N-Myc Downstream-Regulated Gene 2 (*Ndrg2*): A Critical Mediator of Estrogen-Induced Neuroprotection Against Cerebral Ischemic Injury

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Research Article

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Abstract

Growing evidence indicates that estrogen plays a pivotal role in neuroprotection against cerebral ischemia, but the molecular mechanism of this protection is still elusive. N-myc downstream-regulated gene 2 (*Ndrg2*), an estrogen-targeted gene, has been shown to exert neuroprotective effects against cerebral ischemia in male mice. However, the role of *Ndrg2* in the neuroprotective effect of estrogen remains unknown. In this study, we first detected NDRG2 expression levels in the cortex and striatum in both female and male mice with western blot analyses. We then detected cerebral ischemic injury by constructing middle cerebral artery occlusion and reperfusion (MCAO-R) models in *Ndrg2* knockout or conditional knockdown female mice. We further implemented estrogen, ERα or ERβ agonist replacement in the ovariectomized (OVX) *Ndrg2* knockouts or conditional knockdowns female mice, then tested for NDRG2 expression, glial fibrillary acidic protein (GFAP) expression, and extent of cerebral ischemic injury. We found that NDRG2 expression was significantly higher in female than in male mice in both the cortex and striatum. *Ndrg2* knockouts and conditional knockdowns showed significantly aggravated cerebral ischemic injury in female mice. Estrogen and ERβ replacement treatment (DPN) led to NDRG2 upregulation in both the cortex and striatum of OVX mice. Estrogen and DPN also led to GFAP upregulation in OVX mice. However, the effect of estrogen and DPN in activating astrocytes was lost in *Ndrg2* knockouts OVX mice and primary cultured astrocytes, but partially retained in conditional knockdowns OVX mice. Most importantly, we found that the neuroprotective effects of E2 and DPN against cerebral ischemic injury were lost in *Ndrg2* knockouts OVX mice but partially retained in conditional knockdowns OVX mice. These findings demonstrate that estrogen alleviated cerebral ischemic injury via ERβ upregulation of *Ndrg2*, which could activate astrocytes, indicating that *Ndrg2* is a critical mediator of E2-induced neuroprotection against cerebral ischemic injury.

Introduction

Ischemic stroke is one of the leading causes of death and disability worldwide and is widely accepted to be sexually dimorphic [1]. A number of both clinical trials and basic research studies have shown that the incidence of stroke is higher and stroke outcomes are worse in men compared to women [2,3]. Women's relative protection against strokes can be explained in part by serum levels of the neuroprotective ovarian hormone 17β-estradiol (estradiol or E2) [4]. Our previous studies demonstrated that estrogen exerted significantly neuroprotective effects against cerebral ischemia in female mice [5,6,7]. However, the underlying mechanisms of estrogen neuroprotection in female mice remain unclear.

Myc downstream-regulated gene 2 (*Ndrg*2), which plays vital roles in cell proliferation, differentiation, apoptosis, and stress responses [8], is widely expressed throughout the brain and is specifically expressed in astrocytes [9,10]. We previously first found that *Ndrg2*-knockout male mice had much larger cerebral infarction volumes compared with wild-type male mice [11]. We further found that *Ndrg2* knockouts male mice aggravates injury due to brain edema following cerebral ischemia [12]. These results demonstrate that *Ndrg2* could be a potential target for the neuroprotection against cerebral

ischemia in male mice. However, the role of *Ndrg2* in estrogen neuroprotection in female mice has not been reported.

In a previous study, we first found that estrogen and estrogen receptor β (ERβ) agonist DPN could up-regulate NDRG2 mRNA and protein expression in the mouse hippocampus, demonstrating that astrocytic *Ndrg2* is a target gene of estrogen and ERβ [9]. The present study aims to explore a previously unstudied area: the role of *Ndrg2* in estrogen-induced neuroprotection against cerebral ischemic injury in female mice and the underlying molecular mechanism.

Methods

1. Animals

Thirteen male and twenty-eight female C57BL/6 mice aged eight months old were provided by the Laboratory Animal Center of Chinese PLA General Hospital. Sixty-four $Ndrg2^{+/+}$, sixty-four $Ndrg2^{-/-}$, and one hundred and four $Ndrg2^{flox/flox}$ female mice were constructed by the Shanghai Model Organisms, China. $Ndrg2^{flox/flox}$ mice were crossed with B6.C-Tg(CMV-cre)1Cgn/J mice (Jackson Labs) to obtain $Ndrg2^{-/-}$ mice. The strain was backcrossed to C57BL/6J more than 20 times. The pAAV-CAG-Cre-3flag virus (2 μL, Hanbio, China) was injected into the right lateral cerebral ventricle of $Ndrg2^{flox/flox}$ mice to conditionally knock out the expression of NDRG2 in astrocytes (referred to as AAV-*Ndrg2*). All of the animals were maintained under the following standard conditions: 12/12 h light/dark cycle, 50–60% environmental humidity, 25 ± 1 °C, and free access to food and water. The experimental protocols were reviewed and approved by the Ethics Committee of the Chinese PLA General Hospital, China.

The detailed experiment design and group were shown in the results.

2. OVX and hormone replacement treatment

Ovariectomy (OVX) was performed by dorsolateral incisions as previously described [13]. After one week, the ovariectomized female mice then received daily subcutaneous injections of 0.1 ml sesame oil, E2 (100 μg/kg/day), estrogen receptor α agonist (PPT) (2 mg/kg/day), or DPN (8 mg/kg/day) for three weeks. These concentrations selected in this study were found to be effective in prior studies [6,9]. The levels of serum E2 were measured to confirm the effect of E2 replacement. As shown in Supplementary Fig. 1, the serum E2 levels in the Con group were 23.0 ± 2.2 pg/ml, however, serum E2 levels in the OVX group were decreased by 10.5 ± 1.1 pg/ml compared with the Con group ($^{*}p < 0.05$), and E2 replacement increased the serum E2 levels to 61.5 ± 2.7 pg/ml ($^{\#}p < 0.05$).

3. Middle cerebral artery occlusion and reperfusion (MCAO-R)

The mice received MCAO-R injury as previously described [14]. Following 1 h of transient occlusion, cerebral blood flow was restored by removing the suture for 24 h. Physiological parameters were monitored, including rectal temperature, blood pressure, blood gas, and glucose levels (Supplementary

Table 1). Regional cerebral blood flow was monitored by laser-Doppler flowmetry. Only the mice whose mean cortical cerebral blood flow decreased to 15% of the preischemic value during occlusion then recovered to 70% of the baseline after reperfusion were used for further data analysis.

