Abstract. The aim of the present study was to investigate the role of G-protein-coupled receptor 30 (GPR30) in long-term 17ß-estradiol (E2) deprivation (LTED) in a rat model with global cerebral ischemia (GCI), and its therapeutic target for ischemic stroke in the clinical setting. Following bilateral ovariectomy, GCI was induced in rats 1 or 10 weeks post-surgery. To determine the protein and mRNA expression levels of GPR30 in the hippocampal CA1 region of LTED rats, short-term E2 deprivation (STED) rats and naturally aging rats, western blot analysis and reverse transcription-quantitative polymerase chain reaction were performed. The results of the present study demonstrated that E2 treatment revealed significant neuroprotection post-GCI in STED rats, but not in LTED rats, as well as a decrease in the expression levels of GPR30 in the hippocampal CA1 region. In LTED rats, Notably, no effects were observed on the ubiquitination of GPR30 following investigation in STED or LTED rats. While the protein and mRNA expression levels of GPR30 were also decreased in the hippocampal CA1 region of female 24-month-old rats compared with 3-month-old rats. E2 treatment initiated for the entire ovariectomy period elevated GPR30 mRNA and protein expression levels, and attenuated the loss of hippocampal neurons in the GCI-induced CA1 region, indicating that E2 treatment exerted robust neuroprotection within LTED rats. However, the neuroprotective effect of E2 may be blocked by G15. The results of the present study revealed that downregulation of GPR30 expression may attenuate the neuroprotection of E2 within LTED conditions in rats post-ovariectomy by leading to neuronal insensitivity to E2 neuroprotection following cerebral ischemia. These results provide evidence that GPR30 may have potential as a novel therapeutic target for the treatment of clinical ischemic stroke.

Introduction

Previous studies have demonstrated that 17ß-estradiol (E2) exhibits a neuroprotective effect in cerebral stroke and neurodegenerative diseases such as ischemic stroke and Alzheimer's disease (1-3). However, the Women's Health Initiative (WHI) trial did not reveal any decrease in stroke-associated morbidity in women that received hormone therapy (4,5). An average age of 63.3 years for participants in the WHI is the most important criticism for WHI, which far exceeds the menopause age (6). The ‘critical period hypothesis’ has been proposed, which describes a precise window of opportunity for beneficial hormone therapy following menopause (7,8). Estrogen may be administered prior to or during perimenopause to protect the neural and cardiovascular systems (7,9). Previous studies in animals also provided supporting information for the period hypothesis and evidence to support neurological consequences of long-term E2 deprivation (LTED; 10 weeks) (10-13). G-protein-coupled receptor 30 (GPR30), also termed G-protein-coupled estrogen receptor 1, is a recently identified G‑protein‑coupled receptor that closely binds estrogen and is characterized as an estrogen receptor within the membrane. The GPR30 results in rapid non-genomic signaling events and transcriptional regulation (14). Previous studies have demonstrated the neuroprotective effects of GPR30 using the GPR30-selective agonist, G1 (15,16). In vivo studies in female mice using global cerebral ischemia (GCI) and middle cerebral artery occlusion models also demonstrated that G1 exhibited neuroprotective effects against cerebral ischemia (17,18). Furthermore, a
previous study that employed an *in vivo* antisense oligodeoxy-
nucleotide knockdown approach revealed the important role of
GPR30 in mediating rapid kinase activation and neuroprotection
by E2 in an animal model of GCI (19). In addition, the
rapid signaling effects of G1 in the activation of the prosur-
vival kinases Akt and extracellular signal-regulated kinases
(ERKs), and in the inhibition of the proapoptotic signaling
kinase, c-Jun N-terminal kinase, in the CA1 region of the
hippocampus post-GCI were demonstrated (19). C-terminus of
heat-shock cognate protein 70-interacting protein-mediated
estrogen receptor (ER)-α degradation was reported within
the CA1 region of the hippocampus following aging and
LTED, which was associated with loss of estrogen-mediated
neuroprotection (20).

However, alterations in the GPR30 expression levels
and the effect of GPR30 on the loss of estrogen-mediated
neuroprotection in LTED and naturally aging female rats
remain largely unknown. Therefore, the present study aimed
to investigate the role of GPR30 in LTED in a GCI rat model
and its potential in the treatment of clinical ischemic stroke.

