

# $\beta$ -estradiol alleviates Hypoxic-ischemic brain damage in neonatal rats through the GPER1 regulating AKT/NF- $\kappa$ B signal pathway

---

## Keywords

NF- $\kappa$ B, E2, AKT, OGD, HIBD, GPER1, G15

---

## Abstract

### Introduction

Research has established that estradiol (E2) offers neuroprotection against hypoxic-ischemic brain damage (HIBD) in neonatal rats, yet the underlying mechanisms are not fully understood. This study seeks to delineate whether E2's neuroprotective effects in neonatal HIBD are mediated through astrocytes by modulating the G Protein-Coupled Estrogen Receptor 1 (GPER1) receptor and the subsequent AKT Serine (AKT)/NF- $\kappa$ B signaling cascade.

### Material and methods

We developed an in vivo HIBD model in neonatal rats and established primary cultures of astrocytes subjected to oxygen-glucose deprivation-reoxygenation (OGD-R) as an in vitro model. E2 and the GPER1 inhibitor (G15) were administered according to the experimental design. Protein expression levels of GPER1, phosphorylated AKT (p-AKT), NF- $\kappa$ B p65, and cleaved-caspase3 were examined using Western blot analysis. Apoptosis was assessed via the TUNEL assay, and the presence of TNF- $\alpha$  and IL-1 $\beta$  in the cell supernatant was quantified by ELISA. The localization of p-AKT and NF- $\kappa$ B p65 was determined through immunofluorescence.

### Results

Our findings indicate that E2 treatment significantly reduced the volume of brain infarction and astrocyte apoptosis. E2 upregulated GPER1 and p-AKT expression while downregulating NF- $\kappa$ B p65 and cleaved-caspase3 levels in astrocytes and neonatal rats post-HIBD. Additionally, E2 diminished the secretion of TNF- $\alpha$  and IL-1 $\beta$  in the cell supernatant. The G15 inhibitor notably reversed the neuroprotective effects of E2 and the associated molecular changes.

### Conclusions

These results suggest that E2 may exert neuroprotection in neonatal rats with HIBD by inhibiting astrocyte apoptosis and modulating the expression of GPER1, p-AKT, and NF- $\kappa$ B, thereby providing a potential therapeutic strategy for HIBD.

# $\beta$ -estradiol alleviates Hypoxic-ischemic brain damage in neonatal rats through the GPER1 regulating AKT/NF- $\kappa$ B signal pathway

Guangyun Zhang<sup>1,2</sup>, Dawei Yuan<sup>2</sup>, Tiegang Lv<sup>2</sup>, Huafeng Li<sup>3</sup>, Yanli Zhang<sup>2</sup>, Yong Ding<sup>4</sup>, Juan Cui<sup>2</sup>, Min Li<sup>5</sup>, Jing Yu<sup>6</sup>, Jing Chen<sup>2\*</sup>, Xiangping Xu<sup>1\*</sup>

1. Department of Pediatrics, The First Affiliated Hospital of Harbin Medical University, Harbin, 150001, China
2. Department of Pediatrics, Linyi Maternal and Child Health Hospital, Linyi, 276000, China
3. Department of Genetic Laboratory, Linyi Maternal and Child Health Hospital, Linyi, 276000, China
4. Department of Key Laboratory of Birth Defects Prevention and Control, Linyi Maternal and Child Health Hospital, Linyi, 276000, China
5. Department of Neonatal, Linyi Maternal and Child Health Hospital, Linyi, 276000, China
6. Department of reproductive, Linyi Maternal and Child Health Hospital, Linyi, 276000, China

\*Correspondence: xxp20246161@163.com

## Abstract

**Introduction:** Research has established that estradiol (E2) offers neuroprotection against hypoxic-ischemic brain damage (HIBD) in neonatal rats, yet the underlying mechanisms are not fully understood. This study seeks to delineate whether E2's neuroprotective effects in neonatal HIBD are mediated through astrocytes by modulating the G Protein-Coupled Estrogen Receptor 1 (GPER1) receptor and the subsequent AKT Serine (AKT)/NF- $\kappa$ B signaling cascade.

**Methods:** We developed an in vivo HIBD model in neonatal rats and established primary cultures of astrocytes subjected to oxygen-glucose deprivation-reoxygenation (OGD-R) as an in vitro model. E2 and the GPER1 inhibitor (G15) were administered according to the experimental design. Protein expression levels of GPER1, phosphorylated AKT (p-AKT), NF- $\kappa$ B p65, and cleaved-caspase3 were examined using Western blot analysis. Apoptosis was assessed via the TUNEL assay, and the presence of TNF- $\alpha$  and IL-1 $\beta$  in the cell supernatant was quantified by ELISA. The localization of p-AKT and NF- $\kappa$ B p65 was determined through immunofluorescence.

**Results:** Our findings indicate that E2 treatment significantly reduced the volume of brain infarction and astrocyte apoptosis. E2 upregulated GPER1 and p-AKT expression while downregulating NF- $\kappa$ B p65 and cleaved-caspase3 levels in astrocytes and neonatal rats post-HIBD. Additionally, E2 diminished the secretion of TNF- $\alpha$  and IL-1 $\beta$  in the cell supernatant. The G15 inhibitor notably reversed the neuroprotective effects of E2 and the associated molecular changes.

**Conclusion:** These results suggest that E2 may exert neuroprotection in neonatal rats with HIBD by inhibiting astrocyte apoptosis and modulating the expression of GPER1, p-AKT, and NF- $\kappa$ B, thereby providing a potential therapeutic strategy for HIBD.

**Keywords:** HIBD; OGD; E2; GPER1; G15; AKT; NF-  $\kappa$  B

## Introduction

Neonatal hypoxic-ischemic brain damage (HIBD) results from inadequate oxygen and blood supply around the time of birth and can lead to severe morbidity and mortality [1]. Up to 25-60% of survivors still have death or severe disabilities, such as mental retardation, cerebral palsy, epilepsy or learning disabilities [2]. While mild hypothermia is currently the standard treatment for perinatal HIBD, its efficacy varies, and many infants do not respond adequately, underscoring the need for alternative treatments [3,4]. However the clinical trials show that full-term infants affected by mild hypoxic-ischemic encephalopathy show the best response to hypothermia therapy, while newborns affected by moderate to severe encephalopathy do not seem to show high efficiency or no response at all, about 55% of newborns receiving mild hypothermia therapy are still not effectively protected [5]. Therefore, it is an urgent problem to develop more effective methods to treat HIBD.

A wealth of evidence from in vivo and in vitro studies has highlighted the neuroprotective properties of estradiol (E2), which include anti-apoptotic, anti-inflammatory, and anti-oxidative effects. Astrocytes, the most abundant glial cells in the CNS, provide crucial support to neurons and represent a potential target for therapeutic intervention following cerebral ischemia [6]. As early as 15 years ago, researchers proved that 17 $\beta$  estradiol can significantly reduce hypoxic-ischemic brain damage and reduce infarct area in neonatal rats [7], but its specific mechanism is still unclear.

Astrocytes are the most abundant cell types in the central nervous system [8]. Under normal physiological conditions and after ischemia, astrocytes support neurons by providing antioxidant protection, eliminating metabolic substrates and glutamic acid of neurons, and providing energy for neurons through lactic acid shuttle [9]. So, astrocytes play an important role in nutritional support for neurons [10,11]. Although astrocytes are sometimes more elastic than neurons under hypoxic-ischemic conditions, even if astrocytes are not dead, anoxia and ischemia will lead to the impairment of astrocyte function, which will amplify the death of neurons [10,12]. It is found that improving the function of astrocytes after ischemic brain damage can improve the survival rate of neurons [10,13]. Exploring the improvement of astrocyte function after cerebral ischemia and enhancing support for neurons may become a new target for treating ischemic brain damage.

GPER1, a specific estrogen receptor, plays a critical role in rapid estrogen signaling and has been implicated in the neuroprotective effects of E2<sup>[14]</sup>. A lot of studies have explored its role in the neuroprotective mechanism of hormones. For example, the agonist G1 of GPER1 upregulates the expression of GPER in hippocampus of aged female rats, and counteracts the effects of aging on animal anxiety and depression<sup>[15]</sup>; GPER1 also involved in the neuroprotective effect of E2 in local and global ischemic models, which is manifested by reducing the infarct volume and the release of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in ischemic penumbra<sup>[16]</sup>; In addition, GPER mediates the up-regulation of glutamate transporter GLT-1 and the uptake of glutamate by astrocytes<sup>[17]</sup>, which may reduce excitotoxic nerve damage.

As one of the downstream signaling pathways of GPER1,

The PI3K/AKT pathway, a key downstream signaling route of GPER1, is known to promote cell survival and inhibit apoptosis, with NF- $\kappa$ B being a significant player in the inflammatory response and cell death following ischemic injury<sup>[18]</sup>. When cells are exposed to oxidative stress, PI3K/AKT signaling pathway is activated, which will subsequently change the downstream signal cascade and activate downstream signal factor NF- $\kappa$ B<sup>[19]</sup>. After being activated, NF- $\kappa$ B participates in regulating inflammatory reaction, apoptosis and ischemia-reperfusion damage<sup>[20]</sup>, many inflammatory factors also can activate NF- $\kappa$ B in inflammatory reaction, such as TNF- $\alpha$ , IL-1 and IL-6. It has been observed in many studies that E2 can play a neuroprotective role by activating PI3K/Akt pathway. For example, it is found that E2 promotes the development of midbrain neurons through PI3K/Akt pathway<sup>[21]</sup>; It has also been reported that E2 can activate PI3K/Akt pathway in adult rat global cerebral ischemia model, reduce apoptosis of hippocampal neurons and play a role in brain protection<sup>[22]</sup>. Yang et al found that the activation of GPER1 can protect neurons from excitotoxicity through NF- $\kappa$ B signaling<sup>[23]</sup>.