4. Garcia Test

Neurological deficits were evaluated 24 h after reperfusion by a blinded observer based on the Garcia Test (n = 10) [15] (Supplementary Table 2). This results in a score for each mouse that integrates six parameters: (1) spontaneous activity, (2) symmetry of limb movement, (3) forepaw outstretching, (4) cage climbing, (5) body proprioception, and (6) vibrissae reaction. The cumulative minimum score is 3 (severe impairment) and the maximum is 18 (no impairment).

5. Assessment of infarct volume

After neurological scoring, infarct volume was assessed via 2,3,5-triphenyltetrazolium chloride (TTC) staining as previously described (n = 10) [16]. Brain slices were photographed and infarct volume (the unstained areas) was measured by the Image J software. Corrections were made for swelling, and relative infarct size was determined based on the following equation: relative infarct size = (contralateral area − ipsilateral non-infarct area)/contralateral area.

6. Western blot analysis

The corresponding brain tissues (the cortex, striatum or the corresponding ischemic penumbra region) or cells were collected for western blot analysis as previously described (n=3) [15]. Briefly, the tissues or cells were lysed using RIPA buffer (Beyotime, China), then protein concentration was measured using the bicinchoninic acid (BCA) protein assay. The proteins were separated using SDS-PAGE gel electrophoresis and then transferred to a PVDF membrane. Primary antibodies were as follows: rabbit anti-NDRG2 (1:1000, Cell Signaling Technology, USA), mouse anti-GFAP (1:1000, Cell Signaling Technology), and mouse anti-GAPDH (1:1000, Cell Signaling Technology). The membranes were then incubated with the corresponding HRP-conjugated secondary antibody for 2 h. Protein bands were visualized using the LI-COR Odyssey System (LICOR Biotechnology, USA).

7. Immunofluorescence (IF) staining

Immunofluorescence staining was performed on frozen coronal sections of mouse brains (n = 3). Briefly, the mice were anesthetized as described above, and the brains were fixed via transcardial perfusion with 0.9% cold heparinized saline and 4% paraformaldehyde. After post-fixation and concentration gradient dehydration, the brains were cut into 10-μm-thick sections using a Leica CM1900 frozen slicer (Leica, Germany). The sections were washed three times with phosphate-buffered saline (PBS), then incubated overnight at 4 °C in a humidified atmosphere with mouse anti-GFAP antibodies (1:200; Cell Signaling Technology). Samples were then incubated with Alexa-488 (green, Invitrogen, USA)-conjugated donkey anti-mouse secondary antibodies for 2 h in the dark at room temperature. The sections were mounted with 50% glycerol and examined under a fluorescence microscopy (BX51; Olympus, Tokyo, Japan).

8. Mouse genotyping

Genotypes of the homozygous *Ndrg2* deletion (*Ndrg2*$^{-/-}$) and wild-type (*Ndrg2*$^{+/+}$) mice were confirmed via PCR. The primers used were as follows: Wild type forward: *Ndrg2*$^{+/+}$ forward: CTTCCCCAGCCTCGGTGTC; *Ndrg2*$^{+/+}$ reverse: GGGCTCCCTTGTACAGTGTC; *Ndrg2*$^{-/-}$ forward: CTTCCCCAGCCTCGGTGTC; *Ndrg2*$^{-/-}$ reverse: GGCAAGATGGCTCAGCAGTCAAGA.

9. Primary astrocyte culture

Primary mouse astrocytic cultures were harvested from the cortex of 1- to 2-day-old *Ndrg2*$^{-/-}$ pups and their wild-type littermates (*Ndrg2*$^{+/+}$ pups). Briefly, after removal of the meninges and hippocampus, the cortical tissues were subjected to enzymatic digestion and mechanical isolation. The resulting mixed cortical cells were passed through a 70-μm nylon mesh cell strainer and seeded into a cell culture flask in Dulbecco's Modified Eagle Medium (DMEM, HyClone, USA) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin. When cells had reached confluence, the mixed glial cultures were shaken on an orbital shaker at 220 rpm for 18 h. The cultures were incubated at 37 °C in a 95/5% mixture of atmospheric air/CO_2. The purity of the astrocyte culture was greater than 95%, as confirmed by staining with the astrocytic marker GFAP (Supplementary Fig. 2).

10. Statistical analysis

All data were analyzed by an observer who was blind to the experimental protocol. Statistical calculations were performed with GraphPad Prism software, version 8.0. Comparisons between two groups were performed using Student's *t*-test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Neurological deficit scores are presented as median with interquartile range and were analyzed using two-tailed Mann-Whitney *U* test, and the other values are presented as the mean ± SD. $p < 0.05$ were considered statistically significant.

Results

1. Gender differences in cerebral ischemic injury and NDRG2 expression

First, we assessed infarct volume and neurological scores to verify gendered differences in cerebral ischemic injury. As shown in Fig. 1 A(a) and (b), the mean infarct volume in male mice was 50.2 ± 1.7%, whereas female mice exhibited a smaller mean infarct volume of 32.2 ± 1.9% ($^{*}p < 0.05$). Neurological scores were also significantly lower in male than in female mice, demonstrating more severe neurological damage ($^{*}p < 0.05$).

We next detected NDRG2 expression in the cortex and striatum of male and female mice. As shown in Fig. 1 B(a) and (b), NDRG2 expression was significantly higher in female mice than in male mice in both the cortex ($^{*}p < 0.05$) and striatum ($^{\#\#}p < 0.01$).

2. Ndrg2 deficiency significantly aggravates cerebral ischemic injury in female mice

To investigate whether *Ndrg2* is necessary for neuroprotective effects against cerebral ischemic injury in female mice, we used *Ndrg2* systemic knockout female mice to examine infarct volume and neurological scores after MCAO-R injury. First, immunoblotting was performed to detect NDRG2 expression and PCR was used to confirm the genotypes in the brain of $Ndrg2^{+/+}$ and $Ndrg2^{-/-}$ mice. As shown in Fig. 2 A(a) and (b), $Ndrg2^{+/+}$ mice did and $Ndrg2^{-/-}$ mice did not express NDRG2. Moreover, following cerebral ischemic injury, the infarct volume in the $Ndrg2^{+/+}$ mice was 32.3% ± 1.8%, whereas *Ndrg2* knockouts significantly increased the infarct volume to 48.0% ± 1.6% (Fig. 2 B(a) and (b), $^{*}p < 0.05$). Compared with the $Ndrg2^{+/+}$ mice, the $Ndrg2^{-/-}$ mice exhibited markedly worse neurological deficit scores (Fig. 2 B(c), $^{*}p < 0.05$).