**Materials and methods**

**Animal preparation.** A total of 322 adult female
Sprague-Dawley rats (12-weeks old, 250-300 g) were
purchased from the Experimental Animal Center of the
Fourth Military Medical University (Xi’an, China). The study
protocol was approved by the Animal Care and Use Committee
of the Fourth Military Medical University and animal use
conformed to the National Institutes of Health Guidelines
for the Care and Protection of Laboratory Animals (21). Rats
were randomly divided into sham (n=21), STED (n=49), LTED
(n=181), 3-month-old (n=22), female Sprague-Dawley rats,
250-300 g) and 24-month-old (n=49), female Sprague-Dawley
rats, 450-500 g; both 3 and 24-month-old rats were purchased
from the Experimental Animal Center of the Fourth Military
Medical University) groups. Rats were allowed free access to
water and food until optimal operation conditions and were
maintained under a 12 h light/dark cycle at room temperature
(RT) and 50% humidity.

**Ovariectomy and GCI.** Following bilateral ovariectomy,
GCI was induced with four-vessel occlusion in rats at 1 week
(for STED group) or 10 weeks (for LTED group) post-surgery,
as previously described (22-24). Briefly, the rats were
anesthetized with 10% chloral hydrate (350 mg/kg, intraperi-
toneal injection; no signs of peritonitis were observed) and the
vertebral arteries were electrocauterized with a monopolar
coagulator and the common carotid arteries (CCA) were
exposed. After 24 h, CCA were re-exposed and clipped for
10 min and rats were subsequently reperfused. The rats with
loss of righting reflex, loss of response to light and dilated
pupils within 30 sec during ischemia were selected for
subsequent experiments. During ischemia, a temperature of
37±0.5°C was maintained using a thermal blanket. The same
surgical procedures were performed for animals in sham
group, however, the CCA was not occluded.

**Drug administration.** In certain rats, an E2 Alzet minipump was
implanted under the skin in the upper region of the mid-back
under anesthesia. The LTED rats were received E2 treatment
(0.025 mg/day) for 10 weeks immediately post-ovariectomy, or
for 1 week after 10 weeks post-ovariectomy, while the STED
rats were administrated with once intracerebroventricular
treatment of E2 (10 µM in 5 µl normal saline) 1 h pre-GCI
induction. In certain STED or LTED rats, G1 (B5455; ApexBio, Houston, TX, USA; 50 µg in 5 µl dimethyl sulfoxide;
DMSO), G15 (B5469; ApexBio; 100 µg in 5 µl DMSO) or
E2-bovine serum albumin (E2-BSA; RPU50787; Biomatik,
Wilmington, DE, USA; 10 µM in 5 µl normal saline) was bilat-
erally administered using an intracerebroventricular injection
1 h pre-GCI (19). The drug procedures were same as above
for 3-month and 24-month rats. In total, the injections were
performed at 1 µl/min rate and the needle was withdrawn after
5 min. During the whole operation, a temperature of 37±0.5°C
was maintained. At the indicated time points, rats were sacrifi-
ced by decapitation under deep anesthesia and their brains
were removed for the following experiments.