E2, GPER1 and astrocytes play an important role in ischemic brain damage and other central nervous system injuries. However, the relationship among astrocytes, GPER1 and AKT/NF- $\kappa$ B pathway during the neuroprotective effect of E2 on neonatal rat after HIBD is unclear. Understanding the interplay among E2, GPER1, astrocytes, and the AKT/NF- $\kappa$ B pathway may uncover new avenues for enhancing astrocyte function post-ischemia and present novel therapeutic approaches for HIBD.

## **2. Materials and methods**

### **2.1 Animal experiments**

2.1.1 The protocol for animal experimentation received approval from the Experimental Animal Committee of Linyi Maternal and Child Health Hospital (approval number: [2022]006). Sprague-Dawley (SD) neonatal rats were procured from Beijing Huaifu Biotechnology Co., Ltd. (license number: SCXK (Beijing) 2019-0008) and were housed at the Experimental Animal Center of Linyi Maternal and

Child Health Hospital (license number: SYXK [Lu] 2021-0007). The animals were reared under a 12-hour light-dark cycle, at a controlled ambient temperature of 25±2°C and a relative humidity of 60%-80%. Reverse osmosis (RO) purified water was provided ad libitum.

#### 2.1.2 experimental grouping and drug intervention

Neonatal SD rats were randomly assigned to one of eight groups: Sham+vehicle, Sham+E2, Sham+G15, Sham+E2+G15, HIBD+vehicle, HIBD+E2, HIBD+G15, and HIBD+E2+G15. On the second day postpartum, G15 was administered intraperitoneally at a dose of 1.46 mg/kg (stored at -80°C, with a DMSO concentration under 10%, and diluted with PEG300+TW80+0.9%NS, with the final injection volume being less than 0.1ml). E2 was injected intraperitoneally from the fifth day postpartum until 24 hours post-surgery at a dose of 0.1mg/kg (E2 dissolved in DMSO and stored at -80°C). Vehicle groups received a mixture of 10% DMSO+40% PEG300+5% TW80+45% NS as a control.

#### 2.1.3 HIBD model construction

Corresponding to human full-term neonatal brain development, 7-day-old neonatal SD rats were utilized. The HIBD model was generated using the modified Rice-Vannucci method<sup>[24,25]</sup> This entailed the double ligation of the left common carotid artery (CCA). The sham-operated group had the CCA exposed without ligation or hypoxic treatment. Following anesthesia, the rat's neck was disinfected, and a midline incision allowed for CCA ligation. Post-operative rats were returned to their dams for a 1-hour recovery before being subjected to a hypoxic environment (8% O<sub>2</sub> and 92% N<sub>2</sub>, flow rate of 1.5 L/min, at 37°C and over 75% humidity) for 4 hours. Subsequently, rats were returned to their dams for continued care.

#### 2.1.4 Neurobehavioral assessment

On the third day post-hypoxia, the neurobehavioral function of the neonatal rats was assessed in a tranquil environment employing established methods, including the righting reflex, negative geotaxis, and grip strength tests. Each test was performed thrice, with the mean score being recorded.

#### 2.1.5 TTC staining.

Brain infarct volume was evaluated using 2,3,5-triphenyltetrazolium chloride (TTC) staining 72 hours post-HIBD. Anesthetized rats were decapitated, and the brains were rapidly frozen at -80°C for 3 minutes, then coronally sectioned and incubated in 1% TTC at 37°C for 15 minutes in the dark. Viable brain tissue appeared red, whereas infarcted areas remained pale. Infarct volume was calculated using the formula: [(volume of the contralateral hemisphere - volume of non-infarcted ipsilateral hemisphere)/volume of the contralateral hemisphere] × 100%<sup>[26]</sup>.

#### 2.1.6 tissue protein extraction and Western Blot detection.

Brain tissues were collected 72 hours post-HIBD under deep anesthesia, homogenized in RIPA buffer, and centrifuged to obtain the supernatant. Protein concentration was determined using the BCA assay. For Western blot analysis, proteins were separated by SDS-PAGE, transferred onto PVDF membranes, and probed with primary antibodies against GPER1 (1: 1000, GeneTex, USA, cat# GTX107748), Phospho-Akt(1:1000, CST, USA, cat#4060S), NF-kappaBp65 (1:1000, CST, USA, cat#8242S), cleaved-caspase3 (1:1000, Affinity, China, cat#AF7022), caspase-3 (1:1000, Abcam, UK, cat #13847), goat anti-rabbit IgG antibody (1: 8000, genetex, USA, cat# gtx213110-01),  $\beta$ -actin(1:4000, Beyotime, China, cat# AF5003), Histon H3 (1: 2000, Proteintech, USA, cat#17168) followed by appropriate secondary antibodies. Chemiluminescent detection was used for visualization (Beyotime, china, cat# P0027).

#### 2.1.7 Preparation of tissue sections.

Brain tissues were perfused and fixed with 4% paraformaldehyde post-HIBD, embedded, and sectioned. Sections were processed for histological analysis.

#### 2.1.8 TUNEL staining of tissues

TUNEL cell apoptosis detection kit (chromogenic method) (Beyotime, China, cat# C1098) was used to detect apoptotic cells in ischemic penumbra area. The brain tissue sections were put into a drying oven (70°C) to melt wax for 30 minutes in advance. After dewaxing and hydration, follow-up operations were according to the instructions of the kit. Finally, after DAB staining, the cell nucleus was stained with hematoxylin, the sections were sequentially dehydrated with alcohol gradient and transparent with xylene before sealing and observation.

### 2.2 Cell experiment

#### 2.2.1 Culture, purification and identification of astrocytes

Astrocytes were isolated and purified according to previous research<sup>[27]</sup>. When the cells were transferred to the third generation, they were inoculated on a 24-well culture plate for GFAP (Biyuntian, China, Cat#AG259) immunofluorescence staining to identify the purity of the cells.

2.2.2 Experimental grouping and drug intervention: Before drug intervention, the cells were divided into the following groups: Sham+vehicle, Sham+E2, Sham+G15, Sham+E2+G15, OGD+vehicle, OGD+E2, OGD+G15, OGD+E2+G15. According to the grouping, G15 intervention 48 hours in advance until OGD ends. E2 intervention 24 hours in advance until the end of OGD, the concentration of E2 working solution was 10nM (dissolved in DMSO and stored in a refrigerator at -80°C, the concentration of DMSO working solution was less than 0.1%). Sham+vehicle and HIBD+vehicle groups used DMSO with the same concentration as E2 as negative control.

#### 2.2.3 Oxygen glucose deprivation/reperfusion (OGD/R)

After drug intervention, astrocytes were exposed to an anoxic and glucose-deficient environment to simulate anoxic and ischemic conditions. In short, the basal medium was replaced by glucose-free medium, then the cells were put in three-gas culture for 12 hours (1% oxygen +99% nitrogen). After that, they were replaced by normal complete medium and kept in normal culture conditions for 24 hours.

2.2.4 CCK-8 was used to detect cell viability, the relative expression of TNF- $\alpha$  and IL-1 $\beta$  in cell supernatant was detected by Elisa

Cells were inoculated into 96-well plates, with about  $10^4$  cells in each well. CCK-8 (Biyuntian, China, cat#C0041) was used to detect cell viability 24 hours after OGD-R, TNF- $\alpha$ (Elabscience, China, cat#E-EL-R2856c) and IL-1 $\beta$ (Elabscience, China, cat#E-EL-R0012c) in cell supernatant of each group were detected according to the operating instructions of ELISA kit.

2.2.5 TUNEL for detecting apoptotic cells

Cells were inoculated into a 24-well plate containing cell slides. After 24 hours of cell culture, drug intervention based on cell grouping. After 24 hours of OGD-R, the apoptosis of cells in each group was detected according to the instructions of TUNEL kit (crondabio, China, Cat#KCD-T1006). In short, 4% paraformaldehyde fixed cell slides for 30min, 1% Triton X-100 membrane rupture for 5 minutes, then DNase I reaction solution, TdT enzyme reaction solution and Streptavidin-TRITC labeling solution were added dropwise, and finally DAPI staining solution was used to stain the nucleus and seal the film for observation.

2.2.6 The expression of p-AKT and NF- $\kappa$ Bp65 was detected by immunofluorescence. Cells were inoculated into a 24-well plate containing cell slides, 24 hours after OGD-R, fixed with 4% paraformaldehyde at 4 $^{\circ}$ C for 30 min, exposed with 0.1% Triton X-100 at room temperature for 20min, blocked with 5%BSA for 2 hours, incubated with primary antibody (1: 400, CST, USA, CAT # 4060s) at 4 $^{\circ}$ C overnight, Fluorescent secondary antibody (1:200 bioshap China cat # BLO33A) was incubated at room temperature in dark for 1 hour, stained with DAPI for 10 minutes, finally observed and photographed.