To further explore the role of *Ndrg2* in cerebral ischemic injury, we constructed $Ndrg2^{flox/flox}$ mice and adeno-associated virus (AAV), pAAV-CAG-Cre-3flag, to conditionally down-regulate the expression of NDRG2. The adeno-associated virus was injected into the right lateral cerebral ventricle of $Ndrg2^{flox/flox}$ mice to generate the AAV-*Ndrg2* group. Three weeks later, NDRG2 expression was detected in different brain regions by immunoblotting. As shown in Fig. 2 C(a) and (b), NDRG2 was significantly down-regulated in the AAV-*Ndrg2* group compared with the control (AAV-Con) group in both the cortex and striatum ($^{*}p < 0.05$, $^{\#}p < 0.05$). After MCAO-R injury, the infarct volume was 31.5% ± 1.5% in the AAV-Con group and significantly higher (40.0% ± 1.4%) in the AAV-*Ndrg2* group (Fig. 2 D(a) and (b), $^{*}p < 0.05$). Furthermore, the mice in the AAV-*Ndrg2* group exhibited worse neurological outcomes (Fig. 2 D(c), $^{*}p < 0.05$).

3. Estrogen and ERβ agonist DPN treatment upregulate the expression of NDRG2 in cortex and striatum in the OVX mice

As shown in Fig. 3 A(a) and (b), the female mice were divided into five groups: Con (Control), OVX (mice that had undergone ovariectomy for 1 week), E2 (mice treated with estrogen for three weeks, beginning one week after ovariectomy), PPT (mice treated with the estrogen receptor α agonist PPT for three weeks, beginning one week after ovariectomy), and DPN (mice treated with the estrogen receptor β agonist DPN for three weeks, beginning one week after ovariectomy). We found that ovariectomy significantly decreased NDRG2 expression in both the cortex and striatum ($^{*}p < 0.05$ vs. Con-Cortex group, $^{\&}p < 0.05$ vs. Con-Striatum group). Furthermore, E2 and DPN treatment increased NDRG2 expression in both the cortex and striatum ($^{\#}p < 0.05$ vs. OVX-Cortex group, $^{\wedge}p < 0.05$ vs. OVX-Striatum group). However, PPT treatment did not increase NDRG2 protein expression.

4. E2 and DPN did not activate astrocytes in *Ndrg2* knockouts

Next, we detected the expression of GFAP, which indicates activation of astrocytes, in the corresponding ischemic penumbra region of non-ischemic female mice. As shown in Fig. 3 B (a) and (b), GFAP expression in the $Ndrg2^{+/+}$-OVX group was significantly lower than in the $Ndrg2^{+/+}$-Con group ($^{*}p < 0.05$),

and both E2 and DPN treatment significantly increased GFAP expression compared with the *Ndrg2*$^{+/+}$-OVX group ($^{\#}p < 0.05$). GFAP expression was decreased in the *Ndrg2*$^{-/-}$-Con group compared to the *Ndrg2*$^{+/+}$-Con group ($^{*}p < 0.05$). There was no significant difference in GFAP expression among the *Ndrg2*$^{-/-}$-Con, *Ndrg2*$^{-/-}$-OVX, *Ndrg2*$^{-/-}$-E2, and *Ndrg2*$^{-/-}$-DPN groups. GFAP expression was significantly down-regulated in *Ndrg2*$^{-/-}$-E2 group compared with that of the *Ndrg2*$^{+/+}$-E2 group ($^{\wedge}p < 0.05$), and DPN treatment in the *Ndrg2*$^{-/-}$-DPN group exhibited lower GFAP expression compared with that of the *Ndrg2*$^{+/+}$-DPN group ($^{\&}p < 0.05$).

As shown in Fig. 3 C (a) and (b), the primary cultured astrocytes from the cortex of *Ndrg2*$^{-/-}$ pups and *Ndrg2*$^{+/+}$ pups were divided into six groups: *Ndrg2*$^{+/+}$-Ast-Con, *Ndrg2*$^{+/+}$-Ast-E2 (*Ndrg2*$^{+/+}$-astrocytes treated with 10 nM E2 for 24 h), *Ndrg2*$^{+/+}$-Ast-DPN (*Ndrg2*$^{+/+}$-astrocytes treated with 10 nM DPN for 72 h), *Ndrg2*$^{-/-}$-Ast-Con, *Ndrg2*$^{-/-}$-Ast-E2 (*Ndrg2*$^{-/-}$-astrocytes treated with 10 nM E2 for 24 h), and *Ndrg2*$^{-/-}$-Ast-DPN (*Ndrg2*$^{-/-}$-astrocytes treated with 10 nM DPN for 72 h). We found that both E2 and DPN treatment significantly increased astrocyte activation compared to the *Ndrg2*$^{+/+}$-Ast-Con group ($^{*}p < 0.05$). *Ndrg2* knockouts induced a significant decrease in GFAP expression compared to the *Ndrg2*$^{+/+}$-Ast-Con group ($^{*}p < 0.05$). However, neither E2 nor DPN treatment induced astrocyte activation *in vitro*. Moreover, the *Ndrg2*$^{-/-}$-Ast-E2 group showed a markedly reduce in the expression of GFAP compared with the *Ndrg2*$^{+/+}$-Ast-E2 group ($^{\#}p < 0.05$), and the expression of GFAP was significantly down-regulated in *Ndrg2*$^{-/-}$-Ast-DPN group compared with that of the *Ndrg2*$^{+/+}$-Ast-DPN group ($^{\wedge}p < 0.05$).

We next examined whether down-regulation of *Ndrg2* could preserve the effect of DPN on astrocyte activation. As shown in Fig. 3 D (a) and (b), consistent with the results above, GFAP expression was significantly lower in the AAV-Con-OVX group than the AAV-Con-Con group ($^{*}p < 0.05$); both E2 treatment and DPN treatment induced more robust astrocyte activation than the AAV-Con-OVX group ($^{\#}p < 0.05$). The conditional knockdown *Ndrg2* significantly decreased GFAP expression compared to the AAV-Con-Con group ($^{*}p < 0.05$). Interestingly, GFAP expression in the AAV-*Ndrg2*-OVX group was lower than in the AAV-*Ndrg2*-Con group ($^{\&}p < 0.05$), and E2 or DPN treatment significantly increased GFAP expression compared to the AAV-*Ndrg2*-OVX group ($^{\wedge}p < 0.05$).

We then quantified astrocyte activation in the corresponding ischemic penumbra region of non-ischemic female mice by observing astrocyte morphology. As shown in Fig. 3 E, the astrocyte (GFAP-positive) in the *Ndrg2*$^{+/+}$-Con group displayed a normal activated morphology, which is characterized by a large soma and cytoplasmic processes. However, the astrocyte in the *Ndrg2*$^{+/+}$-OVX group showed much smaller soma and thinner, shorter processes than those in *Ndrg2*$^{+/+}$-Con group. Following ovariectomy, E2 or DPN treatment significantly increased astrocyte activation. Interestingly, the astrocytes in the *Ndrg2*$^{-/-}$-Con, *Ndrg2*$^{-/-}$-OVX, *Ndrg2*$^{-/-}$-E2, and *Ndrg2*$^{-/-}$-DPN groups demonstrated small soma and short processes, demonstrating a lack of normal activation. These results provide strong support for the importance of *Ndrg2* in activating astrocytes.