**Western blot analysis and co-immunoprecipitation (co-IP).**
Protein samples were extracted after rats were sacrificed as
previously described (23,24). Briefly, tissues of the hippocampal
CA1 region were microdissected on ice and homogenized
in lysis buffer (1:10 w/v) containing 50 mM 3-morpho-
linopropanesulfonic acid (pH 7.4), 150 mM NaCl, 20 mM
β-glycerophosphate, 3 mM DL-dithiothreitol, 2 mM NaVO₄,
1 mM EGTA, 1 mM EDTA, 1 mM NaF, 1% Triton-X-100,
1% NP-40 and protease inhibitor (0.5 mM phenylmethyl-
anesulfonyl fluoride, 10 mg/ml each of apro tinin, leupeptin,
and pepstatin A) for 10 min. Following centrifugation at
15,000 x g for 15 min at 4°C, the supernatant was collected
and protein was quantified using a bicinchoninic acid protein
assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA).
Total 20 µg proteins were subsequently electrophoresed using
6-15% SDS-PAGE and electro-transferred to polyvinylidene
fluoride membranes. After blocking in 5% nonfat milk for 1 h
at RT, the membranes were incubated overnight at 4°C with
the following primary antibodies: Anti-ubiquitin (1:1,000; cat.
no. ab19247; Abcam, Cambridge, UK), anti-GPR30 (1:500; ab39742; Abcam) anti-ERα (1:500; cat. no. bs-0725R; BIOSS,
Beijing, China), anti-ERβ (1:500; cat. no. bs-0255R; BIOSS)
and anti-and GAPDH (1:5,000; cat. no. BM1623; Wuhan
Boster Biological Technology, Ltd., Wuhan, China). The
anti-mouse/rabbit HRP-conjugated IgG secondary antibodies
(1:5,000; cat. nos. ab6728/ab6721; Abcam) were incubated
for 1 h at RT. The blots were then detected using a Clarity™
western ECL kit (cat. no. 170-5060; Bio-Rad Laboratories,
Inc., Hercules, CA, USA) with an Odyssey Imaging system
(LI-COR Biosciences, Lincoln NE, USA) was used for the
detection of blots and ImageJ software (version 1.47; National
Institutes of Health, Bethesda, MD, USA) was used to quantify
protein expression.

For co-IP detection, the supernatant fractions were
diluted 4-fold with HEPES buffer and were incubated with
anti-GPR30 (1:100; cat. no. ab39742; Abcam) and normal
IgG antibody (5 µg, Cell Signaling Technology, Inc.) for 4 h
at 4°C. Samples were pre-incubated with 20 µl protein A/G
agarose (Santa Cruz Biotechnology, Inc., Dallas, TX, USA)
for 60 min at 4°C, and then centrifuged at 1,000 x g for
30 min at 4°C to remove nonspecifically-adhered proteins.
Western blotting was used to analyze the precipitated protein.

**Immunofluorescence.** Histological assessment was performed in fixed for 2 h (4% paraformaldehyde in 0.01 M PBS, RT) and frozen hippocampal brain sections (25, 26). Briefly, brain sections (25 μm) were permeabilized with 0.4% Triton-X-100 in PBS for 30 min and blocked with 5% normal serum for 30 min at RT. Then the sections were incubated at 4°C overnight with diluted GPR30 antibody (1:50; cat. no. ab39742; Abcam). Following three washes with PBS, the sections were incubated for 2 h at RT with goat anti-rabbit immunoglobulin G-Alexa Fluor 488 secondary antibody (1:300; cat. no. A-11008; Invitrogen; Thermo Fisher Scientific, Inc.). Fluorescent images were captured using a confocal laser microscope system at 488 nm wavelength (FV1000; Olympus Corporation, Tokyo, Japan).

**Nissl staining and neuron survival quantification.** At 7 days post-GCI, 0.9% saline and then 4% paraformaldehyde (ice-cold) in 0.1 M phosphate buffer were used for perfusion. Brains were removed and placed in formalin at 4°C overnight, fixed in 30% sucrose in phosphate buffer at 4°C for up to two days and longitudinally cut into 25-μm sections. Coronal sections were stained with 0.1% cresyl violet for 2 min at RT, dehydrated using a graded alcohol series (100, 90, 80, and 70%), placed in xylene and covered with a coverslip following the addition of Histomount medium (Thermo Fisher Scientific, Inc.). And slices were then mounted with Lab Vision™ Ultramount resin (TA-060-UM; Thermo Fisher Scientific, Inc.). The sections were evaluated using a light microscope (DFC310FX; Leica Microsystems, Inc., Buffalo Grove, IL, USA). Viable CA1 neurons within each rat were counted manually. Cell counts were averaged from bilateral hippocampi on five sections. Data from each group were presented as the mean ± standard deviation.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** The STED (1 week post-surgery), LTED (10 weeks) group, the tail blood samples of rats were collected on the first day pre-surgery and on the first, third, fifth and seventh day post-surgery. The rats were anesthetized by intraperitoneal injection (0.35 ml/100 g) with 10% chloral hydrate. The rats were fixed in the prone position and the tails were soaked in water at 50°C for several minutes until the tail vessels were filling. After alcohol disinfection, the tail was cut with a surgical scissors for 5-10 mm and the blood flowed into the EP tube. Blood specimens of 1 ml were collected each time. After the blood samples were labeled, they were allowed to stand overnight at 4°C and centrifuged at 3,000 x g for 10 min. The supernatant was collected in an EP tube and stored at -80°C freezer. The collected specimens were sent to the laboratory, using Roche E601 electrochemical luminescence automatic analyzer to detect concentration of serum estradiol.