2.3 Statistical analysis. All experiments were repeated three times or more, and the data were expressed as mean standard deviation. The experimental data were statistically analyzed by GraphPad Prism software (version 9.5, USA), the statistical significance was evaluated by one-way ANOVA. If the P value was less than 0.05, the difference was considered significant.

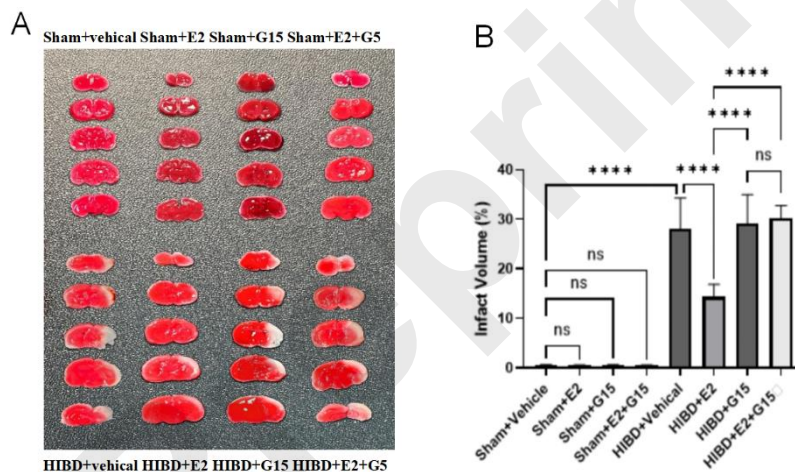
### 3 Results

#### **Estradiol (E2) Intervention Significantly Mitigates Cerebral Infarction Volume and behavioral impairments**

E2 administration markedly reduced the cerebral infarction volume in neonatal rats with HIBD, as indicated by TTC staining. Figure 1 illustrates that the infarction volume percentage in the HIBD+E2 group is significantly less than that in the HIBD+vehicle group. Furthermore, neurological function assessments using the righting reflex, negative geotaxis, and grip strength tests demonstrated that E2 ameliorates neurological impairments in HIBD-affected neonatal rats on the third day post-surgery (Figure 2). GPER1 Inhibitor G15 Reverses the Neuroprotective Effects of E2 Neurobehavioral tests, including the righting reflex, negative geotaxis, and grip strength, indicated that G15 treatment negated E2's neuroprotective benefits (Figure 2A-C).

### E2 increased the GPER1 expression in HIBD models

HIBD induces the decrease of GPER1 expression, when E2 increased its expression. And this effect was partly blocked by G15 (Figure 3).



**Figure 1 Effect of E2 and G15 intervention on cerebral infarction volume of neonatal rats with HIBD** (A) TTC staining was used to detect the infarct area of brain tissue in each group; (B) Quantitative analysis of infarct volume of brain tissue; n=4, \*\*\*\*p<0.0001



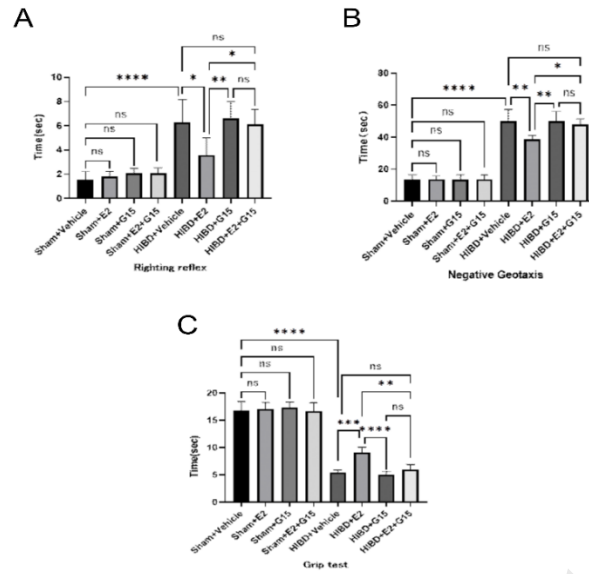


Figure 2 Effect of E2 and G15 intervention on neurological function on the 3rd day after HIBD (A) Righting reflex; (B) Negative geotaxis; (C) Grip test; n=5; \*\*p<0.01, \*\*\*\*p<0.0001

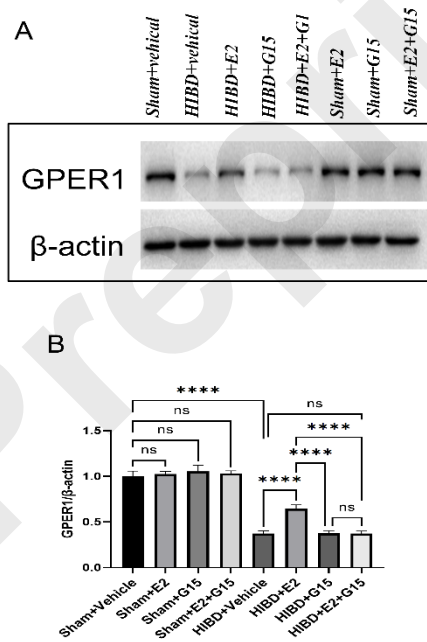


Figure 3  
WB detect GPER1 expression in ischemic penumbra of rodent infarcted brain tissue. n=3, \*\*\*\*p<0.0001

### E2 Promotes p-AKT Expression and Reduces NF-κBp65 in Ischemic Penumbra

To further investigate whether the GPER1-mediated downstream signaling molecule AKT/NF- $\kappa$ Bp65 is involved in E2's neuroprotective effect on HIBD-affected neonatal rats, we conducted Western blot analysis on ischemic penumbra brain tissue from each group (Figures 4 and 5). The findings revealed that the expression of p-AKT was significantly lower in the HIBD+vehicle group compared to the Sham+vehicle group, mirroring the expression trend of GPER1, while NF- $\kappa$ Bp65 was significantly increased. Relative to the HIBD+vehicle group, the HIBD+E2 group exhibited a notable increase in p-AKT expression and a marked decrease in NF- $\kappa$ Bp65 levels. The HIBD+G15 group and the HIBD+E2+G15 group had significantly lower p-AKT expression and upregulated NF- $\kappa$ Bp65 compared to the HIBD+E2 group. No significant differences were observed between the HIBD+vehicle group and the HIBD+E2+G15 group or between the HIBD+G15 group and the HIBD+E2+G15 group (Figures 4 and 5).

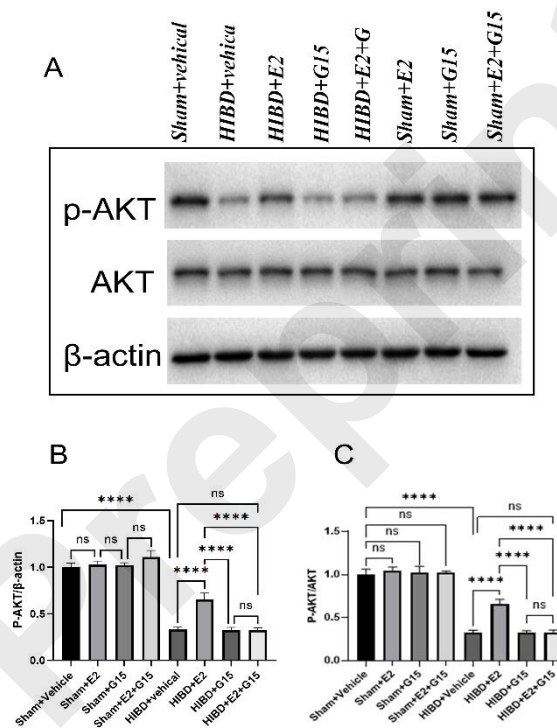


Figure 4 WB detect P-AKT expression in ischemic penumbra of infarcted brain tissue. (A) The representative image of WB; (B) The quantification of WB for p-AKT/  $\beta$  - actin; (C) The quantification of WB for p-AKT/AKT; n=3, \*\*\*\*p<0.0001

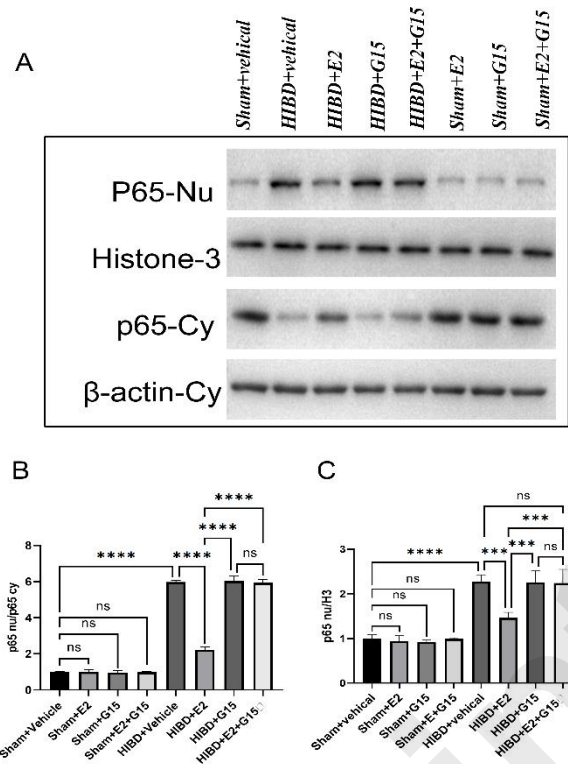


Figure 5 WB detect NF-κBp65 expression in ischemic penumbra of infarcted brain tissue (A) The representative image of WB; (B) The quantification of WB for p65 nu/p65 cy; (C) The quantification of WB for p65 nu/H3; n=3, \*\*\*p<0.0005, \*\*\*\*p<0.0001.