5. Neuroprotective effect of E2 and DPN treatment was lost in *Ndrg2* knockouts but not knockdowns

To further investigate whether *Ndrg2* is a critical mediator in the neuroprotective effects of E2 and DPN, we used *Ndrg2*$^{+/+}$ and *Ndrg2*$^{-/-}$ female mice and divided them into eight groups: *Ndrg2*$^{+/+}$-Con (*Ndrg2*$^{+/+}$-Control), *Ndrg2*$^{+/+}$-OVX (*Ndrg2*$^{+/+}$ mice had undergone ovariectomy one week prior), *Ndrg2*$^{+/+}$-E2 (*Ndrg2*$^{+/+}$ mice treated with E2 for three weeks, beginning one week after ovariectomy), *Ndrg2*$^{+/+}$-DPN (*Ndrg2*$^{+/+}$ mice treated with the estrogen receptor β agonist DPN for three weeks, beginning one week after ovariectomy), *Ndrg2*$^{-/-}$-Con (*Ndrg2*$^{-/-}$-Control), *Ndrg2*$^{-/-}$-OVX (*Ndrg2*$^{-/-}$ had undergone ovariectomy one week prior), *Ndrg2*$^{-/-}$-E2 (*Ndrg2*$^{-/-}$ mice treated with E2 for three weeks, beginning one week after ovariectomy), *Ndrg2*$^{-/-}$-DPN (*Ndrg2*$^{-/-}$ mice treated with the estrogen receptor β agonist DPN for three weeks, beginning one week after ovariectomy). The mice in all groups received MCAO-R injury. As shown in Fig. 4 A(a) and (b), the infarct volume was 32.0% ± 1.5% in the *Ndrg2*$^{+/+}$-Con group, and larger in the *Ndrg2*$^{+/+}$-OVX group (44.6% ± 1.9%, $^{*}p < 0.05$). However, compared to the *Ndrg2*$^{+/+}$-OVX group, E2 and DPN treatment significantly decreased the infarct volume to 35.2% ± 1.5% and 36.0% ± 1.6%, respectively ($^{\#}p < 0.05$). *Ndrg2* knockouts significantly increased the infarct volume to 45.4% ± 2.1% compared to the *Ndrg2*$^{+/+}$-Con group ($^{*}p < 0.05$). The infarct volume was 44.2% ± 2.2% in the *Ndrg2*$^{-/-}$-OVX group, 45.0% ± 1.6% in the *Ndrg2*$^{-/-}$-E2 group, and 45.4% ± 1.5% in the *Ndrg2*$^{-/-}$-DPN group, although none of these differences were significant. However, the infarct volume was larger in the *Ndrg2*$^{-/-}$-E2 and *Ndrg2*$^{-/-}$-DPN groups than in the *Ndrg2*$^{+/+}$-E2 and *Ndrg2*$^{+/+}$-DPN groups, respectively ($^{\wedge}p < 0.05$). Notably, we observed precisely the same trends for each of the groups when we assessed neurological scores. As shown in Fig. 4 A(c), compared with the *Ndrg2*$^{+/+}$-Con group, the *Ndrg2*$^{+/+}$-OVX group had significantly lower neurological scores ($^{*}p < 0.05$). Interestingly, the neurological scores of the mice in the *Ndrg2*$^{+/+}$-E2 and *Ndrg2*$^{+/+}$-DPN groups were dramatically improved compared with those in the *Ndrg2*$^{+/+}$-OVX group ($^{\#}p < 0.05$). Additionally, the neurological scores of the mice in the *Ndrg2*$^{-/-}$-Con group were dramatically reduced compared with the *Ndrg2*$^{+/+}$-Con group ($^{*}p < 0.05$). Furthermore, the neurological scores of mice in the *Ndrg2*$^{-/-}$-E2 and *Ndrg2*$^{-/-}$-DPN groups were much lower compared with the *Ndrg2*$^{+/+}$-E2 and *Ndrg2*$^{+/+}$-DPN groups, respectively ($^{\wedge}p < 0.05$), the mice in the *Ndrg2*$^{-/-}$-DPN group had lower neurological scores than that of *Ndrg2*$^{+/+}$-DPN group ($^{\&}p < 0.05$), which indicating that systemic deletion of *Ndrg2* significantly decreased the neuroprotective effects of E2 and DPN.

Next, we used *Ndrg2*$^{flox/flox}$ female mice and related viruses to construct AAV-Con and AAV-*Ndrg2* mice as described above, then divided them into eight groups: AAV-Con-Con (AAV-Con-Control), AAV-Con-OVX (the AAV-Con mice had undergone ovariectomy one week prior), AAV-Con-E2 (the AAV-Con mice treated with E2 for three weeks, beginning one week after ovariectomy), AAV-Con-DPN (the AAV-Con mice treated with the estrogen receptor β agonist DPN for three weeks, beginning one week after ovariectomy), AAV-*Ndrg2*-Con (AAV-*Ndrg2*-Control), AAV-*Ndrg2*-OVX (the AAV-*Ndrg2* mice had undergone ovariectomy one week prior), AAV-*Ndrg2*-E2 (the AAV-*Ndrg2* mice treated with E2 for three weeks, beginning one week after ovariectomy), AAV-*Ndrg2*-DPN (the AAV-*Ndrg2* mice treated with the estrogen receptor β agonist DPN for three weeks, beginning one week after ovariectomy). After receiving OVX, E2, or DPN treatment, the mice

were all subjected to cerebral ischemic injury. As shown in Fig. 4 B(a) and (b), the mean infarct volume was 32.2% ± 1.9% in the AAV-Con-Con group, and was significantly larger in AAV-Con-OVX group, at 41.8% ± 1.3% ($^{*}p < 0.05$). However, E2 and DPN replacement treatment both markedly attenuated the infarct volume compared to the AAV-Con-OVX group (33.0% ± 1.2% and 33.4% ± 1.1%, respectively; $^{\#}p < 0.05$). Additionally, conditional knockdown of *Ndrg2* significantly increased cerebral infarction volume to 38.0% ± 1.6% compared to the AAV-Con-Con group ($^{*}p < 0.05$). Similarly, ovariectomy significantly increased the infarct volume to 47.0% ± 2.0% compared to the AAV-*Ndrg2*-Con group ($^{\&}p < 0.05$), but E2 treatment and DPN replacement treatment both significantly decreased the infarct volume compared to the AAV-*Ndrg2*-OVX group (40.0% ± 1.6% and 40.8% ± 1.9%, respectively; $^{\wedge}p < 0.05$). As shown in Fig. 4 B(c), the AAV-Con-OVX group had much lower neurological scores than the AAV-Con-Con group ($^{*}p < 0.05$), but treatment with either E2 or DPN significantly enhanced the neurological scores compared to the AAV-Con-OVX group ($^{\#}p < 0.05$). Compared with the AAV-Con-Con group, conditional knockdown of *Ndrg2* significantly decreased the neurological scores in the AAV-*Ndrg2*-Con group ($^{*}p < 0.05$), and the AAV-*Ndrg2*-OVX group had much lower neurological scores compared to the AAV-*Ndrg2*-Con group ($^{\&}p < 0.05$). Again, both E2 and DPN treatment dramatically improved the neurological scores compared to the AAV-*Ndrg2*-OVX group ($^{\wedge}p < 0.05$). Thus, neurological scores and infarct volumes showed similar trends between the different genotypes and treatment groups.