**Statistical analysis.** All data are presented as the mean ± standard deviation. One-way or two-way analysis of variance was performed followed by the Bonferroni/Dunn post-hoc tests to determine group differences using GraphPad Prism software (GraphPad Software 6.0; GraphPad Software, Inc., La Jolla, CA, USA). When only two groups were compared, a Student's t-test was performed for comparison between two groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

No neuroprotection by E2 or GI in the CA1 region of the hippocampus in LTED rats. The effects of E2 or GI on E2-associated neuroprotection in the STED and LTED rats following GCI were analyzed. Hippocampal sections were obtained from rats at 7 days reperfusion post-GCI and Nissl’s staining was conducted to detect surviving neurons. GCI (Pla) led to a marked decrease in the number of surviving neurons within the CA1 region at 7-days-reperfusion in the Pla group, compared with in the sham group (Fig. 1). Neuroprotective effects on the hippocampal CA1 region in STED rats were
observed in the G1 and E2 treatment groups, as indicated by Nissl’s staining and quantification of surviving neurons (Fig.1). However, E2 or G1 treatment post-GCI revealed no neuroprotective effects on the hippocampal CA1 region in LTED.
rats (Fig. 1). These results indicated E2 or G1 exerted robust neuroprotection in STED rats, but exerted no neuroprotection in LTED rats.

**GPR30 expression is reduced in the CA1 region of the hippocampus in LTED rats.** Immunofluorescence and western blot analysis were performed to investigate the protein expression levels of GPR30 in the CA1 region of the hippocampus in STED and LTED rats. Immunofluorescence detected high levels of GPR30 immunoreactivity in the CA1 region of the hippocampus in the sham group; the results demonstrated a marked difference between LTED and STED rats, with reduced levels in the LTED rats (Fig. 2A). Furthermore, western blotting analysis revealed that GPR30 expression levels in the CA1 region of the hippocampus were significantly reduced in LTED rats compared with STED rats (Fig. 2B). The mean serum levels of E2 in the sham, STED (1 week) and LTED (10 weeks) groups are presented in Table I. Levels of E2 within rats that received ovarioctomies (STED and LTED) were markedly lower compared with the sham group (Table I). In short, these results indicated GPR30 decreased in the CA1 region of the hippocampus in LTED rats compared with STED rats.

mRNA levels and ubiquitination of GPR30 in the CA1 region of the hippocampus in LTED rats. mRNA and ubiquitination levels of GPR30 were determined in STED and LTED rats. The RT-qPCR results demonstrated that GPR30 mRNA expression levels within STED rats were ~2 times higher compared with those in LTED rats (Fig. 3A). Further analysis revealed that the ubiquitination of GPR30 exhibited no significant difference in LTED rats compared with STED rats (Fig. 3B). Reverse IP with ubiquitin and blotting for GPR30 indicated marginal alterations in GPR30 ubiquitination within LTED rats compared with STED rats (Fig. 3C). The decrease of Ub GPR30 in Fig. 3C was due to the downregulation of GPR30 expression in hippocampus CA1. There was no significant change of GPR30 ubiquitination among the sham, STED and LTED groups. The decrease of GPR30 was attributable to the decreased mRNA levels but not the ubiquitination of GPR30.