### TUNEL staining shows the apoptosis in HIBD rodents

Animal studies utilizing TUNEL staining disclosed that the HIBD+E2 group exhibited significantly fewer apoptotic cells within the ischemic penumbra than the HIBD+vehicle group, an effect that was negated following G15 treatment. Apoptotic cell counts in the HIBD+G15 group and the HIBD+E2+G15 group were notably higher than in the HIBD+E2 group (Figure 6). Western blot analysis of cleaved-caspase3 paralleled the TUNEL staining results (Figure 7).

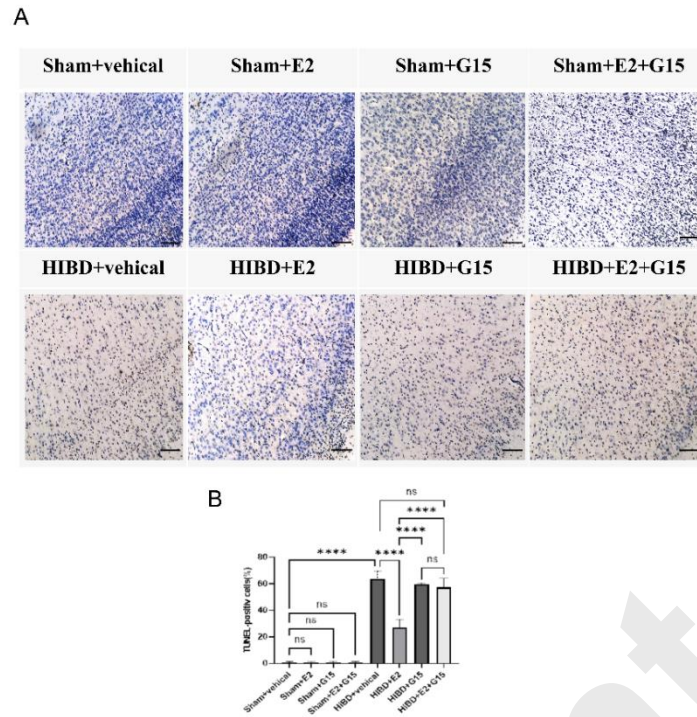


Figure 6 Effect of E2 and G15 intervention on apoptosis of ischemic penumbra in neonatal rats with HIBD (A)TUNEL was used to detect the apoptosis of ischemic penumbra, bar=100um; (B) data quantification analysis, n = 3; \*\*\*\*p<0.0001

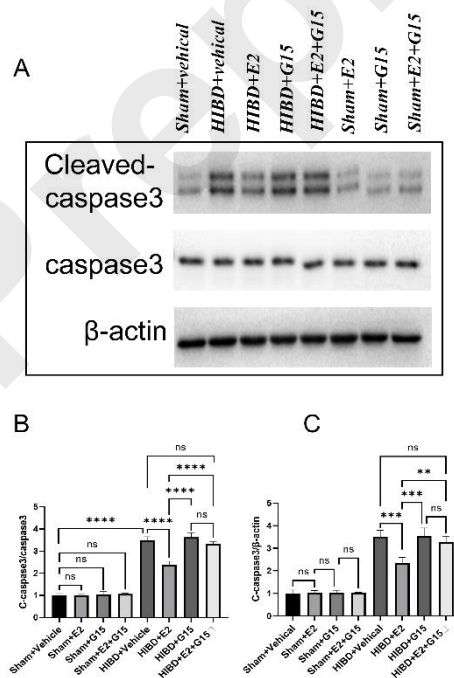


Figure 7 WB detect Cleaved-caspase3 expression in ischemic penumbra of infarcted brain tissue, (A) Representative images; (B&C) quantification results: n=3, \*\*\*p<0.0005, \*\*\*\*p<0.0001

### Relative cell viability and inflammatory factor expression in OGD astrocytes

First, we confirmed the expression of GFAP in in-vitro OGD astrocytes (Figure 8). E2 can increase the reduced cell viability in OGD-R astrocytes, while this effect was partly blocked by G15 (Figure 9). E2 can also reduce the increased TNF- $\alpha$  and IL-1 $\beta$ ; while, G15 can block these inflammatory markers in OGD astrocytes (Figure 10).

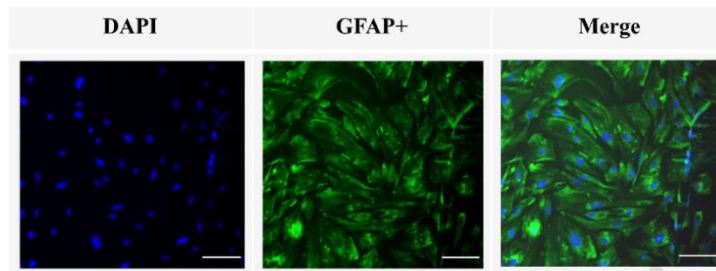


Figure 8 GFAP identification of primary cultured astrocytes n=5, the purity of astrocytes is 97-100%, bar=100um(200 $\times$ ).

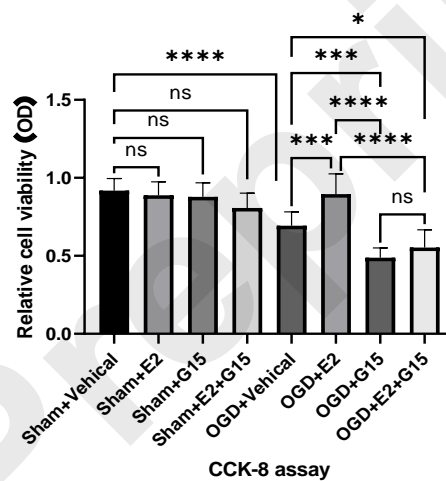


Figure 9. Effects of E2 and G15 intervention on the viability of OGD-R astrocytes. n $\geq$ 5, \*p<0.05, \*\*\*p<0.0005, \*\*\*\*p<0.0001

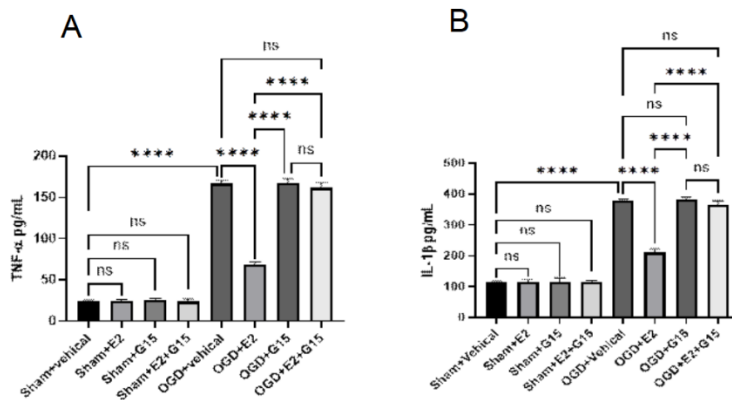
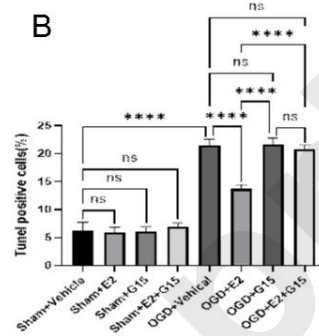
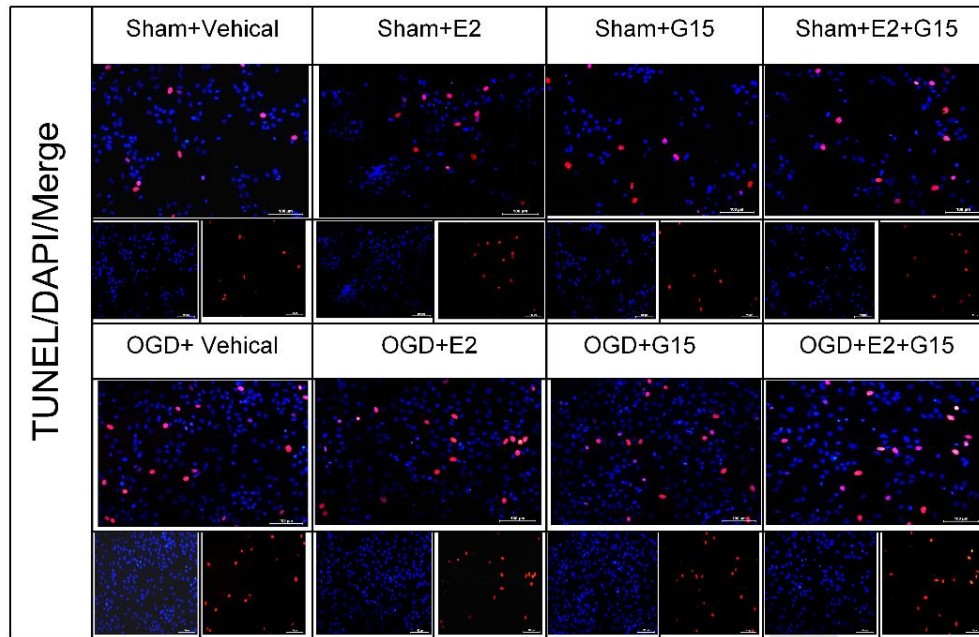


Figure 10 ELISA detect expression of TNF- $\alpha$  and IL-1 $\beta$  in cell supernatant (A) TNF- $\alpha$  ; (B) IL-1 $\beta$  n=5; \*\*\*\*p<0.0001

Cell experiments mirrored these outcomes, as consistent in the TUNEL staining (Figures 6). E2 can reduced the increased TUNEL positive cells percentage in OGD astrocyte while G15 can block these effects (Figures 11). Cell experiments mirrored these outcomes, as seen in cleaved-caspase3 expression (Figures 12). E2 can reduced the cleaved-caspase3 level in OGD-R astrocyte while G15 can block these effects.