Discussion

Ischemic stroke is a cerebrovascular disease with different effects in each sex [17, 18, 19]. Epidemiological studies have reported that the incidence of stroke is higher in men than in women, and that men have worse stroke outcomes [2]. Experimental stroke studies in animals have indicated that male groups have significantly larger infarct volumes than female groups [3]. Although such gendered differences in stroke have been observed, the molecular mechanisms are incompletely characterized.

N-myc downstream-regulated gene 2 (*Ndrg2*) is a member of the NDRG family, which is involved in cell proliferation, differentiation, and stress responses [8]. Growing evidence indicates that *Ndrg2* plays a pivotal role in the pathogenesis of cerebral ischemic injury [20, 21]. A previous study suggested that *Ndrg2*-deficient male mice in models of cerebral ischemic injury induced by MCAO displayed exacerbated infarct damage [22]. A previous study in our lab demonstrated that *Ndrg2* deficiency in male mice aggravated brain edema and elevated permeability of the blood–brain barrier (BBB) at an early phase after ischemia [23]. These results suggest that *Ndrg2* is indispensable for neuroprotection in cerebral ischemic injury. In this study, we found that NDRG2 expression in both the cortex and striatum was significantly higher in female mice than in male mice. These data suggest that gendered differential expression of NDRG2 may be one of the reasons for the gender-based difference in cerebral ischemic injury. Nevertheless, the role of *Ndrg2* in cerebral ischemic injury in female mice has not been explored before. In the current study, we found that either systemic deletion or conditional knockdown of *Ndrg2* in female mice led to significantly increased brain infarct damage and lower neurological scores, consistent with the aforementioned findings in male mice. The results reflect that *Ndrg2* is a key molecule in

neuroprotection against cerebral ischemic injury in female mice. However, further studies are required to clarify the molecular mechanism underlying the regulation of NDRG2 expression.

We previously found that 17β-estradiol (E2) induced NDRG2 mRNA and protein expression in a dose- and time-dependent manner in the mouse hippocampus [9], from which we inferred that *Ndrg2* could be regulated by E2. E2 signaling is primarily mediated by the estrogen-binding receptor proteins estrogen receptor (ER) α and ERβ, which act as nuclear transcription factors [24]. Several studies, including ours, found that estrogen exerted significant neuroprotective effects against cerebral ischemia via activating ERα and ERβ [6, 25, 26]. In the current study, we found that ovariectomy significantly decreased NDRG2 expression in both the cortex and striatum; furthermore, E2 or estrogen receptor β agonist (DPN) treatment, but not estrogen receptor α agonist (PPT) treatment, significantly restored the NDRG2 expression in OVX mice. These results support *Ndrg2* in the brain as the regulatory target of E2 and ERβ.

Astrocytes are an indispensable participant in neuropathological process, especially hypoxia/ischemia, inflammation, and repair. Increased GFAP expression in astrocytes indicates the activation of astrocytes. A previous study found that the activation of astrocytes provides strong support for ischemic preconditioning-mediated neuroprotection [27]. Activated astrocytes improved the ability of neurons to eliminate excitatory neurotransmitters and ions such as glutamate, H^+, and K^+, and the viability of neurons co-cultured with astrocytes was greater than neurons cultured alone [28]. In our study, we found that E2 and DPN preconditioning could activate astrocytes both in OVX mice and in primary cultured astrocytes. Thus, we propose that the neuroprotective effects of E2 and DPN treatment against cerebral ischemic injury in female mice could be mediated by pre-activation of astrocytes. In agreement with these results, our previous study showed that E2 and ERβ treatment alleviate global cerebral ischemia (GCI) and reperfusion in female mice by the activation of astrocytes [5]. *Ndrg2* has been identified as a specific marker for astrocytes; it is mainly expressed in central nervous system astrocytes and plays a pivotal role in astrocyte function [10, 29]. In this study, we found that the effect of E2 and ERβ in activating astrocytes disappeared both in *Ndrg2* systemic knockout female mice and primary cultured astrocytes, but was partially retained in *Ndrg2* conditional knockdown female mice. Altogether, the findings suggest that *Ndrg2* plays an irreplaceable role in the neuroprotection of cerebral ischemia via pre-activation of astrocytes by E2 and ERβ treatment.

Most importantly, we found that E2 and ERβ treatment could significantly attenuate cerebral ischemic injury in OVX mice, consistent with our prior findings [5, 6]. However, the molecular mechanisms of E2 and ERβ-induced neuroprotection are still elusive. In this study, we found that the neuroprotection of E2 and ERβ disappeared in *Ndrg2*-deficient female mice, but was partially retained in female *Ndrg2*-knockdown mice following cerebral ischemic injury. Thus, the promising results suggest that *Ndrg2* may be a novel target of E2- and ERβ-induced neuroprotection in female mice in cerebral ischemic injury.

In summary, we conclude that E2-induced neuroprotection in female mice against cerebral ischemic injury involves activation of astrocytes via ERβ up-regulating NDRG2. These findings provide insight into the

roles of *Ndrg2* in cerebral ischemic injury in female mice and provide a novel target of estrogen neuroprotection against cerebral ischemic injury.

Declarations

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LX Zhang, YL Ma, M Liu and M Sun conducted most of the experiments and drafted the manuscript. J Wang and SY Liu contributed to the statistical analysis and manuscript editing. H Guo and XY Zhang contributed to data interruption. WG Hou and YH Liu designed this study and edited the manuscript. All authors read and approved the final manuscript.