Effects of E2 treatment 10-weeks post-ovarioctomy or during the ovarioctomy period on GPR30 expression levels and hippocampal neuronal survival post-GCI in LTED rats. E2-associated effects on the protein and mRNA expression levels of GPR30 were tested about E2 treatment 10 weeks post-ovarioctomy and for the entire ovarioctomy period (10 week). E2 treatment initiated at 10 weeks post-ovarioctomy revealed no effects in preventing reductions in GPR30 protein expression levels within the CA1 region of the hippocampus (Fig. 4A). In rats that received E2 treatment for the whole ovarioctomy period (10 week), E2 prevented a decrease in the GPR30 protein and mRNA expression levels in the CA1 region of LTED rats (Fig. 4B and C). TUNEL analysis was performed at 3 days post-GCI to investigate apoptosis in the hippocampal CA1 region, and the results demonstrated that while the number of apoptotic cells was only slightly decreased by E2 treatment that was initiated at 10 weeks post-ovarioctomy compared with the Pla (GCI) group in LTED rats (Fig. 5A and B), the reduction observed following treatment with E2 immediately post-ovarioctomy and maintained for 10 weeks was greater in LTED rats (Fig. 5C and D). Nissl's staining indicated that E2 treatment initiated ten weeks after ovarioctomy did not exhibit robust neuroprotection within LTED rats following GCI (Fig. 5E). However, in rats treated with E2 immediately after ovarioctomy and maintained for 10 weeks, E2 treatment exhibited marked neuroprotective effects in LTED rats, and the administration of G15 at the end of E2 treatment attenuated the neuroprotective effects of E2, as indicated by Nissl's staining (Fig. 5F). Quantification of surviving neurons confirmed these observations for both E2 treatment time-points (Fig. 5G and H). In conclusion, E2 treatment initiated immediately post-ovarioctomy and maintained for 10 weeks can prevent the decrease of GPR30 and mRNA levels and can exhibit a marked neuroprotective effects in LTED rats.

**Alterations of protein, mRNA and ubiquitination levels of GPR30 and the survival of hippocampal neurons in naturally aging female rats.** Subsequently, the protein expression levels of GPR30, ERα and ERβ were investigated in the CA1 region of the hippocampus between 3- and 24-month-old rats. Western blot analysis of GPR30 revealed that the protein expression levels of GPR30, ERα and ERβ were decreased significantly within 24-month-old rats compared with 3-month-old rats (Fig. 6A). The RT-qPCR results revealed that the mRNA expression levels of GPR30 within the CA1 region of the hippocampus of 24-month-old rats were decreased to ~50% of the levels in 3-month-old female rats (Fig. 6B); however, the ubiquitination of GPR30 revealed no significant differences between 3- and 24-month-old rats (Fig. 6C). Furthermore, Nissl's staining results of the CA1 region of the hippocampus and quantification of neuronal survival demonstrated that G1 or E2 did not exhibit neuroprotection within the 24-month-old female rats (Fig. 6D and E). The decline of GPR30 resulted in the loss of E2 neuroprotection in aging female rats, and these results further confirmed the mechanism of the decrease of GPR30.
Estrogen and its receptor GPR30 have been revealed to exert neuroprotective effects against cerebral stroke and neurodegenerative diseases. However, their roles and mechanisms following ischemic stroke during the critical period remain unknown. The present study confirmed that E2 and G1 exerted marked neuroprotective effects against cerebral ischemia in STED rats following G1, consistent with previous reports (18,28); however, similar effects were not observed within LTED rats. Additionally, GPR30 expression levels were decreased within LTED rats, which may be primarily attributable to the decline of GPR30 mRNA expression levels, and not the degradation of GPR30 via ubiquitination. Importantly, E2 treatment during the entire ovariectomy period for 10 weeks rather than after 10-week ovariectomy exerted robust neuroprotection in LTED rats. Therefore, we hypothesized that the reduction in GPR30 expression may attenuate the neuroprotective effects of E2, which may occur via neuronal insensitivity to E2 following cerebral ischemia during the critical period.

In addition, GPR30 may have potential as an effective therapeutic target against clinical ischemic stroke during the critical period.

**Discussion**

Estrogen and its receptor GPR30 have been revealed to exert neuroprotective effects against cerebral stroke and neurodegenerative diseases. However, their roles and mechanisms following ischemic stroke during the critical period remain unknown. The present study confirmed that E2 and G1 exerted marked neuroprotective effects against cerebral ischemia in STED rats following G1, consistent with previous reports (18,28); however, similar effects were not observed within LTED rats. Additionally, GPR30 expression levels were decreased within LTED rats, which may be primarily attributable to the decline of GPR30 mRNA expression levels, and not the degradation of GPR30 via ubiquitination. Importantly, E2 treatment during the entire ovariectomy period for 10 weeks rather than after 10-week ovariectomy exerted robust neuroprotection in LTED rats. Therefore, we hypothesized that the reduction in GPR30 expression may attenuate the neuroprotective effects of E2, which may occur via neuronal insensitivity to E2 following cerebral ischemia during the critical period.