**Figure.11 Effect of E2 and G15 intervention on apoptosis of OGD-R astrocytes**  
 (A) TUNEL for evaluating cell apoptosis (200×), bar = 100um; (B) Data analysis; n=5, \*\*\*p<0.0001

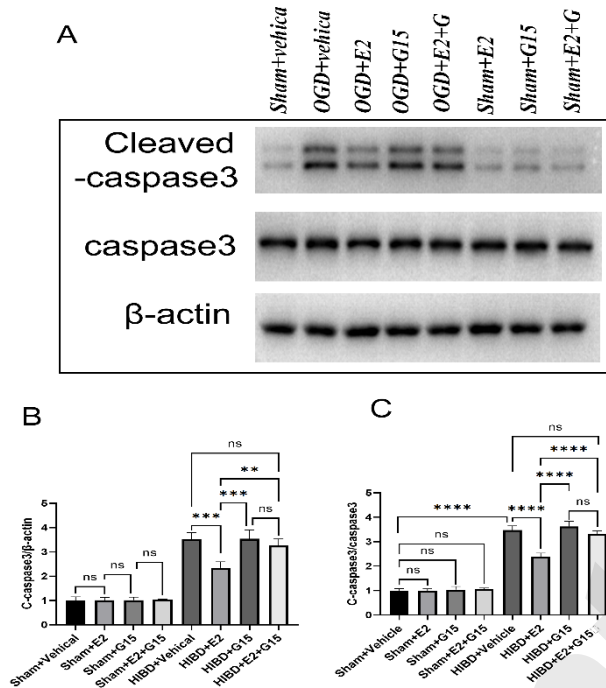


Figure.12 WB detect expression of Cleaved-caspase3 in astrocytes after OGD-R (A) The representative WB images (B&C) The quantification analysis for WB: n=3, \*\*\*\*P<0.0001

In vitro experiments showed that the immunofluorescence and WB result for p-AKT and NF-κBp65 were consistent with the in vivo findings (Figures 13-16), suggesting that GPER1 expression decreased on the third day post-surgery in the HIBD group, accompanied by a decrease in p-AKT expression and an increase in NF-κBp65 expression. E2 intervention promoted GPER1 expression (Figure 17) and was accompanied by an increase in p-AKT expression and a decrease in NF-κBp65 expression, which was reversed following G15 treatment.

HIBD led to a reduction in GPER1 protein expression in both the ischemic penumbra of neonatal rats and in astrocytes post-OGD-R (Figure 17). E2 treatment significantly upregulated GPER1 expression, while G15 effectively inhibited this E2-induced elevation. Astrocyte GPER1 expression post-OGD-R reflected these animal experiment results (Figure 17), suggesting that E2 treatment boosts GPER1 levels, which are suppressed by G15. Hence, astrocytes and GPER1 may play a pivotal role in E2's neuroprotective effect in HIBD-affected neonatal rats.



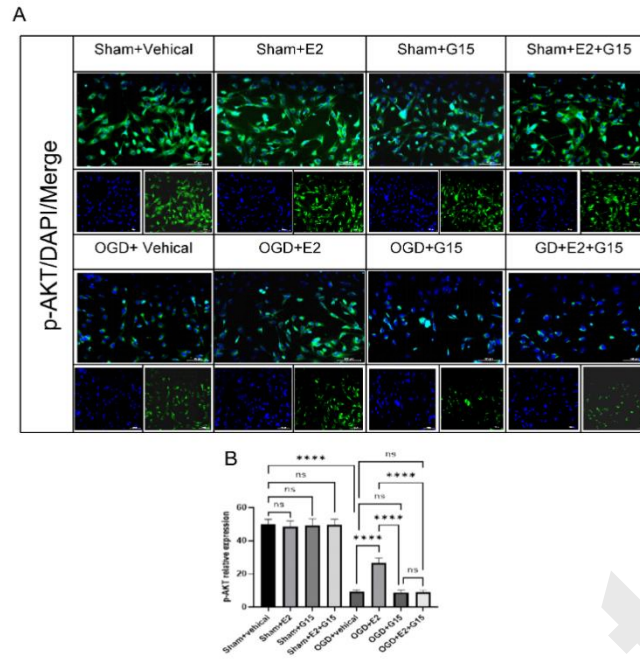


Figure.13 Effect of E2 and G15 intervention on p-AKT expression in OGD-R astrocytes (A) Immunofluorescence detection of expression of p-AKT (200 $\times$ ), bar = 100um; (B) Data analysis; n=6, \*\*\*p<0.0001

A

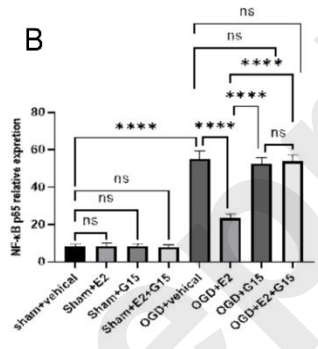
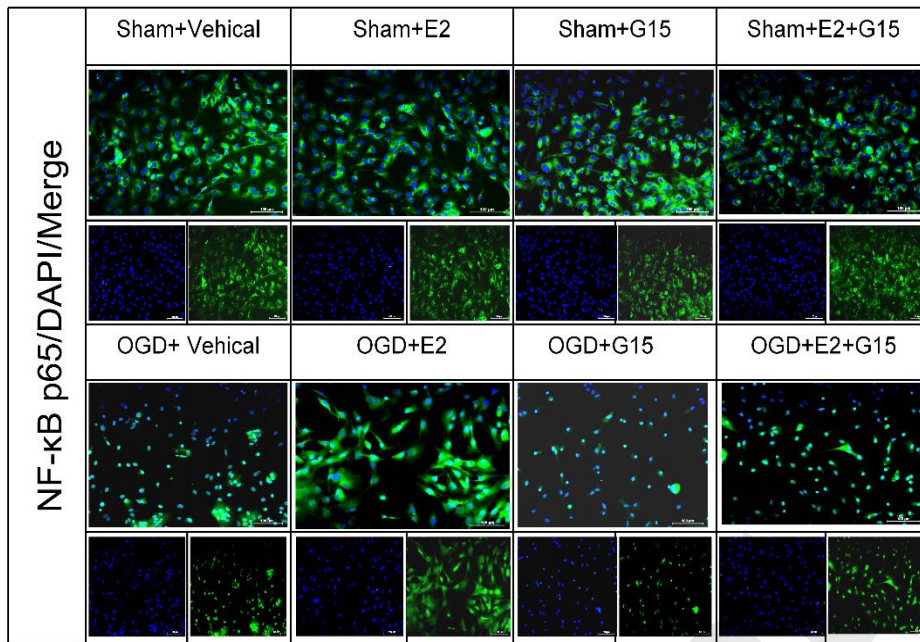


Figure. 14. Effect of E2 and G15 intervention on NF-κB p65 expression in OGD-R astrocytes (A) Immunofluorescence detect expression of NF-κBp65 (200×), bar = 100um; (B) Data analysis, n=6, \*\*\*p<0.0001

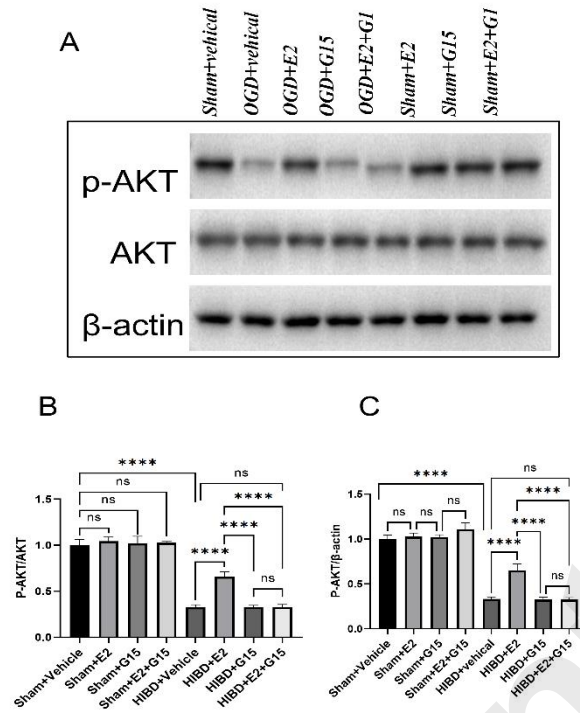


Figure 15 WB detect expression of p-AKT in astrocytes after OGD-R (A) The representative WB images (B&C) The quantification analysis for WB, n=3, \*\*\*\*P<0.0001