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Not applicable

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

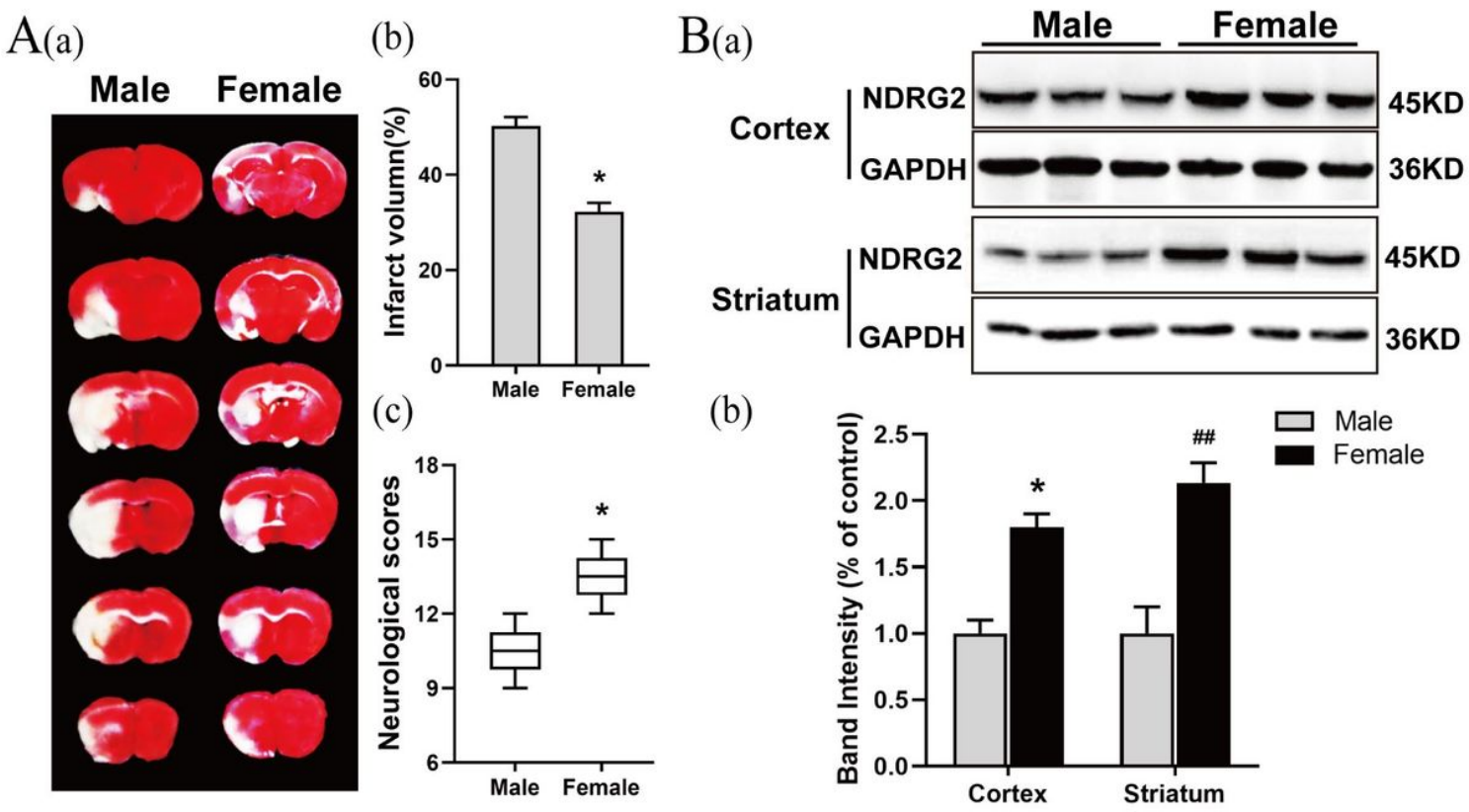

Figure 1

Gendered differences in the severity of cerebral ischemia and expression NDRG2. A(a) Representative photographs of brain slices showing the infarct volume assessed 24 h after reperfusion in male and female mice. A(b) Statistical analysis of infarction volume in male and female mice. Values shown are mean ± standard deviation (SD). *p < 0.05 vs. male mice (n = 10 per group). A(c) Neurological deficit score evaluated 24 h after reperfusion. Values shown are median with interquartile range. *p < 0.05 (n = 10 per group). B(a) Western blot showing the protein expression of NDRG2 in the cortex and striatum in

male and female mice. B(b) Graph of the protein expression of NDRG2. *p < 0.05, ##p < 0.01 (n = 3 per group).