In addition, GPR30 may have potential as an effective therapeutic target against clinical ischemic stroke during the critical period.

The present study provided important evidence for the hypothesis of the beneficial role of the E2 replacement in critical period which states that a precise window of opportunity
Figure 5. Effects of E2 treatment initiated at 10 weeks post-ovariectomy or immediately after ovariectomy on hippocampal neuron survival and apoptosis post-global cerebral ischemia. TUNEL staining was performed at 3 days following reperfusion. (A) Typical photomicrographs of the hippocampal CA1 region from rats that received E2 treatment at 10 weeks post-ovariectomy. TUNEL-positive cells stained red and all other cells were stained blue with DAPI. Scale bar=100 µm. (B) TUNEL-positive cells were quantified in each group, n=8 rats per group. Data is presented as the average number of TUNEL-positive cells in five view fields in hippocampal CA1 region. (C) Typical photomicrographs of the hippocampal CA1 region from rats that received E2 treatment immediately post-ovariectomy. TUNEL-positive cells stained red and all other cells were stained blue with DAPI. Scale bar=100 µm. (D) TUNEL-positive cells were quantified in each group, n=8 rats per group. Data is presented as the average number of TUNEL-positive cells in five view fields in hippocampal CA1 region. For parts B and D, ***P<0.001 vs. sham group; #P<0.05 and ###P<0.001 vs. Pla group. Nissl’s staining of the whole hippocampus region was conducted in hippocampal sections from sham rats or rats treated with E2, Pla, E2 + G15 or G15 at (E) 10 weeks post-ovariectomy or (F) immediately after ovariectomy for 10 weeks. Rats were treated with G15 at the end of the E2 treatment in the E2 + G15 group. Scale bar=500 µm (upper) and 50 µm (lower). (G) E2 replacement at 10 weeks post-ovariectomy did not prevent neuronal death and exert neuroprotection, n=6-8 rats per group. (H) Cell counting demonstrated that E2 replacement immediately post-ovariectomy, for 10 weeks, may prevent ischemic damage compared with Pla-treated rats. However, the neuroprotection of E2 was attenuated when G15 was administered at the end of the E2 treatment for 10 weeks, n=6-8 rats per group. For parts G and H, ***P<0.001 vs. sham group; ###P<0.001 vs. Pla group; **P<0.01 vs. E2-only group. E2, 17b-estradiol; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling; Pla, placebo; G15, G-protein-coupled receptor 30-selective antagonist.
exists for beneficial hormone therapy following menopause, and healthy cell bias which suggested that E2 only yields neurological benefit if it was applied to healthy neurons. These hypotheses suggest that E2 must be administered during perimenopausal to observe its neurological benefit within the CA1 region of the hippocampus, which is critical for memory and learning (29). E2 was reported to function in the maintenance of optimal mitochondrial bioenergetics within neurons by promoting their use of glucose as the major energy source via oxidative phosphorylation (30). However, during LTED, E2 was not able to perform this function, which caused neurons to alter their preferred energy source from glucose to ketones (30). In addition, the functions of E2 in enhancing attentional processes, long-term potentiation, choline acetyltransferase activity and rate-limiting enzyme for neurotransmitter acetylcholine synthesis activity were lost during LTED (31).

A previous study further demonstrated a marked decrease in ERβ and ERα following LTED; however, the loss of E2 neuroprotection was associated with reduced ERα, but not ERβ, levels within the hippocampal CA1 region (23). The aforementioned findings may not completely explain the loss of E2-associated neuroprotective effects in the CA1 region of the hippocampus of LTED rats, as reported previously (18,28).