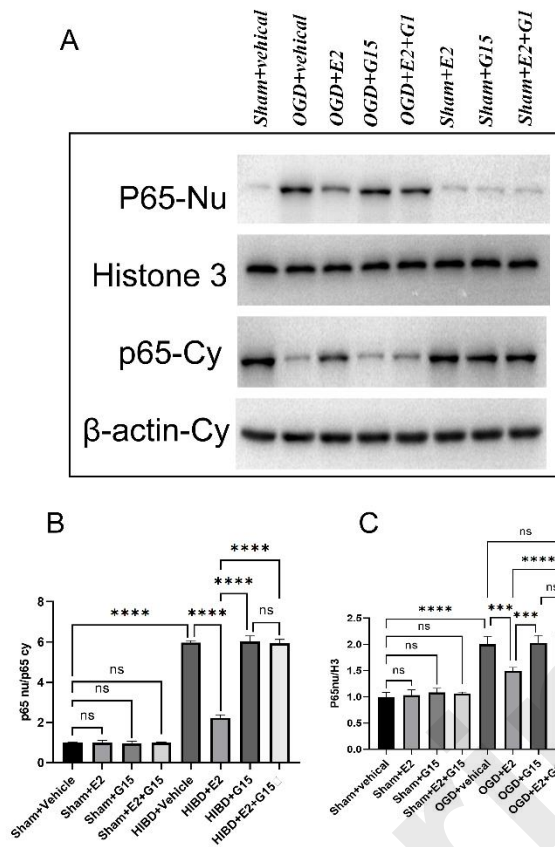


Figure 16 WB detect expression of NF- $\kappa$ Bp65 in astrocytes after OGD-R  
 (A) The representative WB images (B&C) The quantification analysis for WB, n=3,  
 \*\*\*\*P<0.0001

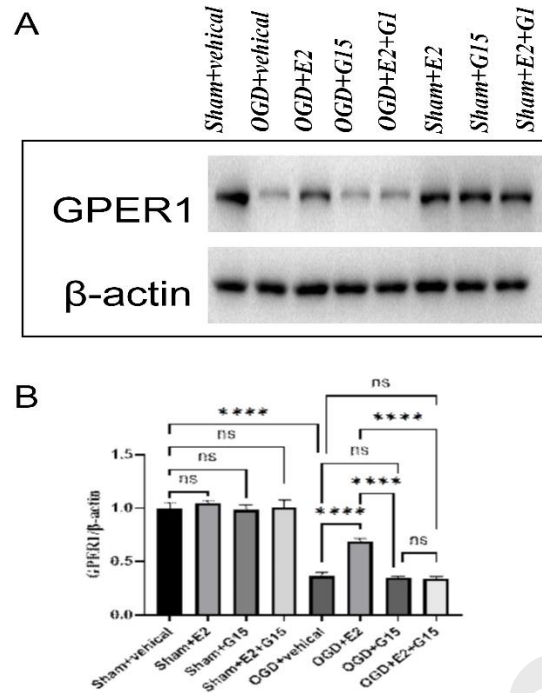


Figure 17 WB detect expression of GPER1 in astrocytes after OGD-R (A) The representative images for WB; (B) The quantification analysis result for WB: n=3, \*\*\*P<0.0001

#### 4 Discussions

Hypoxic-ischemic encephalopathy (HIE) is a leading cause of neonatal death and long-term neurological disabilities such as movement disorders and cognitive impairments. Current treatments like mild hypothermia are inadequate, often resulting in permanent neurological damage. There is a pressing need for new therapeutic approaches.

Astrocytes, the most numerous glial cells in the brain, were once thought to exacerbate nervous system diseases when activated [28]. However, recent research highlights their protective roles, such as glutamate clearance, blood-brain barrier repair, and tissue preservation. Enhancing the resilience of glial cells, therefore, represents a promising direction for neuroprotection strategies [29]; After focal ischemia, GFAP knockout mice showed more significant brain damage than their wild-type mice [30]; Treatment with astrocyte conditioned medium after MCAO can reduce the infarct volume and promote the recovery of blood-brain barrier function [31]. Therefore, alleviating the hypoxic-ischemic damage of glial cells may become an important group of neuroprotective strategies.

Previous studies have shown that estradiol has a significant brain protective effect HIBD [32-33], but its mechanism is still unclear. This study investigated E2's therapeutic potential in neonatal rats with HIBD and in astrocytes post-oxygen-glucose deprivation/reperfusion (OGD-R). In order to explore the possible mechanism of protective effect of E2 on neonatal rats with HIBD, firstly, we validated the neuroprotective effect of E2 in neonatal with rats HIBD and in astrocytes after OGD-R. So far, four estrogens have been found in human body: estrone (E1), 17 $\beta$ - estradiol (E2), estriol (E3) and estriol (E4) [34]. "Estrogen" usually refers to E2, because it is widely distributed in many tissues and organ systems and has active physiological functions [35]. Various clinical studies suggest that E2 can prevent or delay the onset of nerve regeneration diseases, such as stroke, seizure, hypoxia and glucose deficiency and oxidative stress, alleviate the damage of central nervous system [6]. Joseph Nuñez and others found that repeated administration of E2 for three times provided about 70% protection to hippocampus, basal ganglia and amygdala by constructing the same hypoxic-ischemic model. They believed that, E2 as an effective neuroprotective agent, could resist the damage caused by hypoxia-ischemia to the developing brain, the pretreatment of infants at risk of hypoxic-ischemic damage might have clinical prospects [32]; It has also been reported that E2 can reverse the decrease of neuroprotective astrocyte reactivity in the mouse model of global cerebral ischemia and play a role in brain protection [36]. In vitro studies have also found that E2 replacement therapy can alleviate the cell damage caused by oxygen-glucose deprivation and reperfusion in astrocytes [37]; Previous studies have found that E2 plays a powerful neuroprotective role in the ischemic penumbra [38]. Consistent with the above studies, continuous E2 intervention can significantly reduce the expression level of Cleaved caspase3, inhibit apoptosis of cells in the ischemic penumbra and astrocytes after OGD-R, thereby reducing the infarct area of brain tissue and improving motor sensory nerve function, achieving neuroprotective effects. However, G15 intervention reversed this protective effect.

We further explored the role of GPER1, an estrogen receptor, in E2's neuroprotective action. GPER1 is expressed in various brain regions, including the cortex and hippocampus. It mediates estrogen's protective effects in several contexts, such as reducing infarct volume and inflammatory cytokine release in ischemic models [39]; The neuroprotective effect of E2 was also found in primary hippocampal neurons and rat motor neurons exposed to oxygen glucose deprivation [40]; In addition, GPER mediates glutamate to up-regulate the expression of transporter GLT-1 and the uptake of glutamate by astrocytes, which is involved in reducing excitotoxic nerve damage [41]. In vivo, GPER1 reduced the infarct volume and the release of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in ischemic penumbra, participated in the neuroprotective effect of E2 in local and global ischemic models [10]. In this study, we observed that E2 treatment upregulates GPER1 expression, which correlates with reduced infarct size, apoptosis, and improved motor function. Conversely, G15 treatment reduces GPER1 expression and the protective effects of E2, suggesting that GPER1 mediates E2's neuroprotection in neonatal HIBD.

We also investigated whether GPER1 mediates E2's neuroprotective effects in astrocytes by primary culture and OGD-R model construction. Research has shown that astrocytes play a crucial role in synaptic transmission and plasticity, and GPER1 activation in astrocytes can mitigate neuronal damage in ischemic conditions. Our results suggest that E2 treatment post-OGD-R significantly increases GPER1 expression in astrocytes, reducing apoptosis and inflammatory responses. However, G15 treatment inhibits these protective effects, indicating that GPER1 may mediate E2's neuroprotection.

We then explored the potential signaling mechanisms behind GPER1's mediation of E2's protective effects. GPER1, similar to other G protein-coupled receptors, is responsible for rapid estrogen signal transduction and is independent of traditional estrogen receptors ER $\alpha$  and ER $\beta$ . The PI3K/AKT pathway, a downstream signaling pathway of GPER1, is known to influence cell survival and apoptosis. In ischemic diseases, the activation of GPER1 in astrocytes can restore the basic level of autophagy, reduce the release of inflammatory factors and prevent neuronal damage<sup>[42]</sup>. In cerebellar development, S-equol in astrocytes may affect neurons and astrocytes through various signal pathways including GPR30, this protective effect can be inhibited by G15<sup>[43]</sup>.

In the following study, we studied the possible signal mechanism that astrocytes and GPER1 participate in mediating the protective effect of E2 on HIBD in neonatal rats. The structure of GPER1 is similar to other G protein-coupled receptors, and it is mainly composed of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits, which are responsible for rapid estrogen signal transduction<sup>[14]</sup>. Although some ER $\alpha$  and ER $\beta$  are also related to plasma membrane and non-genomic signal transduction, the function and expression of GPER1 are independent of two ER, and it has high affinity for estrogen and a single binding site<sup>[44]</sup>. PI3K/AKT is one of the downstream signal pathways of GPER1. Akt participates in cell apoptosis and cell proliferation by influencing the activation state of various downstream effectors. It has been observed in many studies that GPR30 may regulate PI3K/Akt pathway. For example, it is found that GPR30 activates PI3K/Akt signal, which is an important step to regulate neurite formation and protect cognitive function in the development of hippocampal neurons<sup>[45]</sup>. It is also found that E2 can activate PI3K/AKT signaling pathway through GPR30 to play a neuroprotective role in spinal cord injury model<sup>[46]</sup>; It has also been reported that the neuroprotective effect of estrogen is related to the rapid activation of Akt and can inhibit cell apoptosis<sup>[19]</sup>.