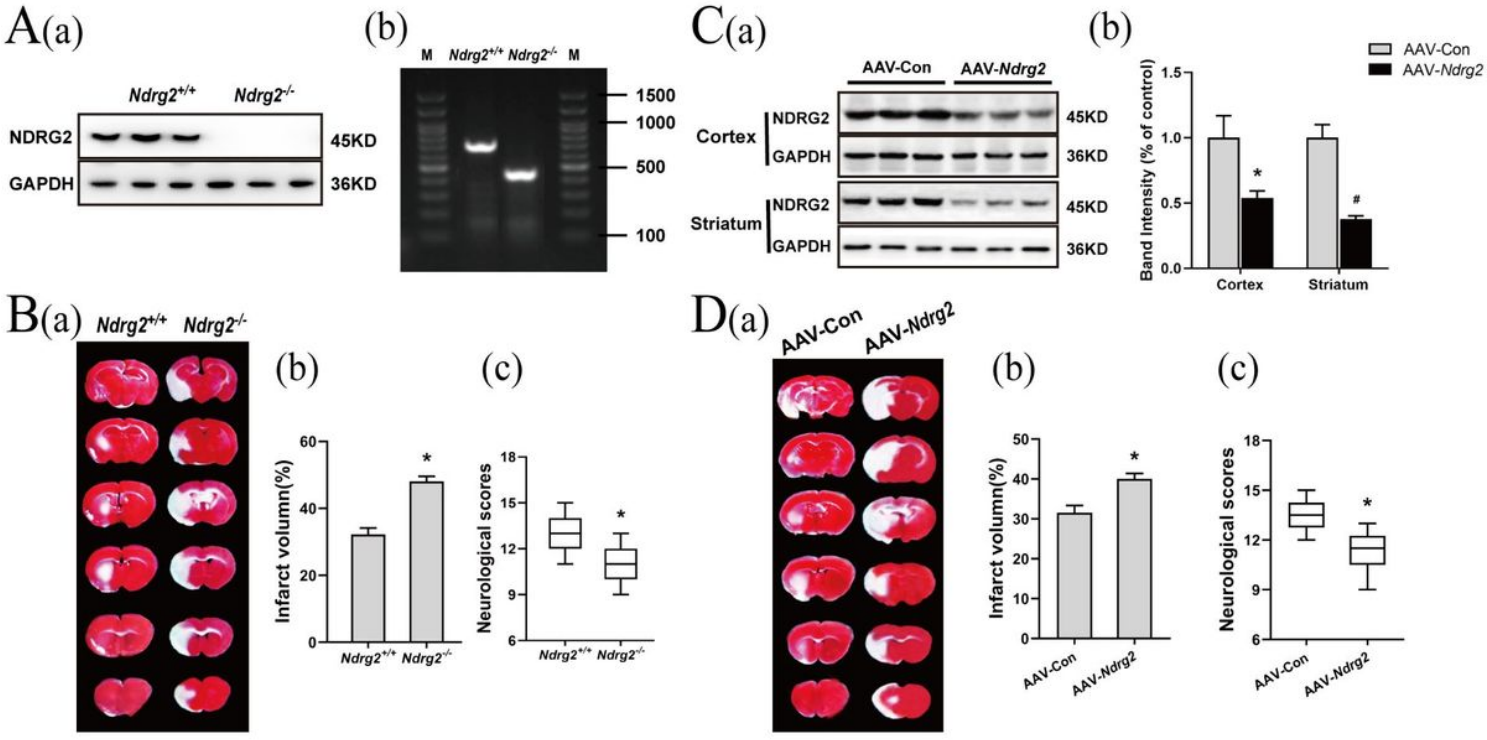

Figure 2

Deletion or down-regulation of Ndrg2 aggravates cerebral ischemic injury. A(a) Western blot showing the protein expression of NDRG2 in Ndrg2+/+ and Ndrg2-/- mice (n = 3 per group). A(b) PCR verification of Ndrg2+/+ and Ndrg2-/- mouse genotypes (n = 3 per group). B(a) Representative photographs of brain slices showing the infarct volume assessed 24 h after reperfusion in Ndrg2+/+ and Ndrg2-/- female mice. B(b) Statistical analysis of infarction volume in Ndrg2+/+ and Ndrg2-/- female mice. *p < 0.05 (n = 10 per group). B(c) Neurological deficit scores evaluated 24 h after reperfusion. Values shown are median with interquartile range. *p < 0.05 (n = 10 per group). C(a) Western blot showing the protein expression of NDRG2 in the cortex and striatum of female mice in the AAV-Con and AAV-Ndrg2 groups. C(b) NDRG2 protein expression levels. *p < 0.05 vs. AAV-Con-Cortex group, #p < 0.05 vs. AAV-Con-Striatum group (n = 3 per group). D(a) Representative photographs of brain slices showing the infarct volume assessed 24 h after reperfusion in female mice in AAV-Con and AAV-Ndrg2 groups. D(b) Statistical analysis of infarction volume in female mice in AAV-Con and AAV-Ndrg2 groups. *p < 0.05 vs. AAV-Con group (n = 10 per group). D(c) Neurological deficit score evaluated 24 h after reperfusion. *p < 0.05 vs. AAV-Con group (n = 10 per group).

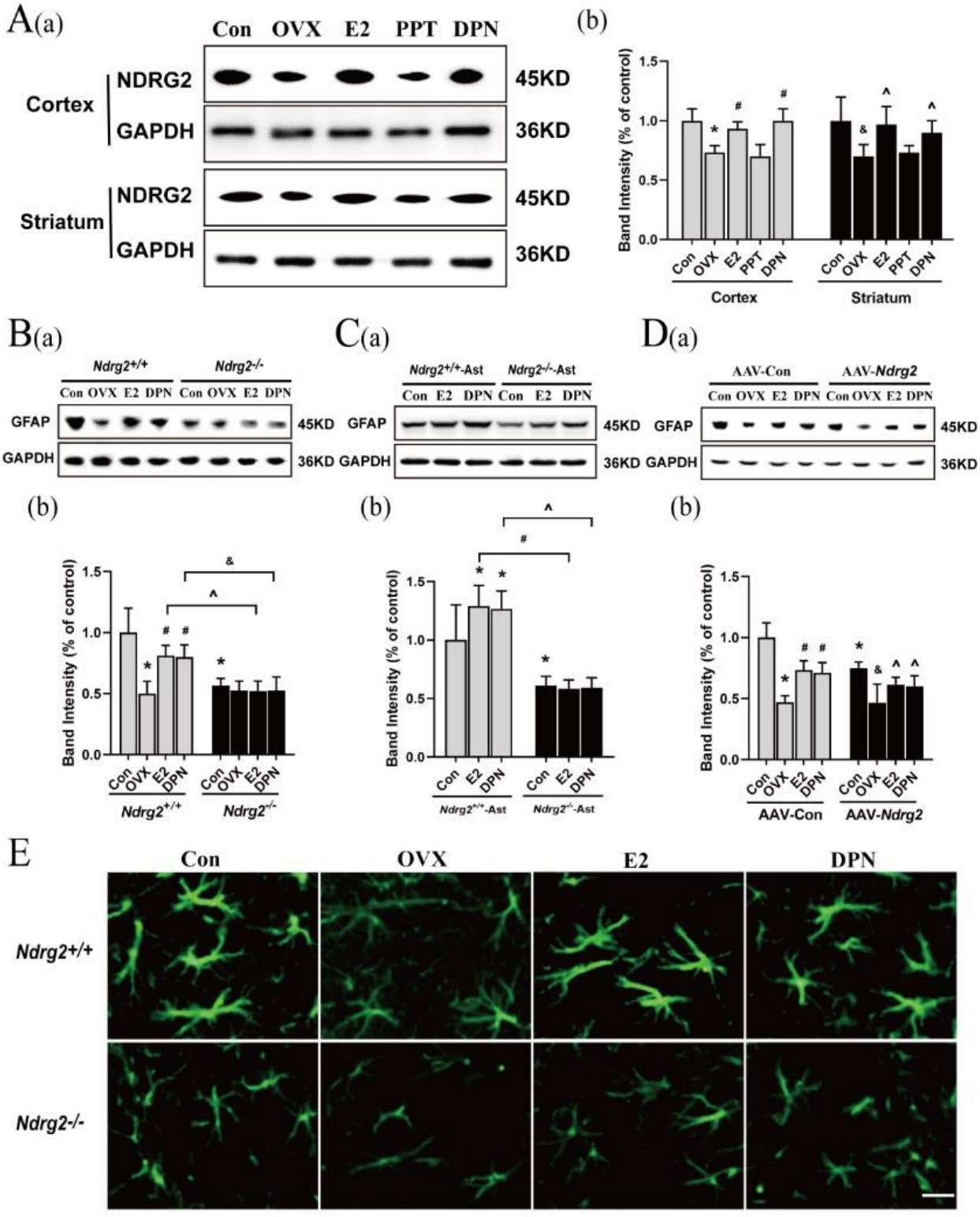

Figure 3

E2-mediated astrocyte activation disappeared in Ndrg2-/- female mice and Ndrg2-/–Ast, but partially remained in AAV-Ndrg2 female mice. A(a) Western blot showing NDRG2 protein expression in the cortex and striatum of female mice in different groups. A(b) NDRG2 protein expression levels. *p < 0.05 vs. Con-Cortex group, #p < 0.05 vs. OVX-Cortex group, &p < 0.05 vs. Con-Striatum group, ^p < 0.05 vs. OVX-Striatum group (n = 3 per group). B(a) Western blot showing GFAP protein expression in the

