curation and analysis; HX and ZH contributed to the drafting of the manuscript; and HX and ZH contributed to the concept and design of the study. All authors approved the publication of the manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.


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Ethical Approval

All mouse protocols were approved by the ethical committee of Xiangya Second Hospital, Central South University.

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Supplemental material

Supplemental material for this article is available online.

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