Finally, we further explored whether NF- $\kappa$ B, a downstream signal molecule of PI3K/AKT, which whether is involved in the protective effect of E2 on the brain of neonatal rats with HIBD, and clarified the relationship between GPER1 and AKT/NF- $\kappa$ B signaling pathway. NF- $\kappa$ B is a multifunctional transcription factor, which is related to the regulation of many different biological phenomena and disease states,

including inflammation, immunity, cell death and stress response<sup>[47]</sup>. When cells are exposed to oxidative stress, PI3K/AKT signaling pathway is activated to regulate the downstream signaling factor NF- $\kappa$ B<sup>[20]</sup>. Studies show that NF- $\kappa$ B promotes inflammation and apoptosis in cerebral ischemia. So, inhibiting NF- $\kappa$ B signaling pathway may be a therapeutic strategy for ischemic cerebral infarction<sup>[48]</sup>. Some studies believe that AKT plays a positive role in regulating NF- $\kappa$ B after activation. For example, Yun Seon Song found that Akt plays its neuroprotective role in cerebral ischemia in mice by activating NF- $\kappa$ B<sup>[49]</sup>; However, some studies have also found that PI3K-Akt pathway can negatively regulate the expression of NF- $\kappa$ B and inflammatory genes<sup>[50]</sup>. For example, Li et al found that G-CSF can reduce neuroinflammation by activating PI3K/Akt signaling pathway and reducing the expression of inflammatory factors such as NF- $\kappa$ B, TNF- $\alpha$  and IL-1 $\beta$  after hypoxia and ischemia in neonatal rats<sup>[51]</sup>. Based on the different results of the above studies, we think that the protective effect of E2 on ischemic brain damage may be affected by many factors. For example, animal species, animal models, degree of brain damage, ways and means of treatment, and even different estrogen levels and sampling time may affect its protective effect and the changes of related receptor expression and signal pathway<sup>[52]</sup>. Although these studies are contradictory, they show the importance of estrogen receptors in ischemic brain damage from different aspects.

However, several limitations need to be explored in future, such as: whether the agonist G1 of GPER1 has the same neuroprotective effect in neonatal rat with HIBD? what is the relationship between GPER1 and classical estrogen receptors? Whether there is a relationship between AKT/NF- $\kappa$ B signaling pathway and classical estrogen receptors?

## 5. Conclusions

In conclusion, our study suggests that E2 treatment can significantly reduce brain injury and astrocyte apoptosis in neonatal HIBD, with GPER1 playing a central role in mediating these effects through the AKT/NF- $\kappa$ B signaling pathway. Further research is needed to understand the relationship between GPER1 and classical estrogen receptors and the detailed mechanisms of the AKT/NF- $\kappa$ B signaling pathway in mediating estrogen's neuroprotective effects.

## Funding

This work was supported by Youth Research Fund of Linyi Maternal and Child Health Hospital(Y202210).

## Data availability statement

Not applicable.

## Ethics statement

This research was approved by the Animal Ethics Committee of Linyi Maternal and Child Health Hospital for implementation



## Author contributions

Guangyun Zhang: data acquisition, analysis, and interpretation, drafted the manuscript. Xiangping Xu: conception, design, and data interpretation, drafted, and critically revised the manuscript. Jing Chen: data analysis, supervision and revised the manuscript. Dawei Yuan: data acquisition and analysis. Tiegang Lv: data acquisition. Huafeng Li: project administration and funding acquisition. Yanli Zhang: project administration and funding acquisition. Yong Ding: formal analysis. Juan Cui, Min Li and Jing Yu: investigation and data curation. All authors gave final approval and agreed to be accountable for all aspects of the work.

## Competing Interests

The authors have declared that no competing interest exists.

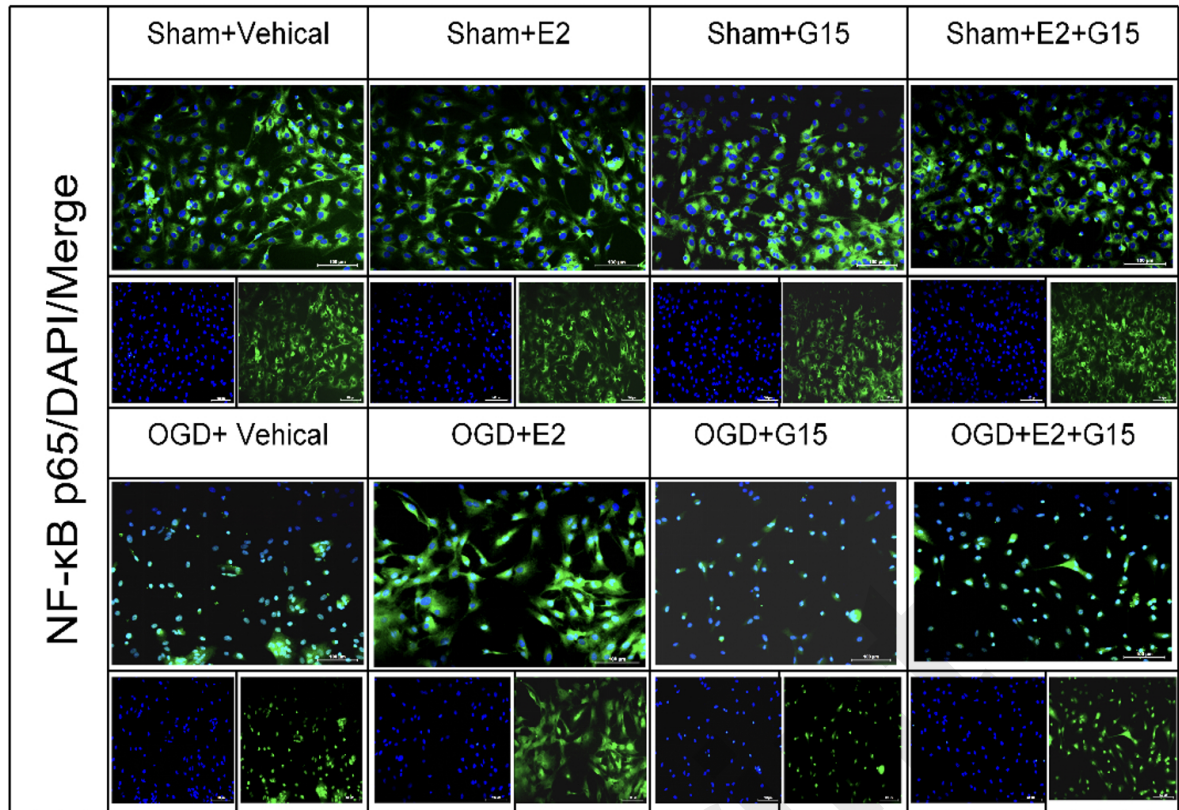
## References

1. She, H.-Q. *et al.* Current analysis of hypoxic-ischemic encephalopathy research issues and future treatment modalities. *Front. Neurosci.* 17, 1136500 (2023).
2. Xu K, Zhang LF, Zhang L, *et al.* MiR-125b-5p enclosed in hypoxic HK2 cell-derived extracellular vesicles alleviates renal ischemia-reperfusion injury by regulating NLRC5. *Archives of Medical Science.* Epub of Print (2024).
3. Silveira, R. C. & Procianoy, R. S. Hypothermia therapy for newborns with hypoxic ischemic encephalopathy. *J. Pediatr. (Rio J.)* 91, S78–S83 (2015).
4. Rao, R. *et al.* Safety and Short-Term Outcomes of Therapeutic Hypothermia in Preterm Neonates 34-35 Weeks Gestational Age with Hypoxic-Ischemic Encephalopathy. *J. Pediatr.* 183, 37–42 (2017).
5. Wang, Z. *et al.* Safety and efficacy of therapeutic hypothermia in neonates with mild hypoxic-ischemic encephalopathy. *BMC Pediatr.* 23, 530 (2023).
6. Brown, C., Suzuki, S., Jelks, K. & Wise, P. Estradiol Is a Potent Protective, Restorative, and Trophic Factor after Brain Injury. *Semin. Reprod. Med.* 27, 240–249 (2009).
7. Liu Y.B, Gao X.T., Huang L.Y., Liu X.L. Clinicopathological characteristics and prognostic factors in invasive micropapillary carcinoma of the breast. *Archives of Medical Science.* 20(2): 428-435 (2024).
8. Markiewicz, I. & Lukomska, B. The role of astrocytes in the physiology and pathology of the central nervous system. *Acta Neurobiol. Exp. (Warsz.)* 66, 343–358 (2006).
9. Kunc M, Gabrych A, Dulak D, Hasko K, *et al.* Systemic inflammatory markers and serum lactate dehydrogenase predict survival in patients with Wilms tumour. *Archives of Medical Science,* 18(5): 1253-1261 (2022).
10. Swanson, R., Ying, W. & Kauppinen, T. Astrocyte Influences on Ischemic Neuronal Death. *Curr. Mol. Med.* 4, 193–205 (2004).
11. Wilson, J. X. Antioxidant defense of the brain: a role for astrocytes. *Surv. Rev.* 75, (1997).