corresponding ischemic penumbra region in female mice in different groups. B(b) GFAP protein expression levels. *p < 0.05 vs. Ndrg2+/+-Con group, #p < 0.05 vs. Ndrg2+/+-OVX group, ^p < 0.05 vs. Ndrg2+/+-E2 group, &p < 0.05 vs. Ndrg2+/+-DPN group (n = 3 per group). C(a) Western blot showing GFAP protein expression in primary astrocytes in different groups. C(b) GFAP protein expression levels. *p < 0.05 vs. Ndrg2+/+-Ast-Con group, #p < 0.05 vs. Ndrg2+/+-Ast-E2 group, ^p < 0.05 vs. Ndrg2+/+-Ast-DPN group (n = 3 per group). D(a) Western blot showing GFAP protein expression in the corresponding ischemic penumbra region in female mice in different groups. D(b) GFAP protein expression levels. *p < 0.05 vs. AAV-Con-Con group, #p < 0.05 vs. AAV-Con-OVX group, &p < 0.05 vs. AAV-Ndrg2-Con group, ^p < 0.05 vs. AAV-Ndrg2-OVX group (n = 3 per group). E Representative immunofluorescence images showing the morphology of astrocytes labeled with GFAP in the corresponding ischemic penumbra region in female mice in different groups. Scale bar = 20 μm.

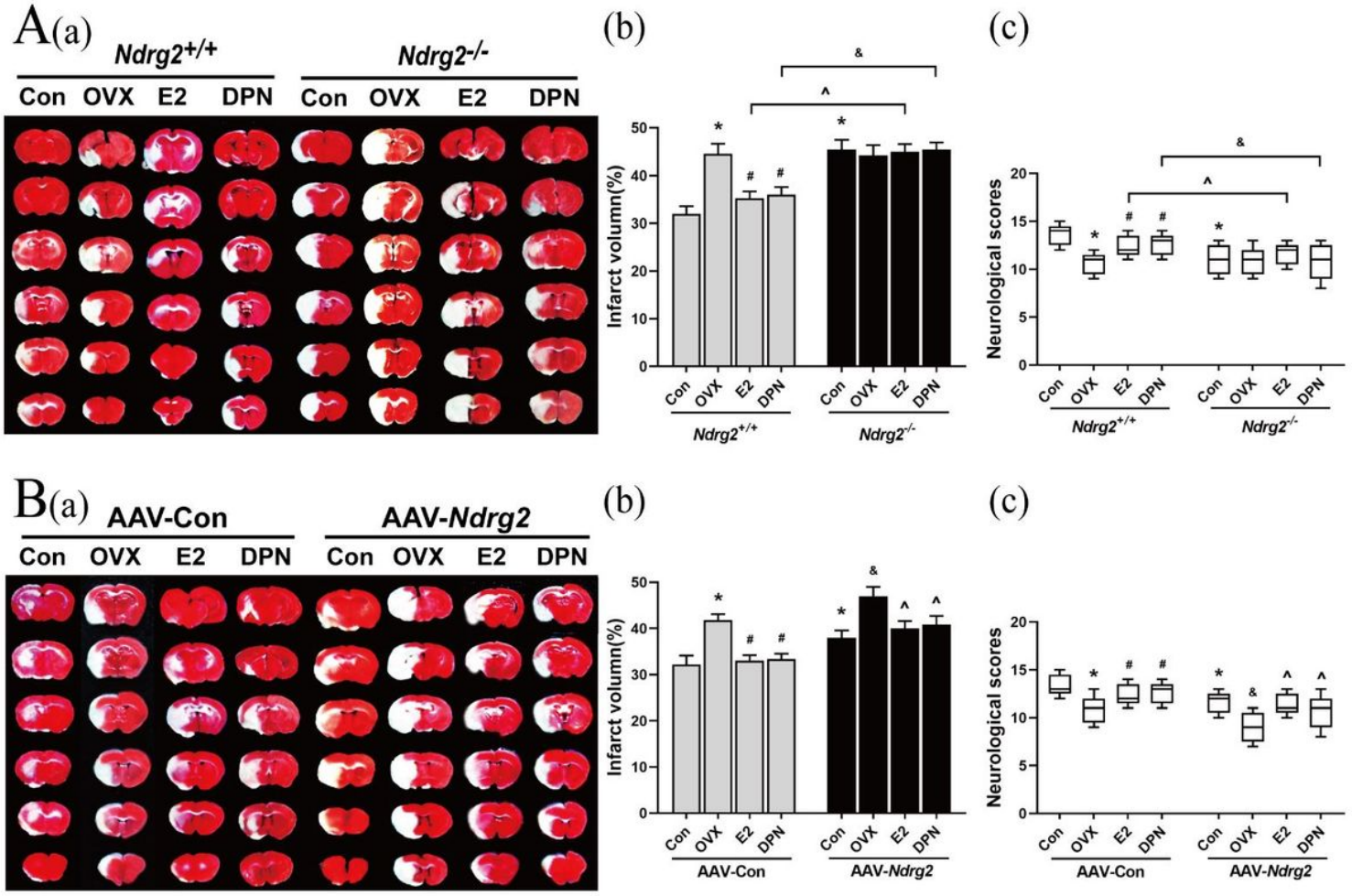

Figure 4

The neuroprotection of E2 and DPN disappeared in Ndrg2-/- female mice, but partially remained in AAV-Ndrg2 female mice. A(a) Representative photographs of brain slices showing the infarct volume assessed 24 h after reperfusion in female mice in different groups. A(b) Statistical analysis of infarction volume in female mice in different groups. *p < 0.05 vs. Ndrg2+/+-Con group, #p < 0.05 vs. Ndrg2+/+-OVX group, ^p < 0.05 vs. Ndrg2+/+-E2 group, &p < 0.05 vs. Ndrg2+/+-DPN group (n = 10 per group). A(c) Neurological deficit score evaluated 24 h after reperfusion. *p < 0.05 vs. Ndrg2+/+-Con group, #p < 0.05

vs. Ndrg2+/+-OVX group, ^p < 0.05 vs. Ndrg2+/+-E2 group, &p < 0.05 vs. Ndrg2+/+-DPN group (n = 10 per group). B(a) Representative photographs of brain slices showing the infarct volume assessed 24 h after reperfusion in female mice in different groups. B(b) Statistical analysis of infarction volume in female mice in different groups. *p < 0.05 vs. AAV-Con-Con group, #p < 0.05 vs. AAV-Con-OVX group, &p < 0.05 vs. AAV-Ndrg2-Con group, ^p < 0.05 vs. AAV-Ndrg2-OVX group (n = 10 per group). B(c) Neurological deficit score evaluated 24 h after reperfusion. *p < 0.05 vs. AAV-Con-Con group, #p < 0.05 vs. AAV-Con-OVX group, &p < 0.05 vs. AAV-Ndrg2-Con group, ^p < 0.05 vs. AAV-Ndrg2-OVX group (n = 10 per group).

Supplementary Files

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