GPR30 was indicated to participate in mediating E2 rapid signaling and neuroprotective effects using in vivo and in vitro models of GCI and middle cerebral artery occlusion or slice cultures (15,16,18,19,32,33). It has previously been reported that the GPR30-selective agonist G1 significantly attenuates glutamate- or oxidative stress-induced neuronal death (15,34). G1 couples to pertussis toxin-sensitive G-proteins and activates multiple signaling pathways, including Src proto-oncogene, mitogen-activated protein kinases and ERKs. Transcriptional regulation via the activation of GPR30 has also been reported (35). However, alterations in GPR30 expression and its role in ischemic stroke during the critical period remain unknown. Furthermore, the difference in GPR30 protein expression levels between the STED and LTED rats remains unclear. The results of the present study indicated that G1 exerted a neuroprotective effect on STED rats, as reported previously (18,28). However, robust neuroprotection exhibited by G1 or E2 within the CA1 region of LTED rats was not detected in the present study.

To investigate the GPR30 decrease in the CA1 region of the hippocampus of LTED rats, mRNA and ubiquitination levels of GPR30 were analyzed. The RT-qPCR results revealed that the GPR30 mRNA expression levels were significantly decreased within the CA1 region of LTED rats compared with STED rats, while no significant difference
of the GPR30 ubiquitination was observed between LTED and STED rats, indicating that decreases in GPR30 expression levels may be primarily attributable to the decline in the mRNA expression levels of GPR30. Additionally, E2 treatment initiated 10 weeks post-ovariectomy exhibited no effects in preventing decreases in GPR30 expression levels; E2 was unable to demonstrate robust neuroprotective effects following GCI. However, E2 treatment for 10 weeks during the entire ovariectomy period was able to prevent reductions in GPR30 expression levels and exhibit robust neuroprotective effects. G15, the specific inhibitor of GPR30, attenuated the neuroprotective effects of E2 within the CA1 region of the hippocampus when administered near the end of E2 treatment for 10 weeks. These results indicated that GPR30 may be an important factor in E2 neuroprotection loss. Collectively, E2 loss following ovariectomy may have contributed to reductions in the mRNA expression levels of GPR30 and E2 sensitivity loss in the CA1 region of the hippocampus. The results of the present study also supported the hypothesis of the critical period as E2 treatment for the entire ovariectomy period was fully neuroprotective.

As ovariectomy in young adult rats removed numerous steroids, including estrogen, progestogen and other factors, LTED did not recapitulate the alterations that occurred in naturally aging female rats. Similar experiments were performed in 3- and 24-month-old female rats in the present study. The 24-month-old female rats were reported to exhibit a decrease in GPR30 levels in the CA1 region of the hippocampus, compared with 3-month-old rats. Furthermore, ERα and ERβ also decreased significantly in the 24-month rats compared with 3-month-old rats, which was consistent with a previous report (20). The mRNA expression levels of GPR30 in 24-month-old female rats in the CA1 region of the hippocampus were reduced by ~50% compared with 3-month-old rats; however, the ubiquitination of GPR30 was not significantly different between the two groups. Further experiments in the present study indicated that neither E2 nor G1 exerted neuroprotective effects in 24-month-old female rats, which may be due to decreases in the protein and mRNA levels of GPR30 in the CA1 region of the hippocampus. These findings indicated that when E2 or G1 was administered in perimenopausal women, the decrease in GPR30 expression levels may be reduced, contributing to robust neuroprotection by E2 treatment.

In conclusion, the present study demonstrated that the decline in GPR30 mRNA expression levels may be responsible for the decrease in GPR30 expression within the hippocampal CA1 region following aging and LTED, which was closely associated with the loss of E2-mediated neuroprotection. In addition, these findings may provide important insights into the underlying mechanism of the critical period hypothesis of E2-associated neuroprotective effects. Although the transcriptional regulation needed further investigation, our present findings suggested GPR30 might exert as a potential novel therapeutic target against ischemic stroke for perimenopausal patients in clinical settings.

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Availability of data and materials
The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors’ contributions
GG and TZ designed the study and wrote the protocol. YW, DF and JL completed the experiments, YW established the animal models. SH performed the western blotting and qPCR. YW and DF wrote the first draft of the manuscript. All authors contributed to and approved the final manuscript.

Ethics approval and consent to participate
The study protocol was approved by the Animal Care and Use Committee of the Fourth Military Medical University.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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