12. Rossi, D. J., Brady, J. D. & Mohr, C. Astrocyte metabolism and signaling during brain ischemia. *Nat. Neurosci.* 10, 1377–1386 (2007).
13. Xu, L., Emery, J. F., Ouyang, Y., Voloboueva, L. A. & Giffard, R. G. Astrocyte targeted overexpression of Hsp72 or SOD2 reduces neuronal vulnerability to forebrain ischemia. *Glia* 58, 1042–1049 (2010).
14. Bessa, A. *et al.* GPER: A new tool to protect dopaminergic neurons? *Biochim. Biophys. Acta BBA - Mol. Basis Dis.* 1852, 2035–2041 (2015).
15. Wang, J. *et al.* G-1 exhibit antidepressant effect, increase of hippocampal ERs expression and improve hippocampal redox status in aged female rats. *Behav. Brain Res.* 359, 845–852 (2019).
16. Zhang, Z. *et al.* The novel estrogenic receptor GPR30 alleviates ischemic injury by inhibiting TLR4-mediated microglial inflammation. *J. Neuroinflammation* 15, 206 (2018).
17. Karki, P., Smith, K., Johnson, J. & Lee, E. Astrocyte-derived growth factors and estrogen neuroprotection: Role of transforming growth factor- $\alpha$  in estrogen-induced upregulation of glutamate transporters in astrocytes. *Mol. Cell. Endocrinol.* 389, 58–64 (2014).
18. Peso, L. D., González-García, M., Page, C., Herrera, R. & Nuñez, G. Interleukin-3-Induced Phosphorylation of BAD Through the Protein Kinase Akt. *Science* 278, 687–689 (1997).
19. Tang, H. *et al.* Reprint of "GPR30 mediates estrogen rapid signaling and neuroprotection". *Mol. Cell. Endocrinol.* 389, 92–98 (2014).
20. Jones, W. K., Brown, M., Ren, X., He, S. & McGuinness, M. NF- $\kappa$ B as an Integrator of Diverse Signaling Pathways The Heart of Myocardial Signaling? *Cardiovasc. Toxicol.* 3, 229–254 (2003).
21. Ivanova, T., Mendez, P., Garcia-Segura, L. M. & Beyer, C. Rapid Stimulation of the PI3-Kinase/Akt Signalling Pathway in Developing Midbrain Neurones by Oestrogen. *J. Neuroendocrinol.* 14, 73–79 (2002).
22. Jover-Mengual, T. *et al.* Acute estradiol protects CA1 neurons from ischemia-induced apoptotic cell death via the PI3K/Akt pathway. *Brain Res.* 1321, 1–12 (2010).
23. Yang, L. *et al.* Activation of microglial G-protein-coupled receptor 30 protects neurons against excitotoxicity through NF- $\kappa$ B/MAPK pathways. *Brain Res. Bull.* 172, 22–30 (2021).
24. Xu, N. *et al.* Adiponectin attenuates neuronal apoptosis induced by hypoxia-ischemia via the activation of AdipoR1/APPL1/LKB1/AMPK pathway in neonatal rats. *Neuropharmacology* 133, 415–428 (2018).
25. Rice, J. E., Vannucci, R. C. & Brierley, J. B. The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann. Neurol.* 9, 131–141 (1981).
26. Gamdzyk, M. *et al.* cGAS/STING Pathway Activation Contributes to Delayed Neurodegeneration in Neonatal Hypoxia-Ischemia Rat Model: Possible Involvement of LINE-1. *Mol. Neurobiol.* 57, 2600–2619 (2020).
27. McCarthy, K. D. & De Vellis, J. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol.* 85, 890–902 (1980).

28. Giovannoni, F. & Quintana, F. J. The Role of Astrocytes in CNS Inflammation. *Trends Immunol.* 41, 805–819 (2020).
29. Kaur, C. & Ling, E. Blood Brain Barrier in Hypoxic-Ischemic Conditions. *Curr. Neurovasc. Res.* 5, 71–81 (2008).
30. Nawashiro, H., Brenner, M., Fukui, S., Shima, K. & Hallenbeck, J. M. High Susceptibility to Cerebral Ischemia in GFAP-Null Mice. *J. Cereb. Blood Flow Metab.* 20, 1040–1044 (2000).
31. Kinoshita, A., Yamada, K., Kohmura, E. & Hayakawa, T. Effect of astrocyte-derived factors on ischemic brain edema induced by rat MCA occlusion. *APMIS* 98, 851–857 (1990).
32. Nuñez, J. *et al.* 17 $\beta$ -Estradiol protects the neonatal brain from hypoxia–ischemia. *Exp. Neurol.* 208, 269–276 (2007).
33. Gerstner, B. *et al.* 17 $\beta$ -estradiol protects against hypoxic/ischemic white matter damage in the neonatal rat brain. *J. Neurosci. Res.* 87, 2078–2086 (2009).
34. Kumar, R. S. & Goyal, N. Estrogens as regulator of hematopoietic stem cell, immune cells and bone biology. *Life Sci.* 269, 119091 (2021).
35. Fuentes, N. & Silveyra, P. Estrogen receptor signaling mechanisms. in *Advances in Protein Chemistry and Structural Biology* vol. 116 135–170 (Elsevier, 2019).
36. Lu, Y. *et al.* Neuron-Derived Estrogen Is Critical for Astrocyte Activation and Neuroprotection of the Ischemic Brain. *J. Neurosci.* 40, 7355–7374 (2020).
37. Ma, Y. *et al.* OPEN Estrogen replacement therapy- induced neuroprotection against brain ischemia-reperfusion injury involves the activation of received:20August2015 accepted: 22 January 2016 Published:19February2016 astrocytes via estrogen receptor. *Sci. Rep.*
38. Wise, P. M., Suzuki, S. & Brown, C. M. Estradiol: a hormone with diverse and contradictory neuroprotective actions. *Dialogues Clin. Neurosci.* 11, 297–303 (2009).
39. DeLeon, C. *et al.* Novel GPER Agonist, CITFA, Increases Neurite Growth in Rat Embryonic (E18) Hippocampal Neurons. *ACS Chem. Neurosci.* 13, 1119–1128 (2022).
40. Zhao, T.-Z. *et al.* GPER1 mediates estrogen-induced neuroprotection against oxygen-glucose deprivation in the primary hippocampal neurons. *Neuroscience* 328, 117–126 (2016).
41. Karki, P., Smith, K., Johnson, J. & Lee, E. Astrocyte-derived growth factors and estrogen neuroprotection: Role of transforming growth factor- $\alpha$  in estrogen-induced upregulation of glutamate transporters in astrocytes. *Mol. Cell. Endocrinol.* 389, 58–64 (2014).
42. Wang, X.-S. *et al.* Activation of G protein-coupled receptor 30 protects neurons by regulating autophagy in astrocytes.
43. Ariyani, W., Miyazaki, W. & Koibuchi, N. A Novel Mechanism of S-equol Action in Neurons and Astrocytes: The Possible Involvement of GPR30/GPER1. *Int. J. Mol. Sci.* 20, 5178 (2019).
44. Sandén, C. *et al.* G Protein-Coupled Estrogen Receptor 1/G Protein-Coupled Receptor 30 Localizes in the Plasma Membrane and Traffics Intracellularly on Cytokeratin Intermediate Filaments. *Mol. Pharmacol.* 79, 400–410 (2011).

45. Ruiz-Palmero, I., Hernando, M., Garcia-Segura, L. M. & Arevalo, M.-A. G protein-coupled estrogen receptor is required for the neuritogenic mechanism of 17 $\beta$ -estradiol in developing hippocampal neurons. *Mol. Cell. Endocrinol.* 372, 105–115 (2013).
46. Chen, J. *et al.* G-protein-coupled receptor 30-mediated antiapoptotic effect of estrogen on spinal motor neurons following injury and its underlying mechanisms. *Mol. Med. Rep.* 12, 1733–1740 (2015).
47. Ozcan, L. *et al.* Selective Nuclear Factor Kappa b (NF $\kappa$ B) Inhibitor, Pyrrolidinium Dithiocarbamate Prevents, Long-Term Histologic Damage in Ischemia-Reperfusion Injuries after Delayed Testicular Torsion. *Urol. J.* 13, 2702–2706 (2016).
48. Oliveira-Marques, V., Marinho, H. S., Cyrne, L. & Antunes, F. Role of Hydrogen Peroxide in NF- $\kappa$ B Activation: From Inducer to Modulator. *Antioxid. Redox Signal.* 11, 2223–2243 (2009).
49. Song, Y. S. *et al.* The role of Akt signaling in oxidative stress mediates NF- $\kappa$ B activation in mild transient focal cerebral ischemia. (2008).
50. Guha, M. & Mackman, N. The Phosphatidylinositol 3-Kinase-Akt Pathway Limits Lipopolysaccharide Activation of Signaling Pathways and Expression of Inflammatory Mediators in Human Monocytic Cells. *J. Biol. Chem.* 277, 32124–32132 (2002).
51. Li, L. *et al.* G-CSF attenuates neuroinflammation and stabilizes the blood–brain barrier via the PI3K/Akt/GSK-3 $\beta$  signaling pathway following neonatal hypoxia-ischemia in rats. *Exp. Neurol.* 272, 135–144 (2015).
52. Simpkins, J. W., Singh, M., Brock, C. & Etgen, A. M. Neuroprotection and Estrogen Receptors. *Neuroendocrinology* 96, 119–130 (2012).



Preprint